ORIGINAL INVESTIGATION

Early effects of mood stabilizers on the Akt/GSK-3β signaling pathway and on cell survival and proliferation

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Abstract

Rationale Lithium, some of the anticonvulsants, and several second-generation antipsychotic drugs are common medications widely prescribed to treat bipolar disorder. Molecular targets and cellular events that mediate their effects have been described for these drugs but are only partially unraveled. Few comparative studies have been performed.

Objectives We evaluated seven mood stabilizers (MS) in the same in vitro system and found several differences and similarities in their cellular mechanisms (proliferation and cell survival). As some MS were previously shown to activate the Akt/GSK-3β axis, this pathway was explored for other drugs.

Materials and methods The SH-SY5Y cells were cultured in RPMI-1640 medium. Effects of MS drugs on serum-induced cell proliferation and on slowing of cell death were analyzed. Phosphorylation and expression of Akt-1 and GSK-3β mRNA and protein were assessed for the seven drugs as well.

Results Lithium, Valproate, Olanzapine, and Clozapine enhance proliferation and protect cells against serum withdrawal-induced injury. These drugs also activate Akt-1 and GSK-3β phosphorylation. Interestingly, gene expression of Akt-1 mRNA and protein, but not GSK-3β, was

increased. The other drugs Lamotrigine, Haloperidol, and Carbamazepine did not affect cellular events nor activate Akt/GSK-3β axis.

Conclusion Valproate and atypical antipsychotics (Olanzapine and Clozapine) regulate SH-SY5Y cell proliferation and survival, activate the Akt/GSK-3β axis, and stimulate gene expression of Akt-1 mRNA and protein, as does Lithium. The other medications have no effect. The study shows the importance of the Akt/GSK-3 axis in MS actions but also pinpoints a different dependence of these drugs on this signaling axis.

Keywords Mood stabilizers · SH-SY5Y cell proliferation · Neuroprotection · Akt–GSK-3β · Signaling axis

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Introduction

Lithium and some anticonvulsant drugs such as Lamotrigine (Ltg) or Valproate (VPA) are common medications prescribed to treat patients with bipolar disorder symptomatology and to prevent relapses (Aubry et al. 2007; Yatham et al. 2006). Recently, some atypical antipsychotics such as olanzapine (Ola) or clozapine (CZ) have also been considered as mood stabilizers (MS) to prevent manic and depressive episodes (Brambilla et al. 2003; Yatham et al. 2005). All these medications are recommended by official guidelines. Regarding conventional antipsychotics such as Haloperidol (Hal), although they have a therapeutic effect in manic episode, a prophylactic effect on relapse prevention has not been shown for this class of medication (Gelenberg and Hopkins 1996). Because bipolar disorder and other psychiatric illness are associated with changes in volume of discrete brain regions, there is much interest in potential neurotrophic effects of treatments (Sheline 2003;

Manji and Duman 2001). Furthermore, recent research has shown interest in how mood-stabilizing agents change the activities of signal transduction systems (Chen et al. 1999; Manji and Duman 2001). Our study has focused on cellular events and on protein kinases (Akt-1 and GSK-3β) involved in signaling axes in order to compare effects of different classes of mood stabilizers.

Lithium, the standard mood stabilizer drug, has emerged as a neuroprotective agent against insult and apoptotic stimuli (for review, Jope and Bijur 2002; Manji et al. 1999; Jope and Williams 1994; Li and El-Mallakh 2000). It has also been reported that long-term lithium treatment increases total gray matter volume (Moore et al. 2000). This cation is involved in a number of biochemical systems and tens of enzymatic activities and signal transduction systems, including the inositol monophosphatase, the phospholipase C, adenylate cyclase, and protein kinase C (for review, Manji et al. 1999). Lithium has been shown to be a direct inhibitor of glycogen synthase kinase (GSK-3β), a serine/threonine-specific protein kinase that plays key roles in the regulation of a variety of cellular processes (Klein and Melton 1996; Stambolic et al. 1996). GSK-3β is regulated upstream by another protein kinase, Akt-1, and together these two kinases are part of a signaling pathway regulated by both phosphatidylinositide 3-kinase (PI3K; Cross et al. 1995; Grimes and Jope 2001) and by the Wnt signal (Zeng et al. 2005). Akt and GSK-3β proteins are involved in cell cycle progression, cell survival, neuronal structure, and apoptotic cell death (Lawlor and Alessi 2001; Jope and Johnson, 2004). There is both direct and indirect evidence supporting the involvement of these proteins in the pathophysiology of mood disorders and schizophrenia (Lesort et al. 1999; Hsiung et al. 2003; Karege et al. 2007; Pandey et al. 2009; Beaulieu et al. 2009).

Some of the other drugs of the above-mentioned list, but not all, have been shown to affect these cellular and biochemical changes. Olanzapine produces trophic effects in vitro and stimulates a number of signal transduction such as Akt, ERK, and the mitogen-activated protein kinase p38 (Lu et al. 2004). Moreover, Olanzapine was shown to enhance glucose uptake consistent with that neurotrophic role (Dwyer et al. 2003). Valproate is also known to have diverse cellular effects, such as protecting against apoptotic insults, although this has been suggested to be cell-type-specific (Chen et al. 1999; Chuang 2005). Valproate was initially reported to inhibit GSK-3β activity in SH-SY5Y cells, but these effects in neuronal cells have not been confirmed by all authors (Gurvich and Klein 2002). Clozapine is an atypical or second-generation antipsychotic drug sometimes used in treatment-resistant bipolar disorder and proposed as a mood stabilizer (Ciapparelli et al. 2003). This drug was reported to regulate the phopshorylation of GSK-3β through the Wnt signal mechanism (Kang et al.

2004). For the other drugs, like Lamotrigine, Carbamazepine, or Haloperidol, few studies have been carried out either on cell survival or on potentiation of cell proliferation (Xie and Hagan, 1998). With respect to the Akt/GSK-3β pathway, Haloperidol effect has been examined in rat frontal cortex (Roh et al. 2007). The authors indicated that haloperidol induces transiently both Ser-21/9 phosphorylation of GSK-3βα/β and Ser-473 phosphorylation of Akt, but no study on long-term expression of these proteins was performed in their protocol. Moreover, few studies have compared the effects of these drugs in the same conditions and on the same cellular model. This approach could help to identify shared mechanisms between drugs.

Therefore, the aim of this study was to compare different classes of mood stabilizers and explore possible differences or shared mechanisms in their biochemical effects on the Akt/GSK-3β signaling pathways. As bipolar disorder is associated with changes in structures of some discrete brain regions, there is much interest in the neurotrophic effects of MS. To determine possible effects of these drugs, cells were grown at low serum content (LSC, 5%). Moreover, as bipolar disorder may be due in part to alterations in signal transduction mechanisms, activation and expression of the Akt-1 and GSK-3β protein were assessed. These proteins exist in different forms (Akt-1, Akt-2, Akt-3, and GSK-3α, GSK-3β, respectively). For this study, we have chosen to study Akt-1 and GSK-3β because they are more ubiquitously expressed at high levels than the other isoforms. Furthermore, phosphorylation at serine473-Akt-1 and serine9-GSK-3β, which are the major physiologic mechanisms to activate these proteins, was used to assess rapid drug actions. The Akt/GSK-3β pathway is an important axis in neural plasticity. A better understanding of these processes may result in identification of therapeutically important sites of actions for the development of novel mood-stabilizing drugs.

Materials and methods

Drugs and reagents

All drugs (Lithium Chloride, Valproate, Haloperidol, Carbamazepine, Lamotrigine, Clozapine) and other reagents used (i.e., LY294002) were purchased from Sigma (St Louis, MO, USA). Olanzapine was a gift from Eli Lilly and Co. (Indianapolis, IN, USA).

Cell culture and drug treatment: assessment of proliferation and cell survival

The human-derived neuroblastoma cells, the SH-SY5Y, purchased from ECACC (Salisbury, UK), were cultured in

a humidified atmosphere of 95% air/5% CO_2 at 37°C in RPMI-1640 medium supplemeted with 10% (v/v) fetal calf serum supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Basel, CH). Depending on the experiment, cells were plated at various densities on 100-mm dishes or six-well plates, and where indicated, serum was withdrawn 24 h or few hours before adding the drugs. For the proliferation test, cells grown in the low fetal calf serum (FCS; 5%) medium were plated at the same density and incubated up to 72 h with different drugs. In preliminary tests, different drug levels were used in dose–response assays, but only optimal drug doses (1 mM lithium, 0.6 mM VPA, 10 μM clozapine) were used in different experiments. Cells were counted in each 24-h period to determine their proliferation. For the survival study, cells were seeded at 10^5 cells/well in a six-well plate in serum-free RPMI-1640, and the drugs were added. Two controls without drugs (serum-free and serum-feed medium) were performed. The medium and drugs were changed every 2 to 3 days, and fresh drugs were added. Phase-contrast images were acquired from three random fields from three coverslips at different times up to 10 days, and cells were counted. Cell viability was assessed by the trypan blue staining method and further confirmed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method (Kahle and Maas 1997).

Biochemical assays

Concentration–response and time course effects of different drugs, namely Lithium, Valproate, Carbamazepine, and Lamotrigine, as well as of typical and atypical antipsychotic, Haloperidol, Olanzapine, and Clozapine, on the Akt-1 and GSK-3β were analyzed. The range of drug concentrations was chosen from either the previous in vitro studies, therapeutic doses, or human plasma levels when known (Bowden et al. 1994 and 1996; Perry et al. 1998; Ciapparelli et al. 2000; Aubry et al. 2007; Kim et al. 2008; Heiser et al. 2007). Concentration range of Lamotrigine and Carbamazepine correspond roughly to the therapeutic windows used in bipolar disorder treatment (Calabrese et al. 2008; Simhandl et al. 1993). Both immediate effects ([473]Ser-Akt and [9]Ser-GSK-3β phosphorylation) and late biochemical actions (Akt-1 and GSK-3β proteins and mRNA expression) were determined. In some experiments, blockade of PI3K pathway was performed by prior addition of 20 μM LY2920049, a specific inhibitor of the pathway.

Protein assay by Western immunoblot

All proteins were determined by the Western immunoblot method as previously described in detail (Karege et al. 2007). Cells were homogenized in a lysis buffer (50 mM Tris pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium decolate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin (10 μg/ml each). Homogenates were incubated for 20 min at 4°C with shaking, centrifuged (10,000×g for 10 min, 4°C), and the supernatant was used for the assay samples. The loading buffer contained 0.125 mM Tris (pH 6.8), 20% glycerol, 10% mercaptoethanol, 4% SDS, and 0.02% bromophenol blue. Samples were heated at 95°C for 10 min before gel loading. Protein concentrations had previously been determined using the BCA kit (Pearse Chemical, Rockford, USA). Equal amounts of a soluble fraction of cell proteins (25 μg for GSK-3β or Akt and β-actin or 50 μg for ser[9]-pGSK-3β and [473]ser-pAkt) were electrophoresed on 10% (w/v ratio) SDS-polyacrylamide gel with a Mini Protean system (Bio Rad) with molecular weight standards. After electrophoresis, the samples were electrotransferred overnight onto nitrocellulose membranes (GE Amersham) then blocked for 1 h at room temperature in Tris-buffered saline Tween-20 solution with 5% (w/v) non-fat milk powder and bovine serum albumin, 2%. The blots were incubated overnight at 4°C with primary antibody for Akt-1 (1:1,000) or phospho-Akt (1:500) purchased from either Cell Signaling Technology (Beverly, MA, USA), or Santa-Cruz Technology (Santa Cruz, CA, USA). After washing, membranes were incubated with an anti-rabbit IgG labeled with HRP (Amersham Pharmacia) for 1 h at room temperature. The membranes were washed and developed with the ECL Western Blotting system (Amersham Pharmacia) followed by exposure of the membranes with radiographic films (Hyperfilm ECL, Amersham Pharmacia). In most cases, the membranes were stripped with Reprob Plus (Chemicon, Temecula, CA, USA) and reincubated with β-actin antibody (1:1,000 dilution; Abcam Ltd. Cambridge, UK) as the reference protein. Quantification of the immunoreactivities was performed by densitometric scanning using an image analysis system (Molecular Analyst, BioRad). The optical density (OD) obtained from each band was normalized against the corresponding β-actin band.

Total RNA extraction and quantitative reverse transcription polymerase chain reaction

Approximately $5×10^6$ cells were collected after different periods of drug treatment, washed in phosphate-buffered saline, and then submitted to lysis with the RNAqueous kit from Ambion (Austin, TX, USA). Total RNA concentration was determined from a spectrophotometer optical density measurement (260 and 280 nm), and the purity of RNA was tested on a Bioanalyzer Agilent Technologies instrument (Basel, CH) which yield two sharp bands of 18 S and

28 S RNA. For all samples tested, the ratio between the spectrophotometer readings at 260 and 280 nm (OD 260/OD 280) was between 1.8 and 2.0. Reverse transcription reactions were carried out on 500 ng of total RNA using the High Capacity cDNA Archive enzyme (Applied Biosystems) at 37°C for 2 h.

For real-time reverse transcription polymerase chain reaction (RT-PCR), the ABI Prism 7900 HT Sequence Detection System instrument and the Assay-on-demands Gene Expression Products (TaqMan MGB probes, FAM dye-labeled) from Applied Biosystems (Applera Europe, Rotkreuz) were used to quantify mRNA for target gene and endogenous controls (β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The thermal conditions were given by the supplier : an initial step (2 min at 50°C and 10 min at 95°C) was followed by 40 cycles of amplification (15 s at 95°C and 1 min at 60°C). All samples were run in triplicate. Data were analyzed by the SDS 2.2 software with the comparative Ct method, where mRNA levels of target genes were normalized to the corresponding β-actin or GAPDH gene expression (Livak and Schmittgen 2001). The drug-naive treated samples at time 0 h were used as calibrator samples, and results were expressed in percentage of change with respect to control.

Statistical analyses

Statistical analyses were performed with a statistical software (StatView V, Brain Power, Calabasas, CA, USA). Data from immunoblotting and from quantitative RT-PCR (qRT-PCR) were expressed in relative values (percentages or ratios ± SD) with respect to housekeeping genes (GAPDH or β-actin). Due to transformed values, nonparametric Friedman tests for analysis of variance (ANOVA) was used for variance between groups, and multiple comparisons between was performed with post hoc Mann–Whitney U test. The significance was set at $p < 0.05$.

Results

Cell proliferation and cell survival

Cellular effects of different mood stabilizers were assessed in serum-fed (proliferating cells) and serum-deprived (dying cells) neuroblastoma cells, by applying different doses of mood stabilizers. To reveal possible effects of these drugs, cells were grown at low serum content (LSC, 5%). Figure 1a, b displays a concentration–response study with different mood stabilizer drugs. Only Lithium, Valproate (Fig. 1a), and Olanzapine (Fig. 1b) significantly potentiated serum-induced cell proliferation in a dose–
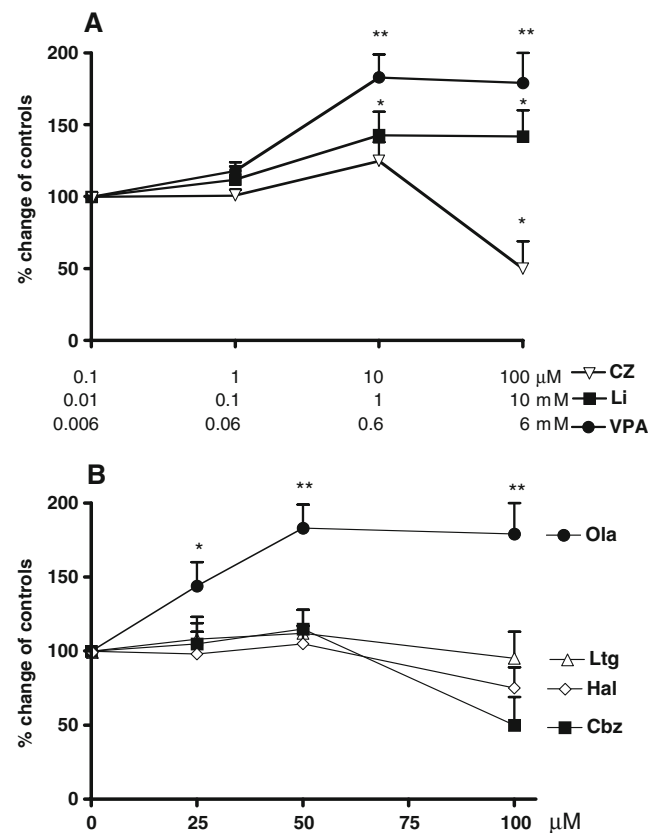

Fig. 1 Concentration–response effect of mood stabilizer on cell proliferation on SH-SY5Y cells cultured in RPMI-1640 with low serum content (LSC, 5% FCS) and complemented with mood stabilizers for a period of 24 h. a Lithium, Valproate, and Clozapine and b Olanzapine, Lamotrigine, Haloperidol, and Carbamazepine. Results are expressed in percentage changes of controls (no drug added to LSC), and data are mean values (±SD) of four to six independent assays. Nonparametric Mann–Whitney tests: *$p < 0.05$; **$p < 0.01$

response manner. For the other drugs, there was either no change or decrease in cell proliferation (high concentrations induced toxicity). The doses with optimal responses were used to assess the time-course effects. Li$^+$ (1 mM), VPA (0.6 mM), and Ola (25 μM) gradually increased cell proliferation both at 24 and 48 h. At 72 h, cells were in a confluence state, both in controls and MS-treated samples (Fig. 2a, b), and the effect of drugs was nulled. The withdrawal of serum induced a progressive death (Fig. 3), and application of drugs on cell cultures prevented or delayed cell death. Lithium, Valproate, Clozapine (Fig. 3a, b), and Ola (Fig. 3c) significantly delayed the cell death. The other drugs (Lamotrigine, Carbamazepine, and Halaperidol) were unable to prevent cell death (Fig. 3c)

Phosphorylation of Akt-1 and GSK-3β proteins

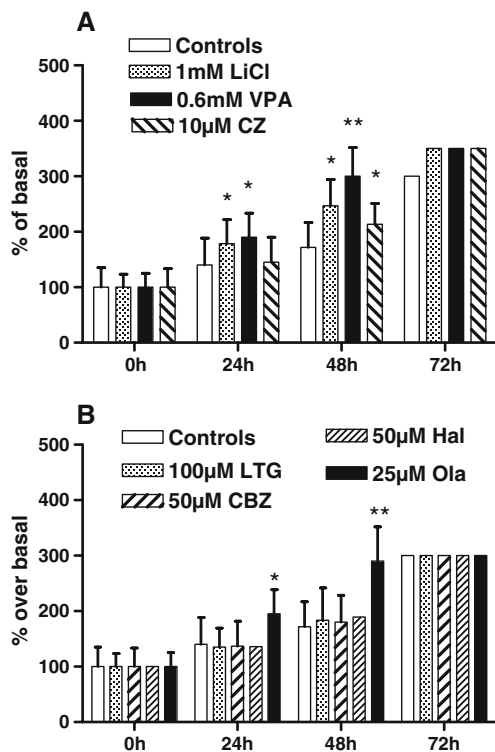A study of immediate effects on the phosphorylation of Akt at serine-473 and GSK-3β serine-9 was performed (Fig. 4),

Fig. 2 Time-course study of mood stabilizer effects on cell proliferation. **a** Li⁺, VPA, and CZ. **b** Ltg, Cbz, Hal, and Ola. Results which show relative growth are expressed in percent of control values (LSC cells). Data are mean values (s ± SD) of four to six independent assays. Nonparametric Mann–Whitney tests: *$p < 0.05$; **$p < 0.01$

and result showed that addition of Lithium (1 mM), Valproate (0.6 mM), and Olanzapine (25 μM) to LSC-grown samples activated GSK-3β and Akt-1 phosphorylation after 30 min. There was also a late activation by Clozapine (not shown), and no other drug did activate the Akt/GSK-3β. Respective t tests on both GSK-3β and Akt were for Lithium ($p < 0.05$ and 0.01, respectively), for Valproate ($p < 0.01$ and 0.01, respectively), and for Olanzapine ($p < 0.01$ and 0.01, respectively). Addition of LY294002 (20 μM), a specific PI3K inhibitor, 10 min before drug application, resulted in decrease or suppression of the Akt-1 phosphorylation in both Lithium (1 mM) and Olanzapine (50 μM)-treated cells with respective to LSC samples (data not shown).

Akt-1 gene expression (mRNA and protein levels)

Figure 5a, b displays data of the real-time qRT-PCR for Akt-1 gene expression. Lithium and Valproate (Fig. 5a) and Olanzapine (Fig. 5b) induced a time-dependent increase in Akt mRNA levels (Friedman test of ANOVA: $\chi^2 = 19.8$ $p < 0.01$, $\chi^2 = 20.2$; $p < 0.01$, and $\chi^2 = 21.6$, $p < 0.01$, respectively). These changes were significant after 12 h for Lithium ($p < 0.05$), Valproate ($p < 0.01$), and Olanzapine ($p < 0.01$)

and progressively increased at 18 and 24 h. For the Clozapine-treated cells, the global changes were not significant, but at 24 h, Akt-1 mRNA was significantly increased compared with control levels ($p < 0.05$). No change was observed in GSK-3β mRNA during the same period, whatever the drug used (data not shown).

Analysis of total Akt-1 and total GSK-3β proteins over a period of 72 h of drug treatment revealed that Akt protein levels, but not GSK-3β protein, significantly increased compared to control levels (time—0 h; Fig. 6b, c). Friedman test of ANOVA yielded for Akt changes: $\chi^2 = 13.5$; $p < 0.05$. Multiple comparison analysis indicated that changes were significant after 24 h for Lithium, Valproate, and Olanzapine ($p < 0.05$). A weak change was also observed for Clozapine but at 48 h ($p < 0.05$; Fig. 6b). No change in GSK-3β protein levels was observed during this period.

Discussion

The major goal of the present study was to determine whether anticonvulsant molecules or typical and atypical antipsychotic drugs could induce cellular effects and regulate molecular pathways, namely the Akt/GSK-3β axis, with comparable efficacy and timing with those of Lithium (Fig. 7). Seven different drugs, most of them used as mood stabilizers, were analyzed in vitro by investigating both cellular and biochemical actions on cultures of SH-SY5Y cells. The potentiation of cell proliferation and the blockade of cell death were also assessed. Phosphorylation of both Akt-1 and GSK-3β protein kinases and expression of mRNA and proteins were analyzed as well. The study yielded the following results:

1. On the cellular level, the low serum content stimulates the cell division and proliferation at a steady pace. Addition of the mood stabilizers to the culture medium results in significant potentiation. The effect was statistically significant after 24 h (Lithium, Olanzapine, and Valproate) and 48 h (Clozapine). In these conditions, the other drugs (Lamotrigine, Carbamazepine, and Haloperidol) have no effect on the proliferation.

The withdrawal of serum initiates the apoptotic cell death. Addition of some mood stabilizers (Li+, VPA, CZ, and Ola) in the serum-free cell culture protects cells by a transient proliferation and a delayed death allowing them to survive a few more days. Lamotrigine, Carbamazepine, and Haloperidol have no effect on the survival of SH-SY5Y cells.

2. On the molecular level, after 30 min, Lithium, Olanzapine, and Valproate induced a significant phosphorylation of ⁹Ser-GSK-3β and ⁴⁷³Ser-Akt-1, com-
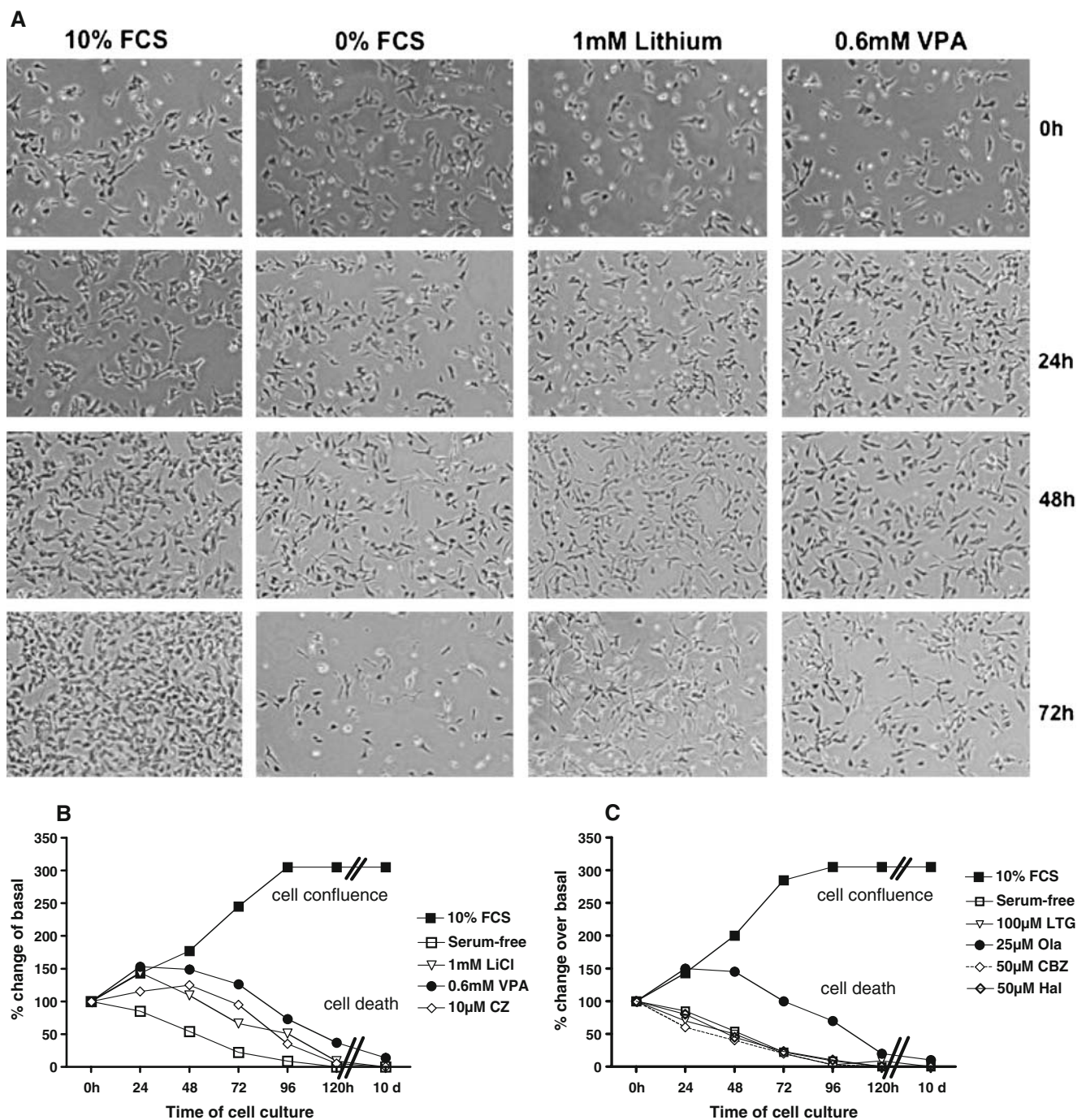
Fig. 3 The neuroprotective effect of mood stabilizers against serum withdrawal-induced cell death. Viable cells were counted in serum-deprived medium where mood stabilizer drugs were supplemented. **a** Contrast images of SH-SH5Y cells in different conditons of culture. **b** Serum-free cells with addition of 1 mM Li+, 0.6 mM VPA, or 25 μM CZ. **c** Serum-free cells with addition of 100 μM Ltg, 50 μM Cbz,

50 μM Hal, or 25 μM Ola. Results are mean values (±SD) of four to six independent assays. In both figures, control cases with 10% FCS-feed and serum-free cells are shown. With respect to serum-free samples, significant differences were found at 48 (Li+, VA, and CZ, $p < 0.05$) and at 24, 48, and 72 h (Ola, $p < 0.05$)

pared with LSC-feed controls. The other drugs did not induce any phosphorylation activity. The effect of these drugs was prevented by a previous application of a PI3K inhibitor (20 μM LY294002), confirming that

Akt-1 was activated through PI3K signaling. At 12 h, Lithium, Olanzapine, and Valproate induced an increase in Akt-1 mRNA levels, compared with LSC samples. No such change was observed for GSK-3β

mRNA. Clozapine effect on Akt mRNA was significant after 24 h, and the other drugs did not induce any change in mRNA levels. After 24 h, increase in Akt-1 protein levels, but not in GSK-3β levels, was observed in Lithium-, Olanzapine-, and Valproate-treated cells. Clozapine effect was significant after 48 h, and no other drug was observed to be effective. After these observations, Lamotrigine, Haloperidol, and Carbamazepine's effects on these cell paradigms were null.

Globally, these data are in accordance with a number of previous individual reports especially the phosphorylation of Akt and GSK-3β and the cellular events here studied (Di Daniel et al. 2005; Kang et al. 2004; Li et al. 2002; Manji and Duman 2001; Kozlovsky et al. 2006). However, we were unable to find a study investigating all these medications in a single protocol. Di Daniel compared the action of four MS drugs (VPA, Li$^+$, Cbz, and Ltg) but on a different signaling axis, i.e. ERK/MAPK. Moreover, these authors did not include a typical antipsychotic drug, such as Haloperidol (Di Daniel et al. 2005). Using animal brain, Kozlovsky et al. (2006) have analyzed the effect of four drugs (VPA, CZ, Hal, and Li+) on the in vivo phosphorylation of GSK-3β and reported similar data. However, they did not test the MS effect on Akt-1 activation. Many other important studies were carried out using either one drug or compared two or three drugs, mostly Ola, CZ, VPA, and Li+ (Kim et al. 2008; Heiser et al. 2007).

The novelty of this study consists in three points: first, for the first time, seven different MS drugs were used to test two cell events (proliferation and survival) and three molecular assays (protein phosphorylation, mRNA, and protein expression) on two important biochemical actors, Akt-1 and GSK-3β. To our knowledge, no other previous assay has tested a so complex mechanism. Second, to our knowledge, no other study has been conducted on the Carbamazepine or Lamotrigine's effects on Akt/GSK-3β pathway. And thirdly, for the first time, we report an increase of the Akt-1 mRNA and protein levels by mood stabilizers. The latter point is the most important result of this study, given the importance of Akt-1 protein in the proliferation processes.

Previous works conducted on animal and neuronal and non-neuronal cells have suggested that psychotropic drugs, including Lithium, Valproate, or other antipsychotics, can enhance cell viability and activate the Akt/GSK-3β signaling pathway (Li and El-Mallakh 2000; Di Daniel et al. 2005; Kang et al. 2004; Manji and Duman 2001; Kozlovsky et al. 2006). However, Shin et al. reported a different effect of CZ with an inhibition of Akt and a dephosphorylation of GSK-3β (Shin et al. 2006). But as they admitted themselves, they used a special cell line, the U-87MG glioblastoma, a cell which lacks a powerful
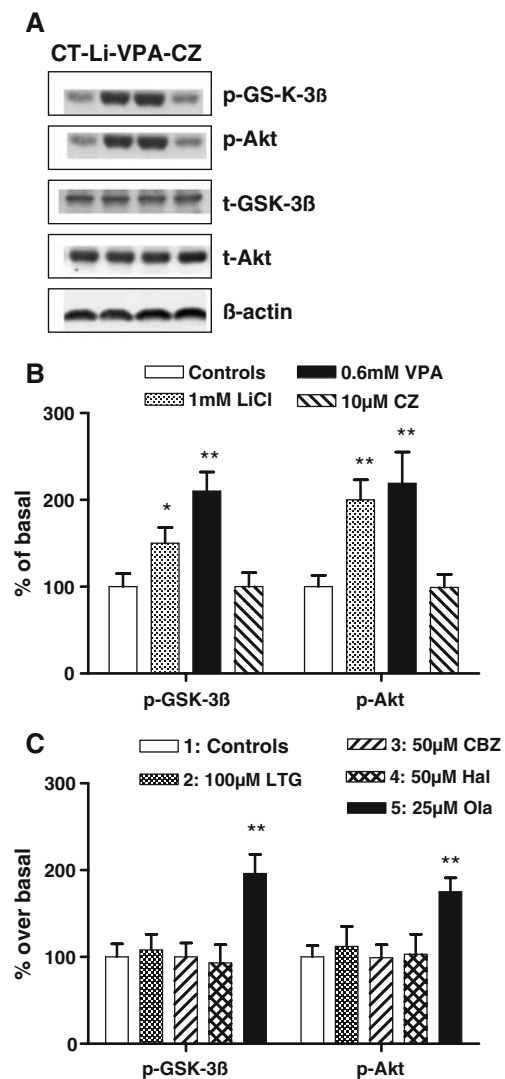

Fig. 4 Effect of mood stabilizer drugs on phosphorylation of ^{473}S-Akt-1 and ^{9}S-GSK-3β. **a** Representative Western blot images. **b** Cells were treated with Li$^+$, VPA, or CZ. **c** Cells were treated with Ltg, Cbz, Hal, and Ola. Relative values of optical densities (ROD) of ^{9}S-pGSK-3β and ^{473}S-pAkt immunoblots expressed as percent of nontreated samples. Data are mean values (±SD) of four to six independent assays, and statistical analysis with *t* test yielded significant change in different samples. Nonparametric Mann–Whitney tests were: *$p < 0.05$ and **$p < 0.01$

regulator of the Akt/GSK-3β axis, the PTEN, and probably mobilizes alternative pathways.

With respect to Carbamazepine, this drug was reported to be involved in signal transduction of cyclic adenosine monophosphate (cAMP) second messenger systems, but no effect on Akt/GSK-3β has been reported up to date (Gould et al. 2004). Lamotrigine has a potent activity dependent on ion channels (i.e., Na+ and Ca+) and could have indirect action on signal transduction (Xie and Hagan 1998). Whether Ltg has direct actions on this intracellular signaling molecules has not been extensively studied to
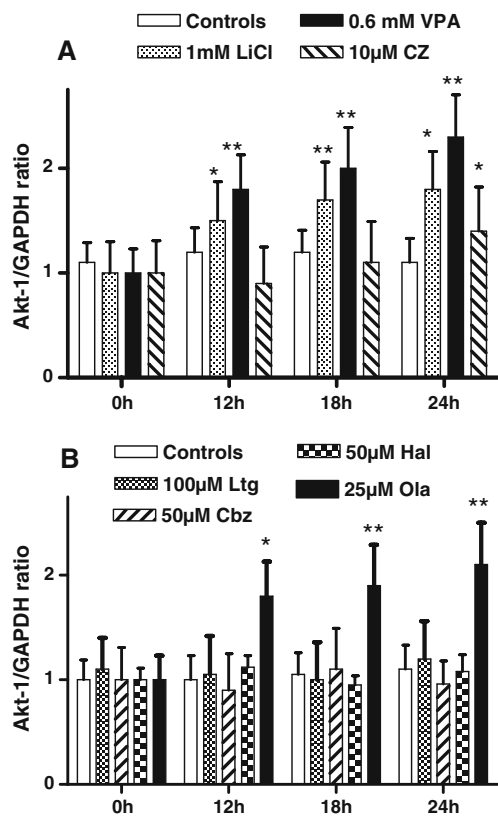
Fig. 5 Time course of Akt-1 mRNA expression in SH-SY5Y cells grown in LSC medium supplemented with mood stabilizers at their respective optimal doses. Akt-1 mRNA was assayed by the real-time quantitative RT-PCR method, and results were expressed in ratio values with respect to a house-keeping gene (i.e., GAPDH). **a** Li$^+$, VPA, and CZ at optimal levels. **b** Ltg, Cbz, Hal, and Ola. Data, expressed as percent of change with respect to controls, are mean values (±SD) of four to six independent assays. Friedman test of ANOVA: $\chi^2=19.8$, $p<0.01$; $\chi^2=20.2$, $p<0.01$; and $\chi^2=21.6$, $p<0.01$, respectively. Post hoc nonparametric Mann–Whitney tests were significant: *$p<0.05$; **$p<0.01$

date. Also, both Haloperidol and Clozapine were shown to induce GSK-3β phosphorylation, but as this study was conducted in rat brain cortex, it could be thought that the effect depends on the cell type (Roh et al. 2007).

Interestingly, Akt protein levels, but not GSK-3β, were gradually increased in parallel to the drug-induced cell proliferation. It seems that the two molecular and cellular processes have a causal relationship. A number of previous reports have demonstrated that Akt is not only involved in cell growth but is also involved in glucose metabolism/ uptake (Hajduch et al. 2001; Lawlor and Alessi 2001). Akt was shown to be a key mediator of signal transduction process and mediates many of the survival signals (Brunet et al. 2001). Therefore, it is possible to speculate that the addition of the mood-stabilizing drugs resulted in inducing more Akt protein levels available for signals that mediate the subsequent cell proliferation. Valproate was the most effective on enhancing Akt-1 protein levels. This effect
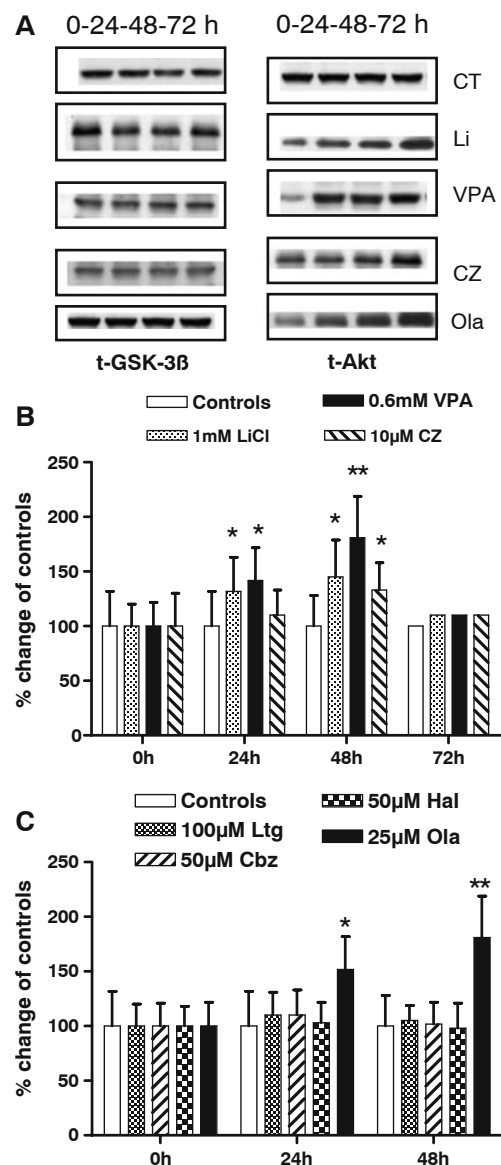

Fig. 6 Time-course of mood stabilizer drugs on Akt-1 protein levels in SH-SY5Y cells grown in LSC medium supplemented with mood stabilizers at their respective optimal doses. Proteins were assayed by the Western immunoblot method, and results are relative optical densities (ROD). **a** Represntative Western blot images. **b** Li$^+$, VPA, and CZ. **c** Ltg, Cbz, Hal, and Ola. Data, expressed as percent of change with respect to controls, are means (±SD) of four to six independent assays. Nonparametric Mann–Whitney tests were significant: *$p<0.05$; **$p<0.01$

could be alternatively explained by its capacity to upregulate gene expression through inhibiting histone-deacetylase, as has been reported (Harwood and Agam 2003; De Sarno et al. 2002).

Our study showed that cellular events and protein synthesis were preceded by rapid phosphorylation and mRNA synthesis. This may suggest that long-term effects must probably be previously sensitized by acute effects, such as protein phosphorylation and mRNA synthesis. To
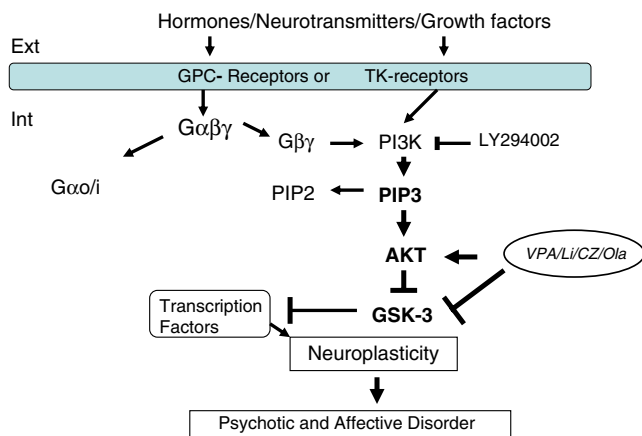
Fig. 7 Simplified overview of the PI3K/Akt/GSK-3β signaling pathway. The potential sites of MS actions are indicated. *GPC-receptors* G-protein coupled-receptors, *TK-receptors* tyrosine kinase-receptors, *PIP3* phosphatidylnositol-triphosphate, *PIP2* phosphatidylinositol-biphospahte

reveal this sequential process, the use of cells with high rates of division such as SH-SY5Y could be an advantage.

Globally, our results indicate that cellular mechanisms observed with Lithium are shared by Valproate, Clozapine, and Olanzapine but not by Carbamazepine, Lamotrigine, and Haloperidol. This could suggest that either the SH-SY5Y cell is not a universal model to investigate these cell events or that the MS drug effects are cell-type-specific. Moreover, all drugs do not activate the Akt/GSK-3β. This indicates that this signaling pathway is not shared by all mood stabilizers.

This study raised some points which require more clarification. First, comparison to clinical situations was allowed by performing at optimal concentrations which correspond nearly to their therapeutically relevant concentrations as reported by different authors (Bowden et al. 1994 and 1996; Perry et al. 1998; Ciapperelli et al. 2003; Aubry et al. 2007). However, the doses used for Olanzapine were higher than the corresponding clinical doses (Olesen and Linnet 1999). The serum concentrations are usually much lower, but according to some authors, the brain levels can contain much higher doses of Olanzapine (Olesen and Linnet 1999; Robertson and McMullin 2000; Lu et al. 2004). This is probably due to the fact that brain cells, especially fatty cells, can accumulate the drug and reach levels sufficient to induce the effects observed in this study (Lu et al. 2004). In addition, active metabolites can also contribute to raise the effective concentration of the drug. This is the case for haloperidol, where postmortem concentrations were found to be ten to 30 times higher than the serum concentrations (Kornhuber et al. 1999).

Second, it remains also to clarify the clinical relevance of such rapid cellular and molecular effect. In clinical context, these drugs are effective in chronic administration where after several days they may enhance the resilience of human neural cells against cell death elicited by diverse insults (Manji and Duman 2001). The in vitro studies remain a remote mirror of what really happen in vivo and in pathological conditions. The increase of Akt-1 gene expression could be an indication of the MS drugs' effect to sustain the lag period for onset action.

Another interesting issue concerns the specificity of this pathway to MS drugs. In other words, do antidepressant drugs have the same effect? There are few reports on the effects of antidepressants on either Akt or GSK-3β. According to Tsai et al. 2008, GSK-3β gene is associated with antidepressant response in a Chinese population. Moreover, administration of imipramine and fluoxetine was found to increase ^{9}S-GSK-3β in mouse brain (Li et al. 2004). These studies show the importance of this pathway which can be activated by both mood stabilizers and antidepressants.

Despite the above-mentioned limitations, this study has shown that in human-derived neuroblastoma SH-SY5Y cells, four drugs used as mood stabilizers or antipsychotics, namely Li+, VPA, Ola, and CZ can mimic the postulated cellular events occurring in mood disorder. These drugs were able to delay cell death and therefore to act as neuroprotectors and to increase serum-induced cell proliferation. The possibility that these drugs may protect cells from injury-induced death or may enhance growth is particularly relevant given the reported morphological deficits associated with bipolar disorder. On a molecular level, these three drugs activate Akt-1 protein by a rapid phosphorylation, resulting in deactivating phosphorylation of GSK-3β. These drugs also induce a time-dependent increase in Akt-1 gene and protein expression but not in GSK-3β. The enhanced expression of Akt-1 was parallel to enhanced cell proliferation, thus confirming its role in anti-apoptotic processes. This study may suggest that long-term effects must probably be previously sensitized by acute effects that only in vitro assays with SH-SY5Ycells can reveal because of their high rate of division. The study provides a framework for understanding how protein kinases involved in signal transduction may contribute to study MS mechanisms of action.

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