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The neuroprotective action of the mood stabilizing drugs lithium chloride and sodium valproate is mediated through the up-regulation of the homeodomain protein Six1

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ABSTRACT

The mood stabilizing agents lithium chloride (LiCl) and sodium valproate (VPA) have recently gained interest as potential neuroprotective therapeutics. However, exploitation of these therapeutic applications is hindered by both a lack of molecular understanding of the mode of action, and a number of sub-optimal properties, including a relatively small therapeutic window and variable patient response. Human neuroblastoma cells (SH-SY5Y) were exposed to 1 mM lithium chloride or 1 mM sodium valproate for 6 h or 72 h, and transcriptomes measured by Affymetrix U133A/B microarray. Statistically significant gene expression changes were identified using SAM software, with selected changes confirmed at transcript (TaqMan) and protein (Western blotting) levels. Finally, anti-apoptotic action was measured by an in vitro fluorescent assay. Exposure of SH-SY5Y cells to therapeutically relevant concentrations of either lithium chloride or sodium valproate elicited 936 statistically significant changes in gene expression. Amongst these changes we observed a large (maximal 31.3-fold) increase in the expression of the homeodomain protein Six1, and have characterized the time- and dose-dependent up-regulation of this gene in response to both drugs. In addition, we demonstrate that, like LiCl or VPA treatment, Six1 over-expression protects SH-SY5Y cells from staurosporine-induced apoptosis via the blockade of caspsase-3 activation, whereas removal of Six1 protein via siRNA antagonises the ability of LiCl and VPA to protect SH-SY5Y cells from STS-induced apoptosis. These results provide a novel mechanistic rationale underlying the neuroprotective mechanism of LiCl and VPA, suggesting exciting possibilities for the development of novel therapeutic agents against neurodegenerative diseases such as Alzheimer's or Parkinsonism.

Introduction

Lithium chloride (LiCl) and the anticonvulsant valproate (VPA) are frontline treatments for the manic phase of bipolar disorder (BD), acting as mood stabilizers (McElroy et al., 1992). The quoted prevalence for BD is approximately one percent of the population (Spearing, 2001), although several factors probably result in under- or misdiagnosis. First, there are many BD phenotypes, complicating diagnosis (Thomas, 2004; MacQueen et al., 2005). Second, from these multiple phenotypes several variants exist in which one or other phase is extremely mild. Third, the combination of the previous two factors results in frequent incorrect diagnosis of BD as unipolar disorder (Dunner, 1992; Spearing, 2001) meaning that the actual prevalence may be considerably higher. In addition to this established therapeutic area, increasing support is being given towards the use of LiCl and VPA as general neuroprotective agents, with potential treatments including disorders such as Alzheimer's or Parkinsonism (Brunello, 2004; Aghdam and Barger, 2007), although the molecular rationale behind this has yet to be elucidated (Di Daniel et al., 2005). This combined therapeutic arena, encompassing both mood stabilization and neuroprotection means that the potential patient population who may be subject to chronic treatment with these therapeutic agents is very large. Unfortunately, in addition to possessing unknown molecular mode of action(s), both LiCl and VPA have several other sub-optimal parameters. First, for treatment of bipolar

Abbreviations: BD, Bipolar disorder; DAVID, database for annotation, visualization and integrated discovery; HCaRG, hypertension related calcium regulated gene; LiCl, lithium chloride; SAM, statistical analysis of microarrays; Staurosporine, STS; VPA, sodium valproate.

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disorder only around 50% of patients respond to LiCl therapy and a similar number (54%) to VPA treatment (Mendlewicz et al., 1999). Second, both LiCl and sodium valproate have relatively small therapeutic indices (Strakowski et al., 2001). Third, both LiCl and VPA require high dosing levels (millimolar circulating concentrations) to achieve efficacy (Jann et al., 1982; Balfour and Bryson, 1994). It is thus clear that in order to optimize the use of these agents and/or develop novel therapeutics it is necessary to fully understand their molecular actions.

Both LiCl and VPA, along with other mood stabilizing agents, elicit wide-ranging effects on neural cells, including a shared ability to protect against apoptotic insults (Chuang, 2004). Several investigations have suggested that inhibition of glycogen synthase kinase 3 beta (GSK3β) is a central component to both their mood stabilization and neuroprotective abilities (Bijur and Jope, 2000; Li et al., 2002b). However, other studies have proposed mechanisms centred around p53 modulation (Lu et al., 1999), phosphatidyloinositol signalling (Ding and Greenberg, 2003), histone deacetylase inhibition (Phiel et al., 2001) or endoplasmic reticulum dysfunction (Kim et al., 2005). There is thus a need for further studies to understand how these individual effects combine to produce the known phenotypic effects of these agents.

To begin to understand the molecular mode of action of both LiCl and VPA in neural cells we have used DNA microarray analysis to examine changes in the transcriptome of human SH-SY5Y neuroblastoma cells in response LiCl and VPA. Concentrations used were within the therapeutic range, with patient serum levels range from 0.8 to 2 mmol/L for LiCl (Sproule, 2002) and 0.3 to 0.8 mmol/L for VPA (Allen et al., 2006), with brain concentrations expected to be similar due to the good ability of both compounds to cross the blood-brain barrier. Both agents elicit a marked rise in the expression of the cell differentiation factor Six1, and we demonstrate that this increase is central to the ability of both LiCl and VPA to exert their neuroprotective effect.

Methods and materials

Cell culture. SH-SY5Y cells (ECACC 94030304), a human neuroblastoma cell line, were obtained from ECACC (Porton Down, Wilts, UK) and Bristol University. Cells were routinely cultured in DMEM:F12 supplemented with 10% foetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. All cell culture consumables were purchased from Invitrogen (Paisley,UK).

As indicated, a Six1 expression plasmid, or control plasmid, was transfected into SH-SY5Y cells using FuGENE 6 (Roche Diagnostics, Lewes, UK), along with a GFP expression plasmid (BD Biosciences) to act as an internal control. Cells were then incubated for 48 h before the addition of 1 μM STS (STS) for 16 h.

For siRNA experiments pre-designed Six1 silencer siRNA (Ambion, Warrington, UK) was transfected into cells using the Silencer siRNA transfection system (Ambion) as per manufacturer's recommendations, with two separate siRNAs against Six1 used to ensure specificity of response. In addition, a pre-designed silence siRNA against HCaRG was used to control for off-target effects.

DNA microarray and analysis. SH-SY5Y cells were seeded at 2.4×10^5 cells/cm^2 in 25 cm^2 flasks and allowed to attach overnight. Cells were dosed daily with 1 mM lithium chloride (Sigma Aldrich, Poole, UK), 1 mM sodium valproate (Sigma Aldrich) or medium control. Following 6 or 72 h of exposure, total RNA was isolated from quadruplicate samples using the RNeasy Mini kit (Qiagen, Crawley, UK) and was quantified using a Nanodrop Agilent 2100 Bioanalyser. Medium was changed 6 h prior to the 72 hour time point to control for gene expression elicited by medium shock. In addition, the quadruplicate biological samples were composed of two samples each from the ECACC and Bristol-sourced SH-SY5Y cells; sourcing cells from two different sites controls for site-specific phenotypic changes in cell lines that can potentially obscure the biologically relevant changes.

Samples were used to interrogate the Affymetrix U133A/B GeneChip set, representing over 47,000 transcripts including approximately 38,500 fully annotated probe sets.

The data generated from the microarray analysis was first normalised using the Affymetrix algorithm that allows reliable comparison of multiple arrays, minimising differences of non-biological origin. Next the SAM (Significance Analysis of Microarrays) package (http://www-stat.stanford.edu/~tibs/SAM/) was used to identify significantly altered gene expressions, and generate false discovery rate (FDR) values for the analysis. The SAM package offers the advantage over other microarray analysis tools in that it does not presume equal variance or independence of genes (or both), scenarios that are often violated in biological systems (Tusher et al., 2001). SAM settings were for a two class unpaired analysis, using one hundred permutations to calculate the differentially expressed gene list. No fold-change threshold was set, with instead the delta function manually altered to provide a false discovery rate of approximately 1%. The exception to this is for 72 h exposure to LiCl, where a false discovery rate of 20% was used due to the very low number of differentially expressed transcripts identified when lower FDRs were used. To examine the potential impact on biological pathways of the identified gene expression changes the DAVID software suite (http://david.abcc.ncifcrf.gov/home.jsp) was used to undertake functional annotation clustering, whereby GO identifiers that are statistically over-represented are clustered according to their biological functions (Dennis Jr et al., 2003).

Transcript level measurement. Primers and TAMRA/FAM dual labelled probe specific for Six1, Bcl2, Bcl2L1 and 18S were designed using the Primer Express software (Applied Biosystems, Warrington, UK) and were purchased from MWG (Milton Keynes, UK).

SH-SY5Y cells were seeded at 2.4×10^5 cells/cm^2 in 25 cm^2 flasks and allowed to attach overnight. Cells were dosed daily with either varying concentrations of lithium chloride, sodium valproate or medium control. At specified time points, total RNA was isolated from quadruplicate samples using the RNeasy Mini kit (Qiagen) and was quantified using a Nanodrop Agilent 2100 Bioanalyser.

Total RNA was treated with RNase-free DNase (Promega, Southampton, UK) to remove genomic contamination. Reverse transcription was primed with random hexamers and carried out by Superscript II (Invitrogen) as per the manufacturer's instructions. To ensure that DNase treated samples were free from genomic contamination an RT− control (lacking enzyme) was carried out for every RNA sample. cDNA generated from 50 ng (Six 1) or 50 pg (18S rRNA) of total RNA was amplified using TaqMan Universal PCR Master Mix with 400 nM primers and 200 nM fluorogenic probe in a total reaction volume of 25 μl. Quantitative polymerase chain reaction (Q-PCR) reactions were run on the ABI7000 SDS instrument and quantitation was carried out using the ABI proprietary software against a standard curve generated from human genomic DNA (Promega).

Protein level measurement. SH-SY5Y cells were seeded at 2.4×10^5 cells/cm^2 in 25 cm^2 flasks and allowed to attach overnight. Cells were dosed daily with either varying concentrations of lithium chloride, sodium valproate or medium control. Following 72 h of exposure total protein was extracted in RIPA buffer (1×PBS pH 7.3, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS), with 1× protease inhibitor cocktail solution (Roche, Lewes, Sussex, UK) added just prior to use.

Total SH-SY5Y protein extracts (5 μg per lane) were resolved on 12% SDS-polyacrylamide gels and then transferred electrophoretically to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Bucks, UK). Membranes were blocked (1 h) in 5% fat free dried milk and then probed with primary antibodies against Six1 (sc-9127; 1:400), cylcinA1 (sc-15383; 1:300) or pax3/7 (sc-7748; 1:300)