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Valproic acid exposure leads to upregulation and increased promoter histone acetylation of sepiapterin reductase in a serotonergic cell line

Diana Balasubramanian [a], Alicia X. Deng [a], Kit Doudney [a], Mark B. Hampton [b], Martin A. Kennedy [a, *]

[a] Gene Structure and Function Laboratory and Carney Centre for Pharmacogenomics, Department of Pathology, University of Otago, Christchurch, New Zealand
[b] Centre for Free Radical Research, Department of Pathology, University of Otago, Christchurch, New Zealand

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ABSTRACT

Valproic acid (VPA) is a widely used antiepileptic drug and first-line treatment in bipolar disorder, although the mechanisms underlying its therapeutic effects are largely unknown. Recently, the recognition of VPA as an epigenetic drug offers new opportunities for understanding its therapeutic actions. In a rat serotonergic cell line (RN46A) we observed that VPA exposure has a strong upregulatory effect on the gene for sepiapterin reductase (SPR), a key enzyme involved in the tetrahydrobiopterin (BH4) synthetic pathway. BH4 is an essential cofactor in the biosynthesis of neurotransmitters like serotonin, dopamine and noradrenalin, and the BH4 pathway may thus be important in mood biology. Using real-time quantitative PCR we show that VPA, at therapeutically relevant doses, increases the expression of the *Spr* gene by about 8-fold in RN46A cells. In addition, Spr protein levels in VPA-exposed cells were elevated, as were the intracellular BH4 levels. HDAC inhibitors (HDACI) trichostatin A and sodium butyrate also upregulated *Spr*, but this was not observed using the VPA-analogue valpromide, which lacks HDAC inhibitory activity. Further examination of this effect revealed that exposure to VPA increased the acetylated histone mark H3K9/K14ac at the *Spr* promoter. The DNMT inhibitor 5′aza-dC also upregulated *Spr* by over 8-fold. However, DNA methylation status across the *Spr* promoter did not change in response to VPA. The BH4 pathway is fundamental to the regulation of neurotransmitters relevant to mood disorders, and this epigenetic effect of VPA at the *Spr* promoter may represent a novel mechanism through which VPA achieves its therapeutic action.

1. Introduction

Bipolar disorder is one of the most common and debilitating psychiatric disorders affecting about 2% of the population worldwide (Merikangas et al., 2011). It is characterized by alternating episodes of mild to severe mania and depression and is typically a recurrent illness with a majority of patients relapsing into depression or mania in spite of treatment (Gitlin et al., 1995; Goldberg and Harrow, 2004). Bipolar disorder is associated with high morbidity and mortality with greatly increased suicide rates compared to the general population (Baldessarini et al., 2006; Goodwin et al., 2003). Pharmacological intervention is considered to be the most effective treatment for bipolar disorders, and mood stabilizers such as lithium and VPA are regularly used as the first line of bipolar therapy (Fountoulakis and Vieta, 2008). Other drugs include the antiepileptic drugs (AEDs) carbamazepine (CBZ) and lamotrigine (LTG), antidepressants and antipsychotics.

VPA is a broad spectrum drug and has multiple effects. It is a standard first line treatment for bipolar disorder, especially bipolar mania (Macritchie et al., 2003), as well as epilepsy (Loscher, 2002). In addition, VPA is used in the treatment of various other conditions including migraine (Calabresi et al., 2007; Rodriguez-Sainz et al., 2013) and neuropathic pain (Cutrer and Moskowitz, 1996; Vorobeychik et al., 2011). Recent research is exploring possible therapeutic roles for VPA in cancer therapy, latent HIV infection and Alzheimer's disease (Terbach and Williams, 2009).

Despite these wide ranging uses, the mechanism of VPA action is still unclear. The anticonvulsant effects of VPA are often attributed to inhibition of voltage-gated sodium and calcium channels (Kelly et al., 1990; McLean and Macdonald, 1986) as well as increased

* Corresponding author.
E-mail address: martin.kennedy@otago.ac.nz (M.A. Kennedy).

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GABA levels (Guidotti et al., 2011; Rho and Sankar, 1999). Other well-documented effects include increase in glutamatergic neurotransmission and modulation of multiple signalling pathways including the GSK3beta/Wnt, MAPK/ERK, phosphoinositol and protein kinase B/Akt pathways (Kostrouchova and Kostrouch, 2007; Monti et al., 2009).

However, one of the most intriguing aspects of VPA is that it is an effective histone deacetylase (HDAC) inhibitor capable of inhibiting class I and class II HDACs (Gottlicher et al., 2001; Phiel et al., 2001). The HDAC inhibitory action of VPA has been implicated in its efficacy in bipolar disorders and neuroprotection as well as its teratogenic side effects (Gurvich et al., 2005; Monti et al., 2009).

Tetrahydrobiopterin (BH4) is a reduced pterin molecule and acts as a vital cofactor for the aromatic amino acid hydroxylases, which are in turn essential in the biosynthesis of neurotransmitters like serotonin, dopamine and noradrenalin (Kaufman, 1964; Thony et al., 2000). BH4 is also crucial for nitric oxide synthesis as a cofactor for all the isoforms of nitric oxide synthase (NOS) (Werner et al., 2011). Nitric oxide (NO) is involved in the release of the excitatory neurotransmitter glutamate (Cardenas et al., 2000). BH4 can thus modulate both the monoaminergic and glutamatergic systems. Dysregulation of these neurotransmitter systems has been implicated in the etiology and pathogenesis of several neuropsychiatric disorders including schizophrenia, Parkinson's disease, Alzheimer's disease and depression (Krishnan and Nestler, 2008; Sanacora et al., 2008). In addition, many monoamine neurotransmitter disorders in humans are caused by deficiency of enzymes involved in the BH4 pathway, most notably dopa-responsive dystonia or Segawa disease (Segawa et al., 1976).

The critical role played by BH4 in the pathobiology of psychiatric disorders led to several studies linking altered BH4 levels with psychiatric disorders including schizophrenia, Parkinson's disease and depression (Foxton et al., 2007; Hashimoto et al., 1994; Richardson et al., 2007). However, very few studies have examined the role of the BH4 pathway genes in psychiatric disorders. We previously showed that after exposure to the antidepressant paroxetine, neural cells derived from mouse embryonic stem cells showed increased levels of the BH4 biosynthetic enzyme sepiapterin reductase (Spr) (McHugh et al., 2008). We also observed association between promoter variants of the *Spr* gene and susceptibility to mood disorders as well as to antidepressant treatment response (McHugh et al., 2009). A similar analysis of promoter variants for another enzyme in the BH4 biosynthetic pathway, GTP-cyclohydrolase I feedback regulator (GFRP), also suggested an association with antidepressant responses (McHugh et al., 2011).

In this paper we set out to evaluate the effects of VPA and other drugs used for treatment of mood disorders on regulation of the BH4 synthetic pathway. Our primary hypotheses were (i) that these drugs would modify expression of the BH4 pathway gene *Spr*, and (ii) that this would be mediated by epigenetic modifications. Therefore we carried out gene and protein expression analyses in the serotonergic precursor cell line RN46A, and examined the epigenetic effects of VPA at this locus.

2. Methods

2.1. Cell culture and drug treatment

RN46A cells (White et al., 1994) were cultured in Dulbecco's Modified Eagle Medium (DMEM/F12 with GlutaMAX™-I) and supplemented with 5% fetal bovine serum and 250 μg/mL Geneticin® (Life Technologies, Carlsbad, CA). For dose–response experiments, cells were seeded in 6-well plates (3.5 cm^2) at a concentration of 3.5×10^4 cells per well, and incubated overnight. Cells were then treated with different concentrations of the drugs or with vehicle for 72 h. All the drugs used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA).

To assess possible drug effects on cell survival, cell cultures were initially exposed to a range of concentrations of each drug for 72 h, and cell numbers were counted using the Trypan blue exclusion method. Numbers were compared between drug exposed and control cultures to determine potential drug toxicity on RN46A cells, and drug concentrations used in this study had no discernable effects on cell survival (Supplementary Table S1).

Stock solutions for 5'aza-dC (100 μM), trichostatin A (TSA) (330 μM), lamotrigine (LTG) (3.9 mM) and valpromide (VPD) (1.10 M) were prepared in 100% DMSO and stored at −80 °C. VPA, sodium butyrate and lithium chloride were readily soluble in water and hence the stock solutions were prepared in ultrapure Milli-Q® (MQ) water (Millipore, MA, USA) and stored at 4 °C. Stock solution of carbamazepine (CBZ) (29.5 mM) was made by dissolving in 2-hydroxypropyl-beta-cyclodextrin (HBC) with heating at 65 °C for 4 h. Various concentrations were then prepared by diluting the stock in culture medium. For drugs dissolved in DMSO, the final concentration of DMSO in the culture medium never exceeded 0.05%. Control (untreated) cells were cultured in 0.05% DMSO. All drug solutions were filter sterilized prior to use.

2.2. RNA extraction and quantitative real time PCR

Total RNA was isolated using Trizol® LS Reagent (Invitrogen, Carlsbad, CA). To prepare cDNA, 1 μg of total RNA was first treated with DNase-I (Invitrogen, Carlsbad, CA) at room temperature for 15 min. The reaction was stopped by adding EDTA and incubating at 65 °C for 20 min. cDNA synthesis was then carried out using Superscript™ III first strand synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The reaction contained ~1 μg RNA, 1 μL of oligo$(dT)_{20}$, 1 μL random hexamers, 1 μL of dNTP mix, 4 μL 5X first-strand buffer, 1 μL of 0.1 M DTT, 1 μL RNaseOUT™ and 1 μL of reverse transcriptase, (Superscript™ III) and deionised water to make up the volume to 20 μL.

Quantitative real time PCR (qPCR) was performed on the LightCycler® 480 System (Roche Applied Science, Mannheim, Germany). The Universal Probe Library (UPL) System (Roche Applied Science) was used for relative quantification assay and primers for each candidate gene were designed using UPL assay design centre (http://www.roche-applied-science.com/shop/products/universal-probelibrary-system-assay-design). All qPCRs were run in triplicate and gene expression differences were measured using normalization to three reference genes (*Actb, Rnf4, G6pd*). NormFinder, an Excel-based algorithm was used to evaluate the stability of the reference genes (Andersen et al., 2004). A calibrator cDNA sample, which remained constant throughout the course of the experiments, was used in each run to normalize for inter-run variation. The primers and UPL probes used in qPCR are listed in Supplementary Table S2.

2.3. Western blot

RN46A cells grown in 6-well culture plates for 72 h were extracted by trypsinization using TrypLE™ (Life Technologies, Carlsbad, CA, USA). Cells from three wells were pooled together after trypsinization and the cell pellet was washed in ice cold PBS. For cell lysis, 500 μL RIPA buffer containing protease inhibitor cocktail (Complete Mini, Roche Applied Science, Mannheim, Germany) was added to the cell pellet in 1.5 mL centrifuge tubes and the tubes were incubated on ice for 30–60 min with intermittent vortexing. The total protein concentration of all samples was

determined using Bio-Rad DC Protein Assay Kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Bovine serum albumin (BSA) was used to create standard curves for the assay.

Separation of proteins was carried out using SDS-PAGE in a Mini-PROTEAN® Tetra Cell apparatus (Bio-Rad, Hercules, CA) with a 12% separating gel and 7% stacking gel. Electrophoresis was carried out at a constant voltage of 180 V for about 1.5 h.

After SDS-PAGE, the separated proteins were transferred onto a polyvinylidene fluoride membrane (Amersham Hybond-P, GE Healthcare) by electroblotting. The membrane was incubated in 2% BSA for 1.5 h before incubating in the primary antibody solution overnight at 4 °C with gentle shaking. The primary mouse polyclonal anti-SPR antibody (Abnova, Taipei City, Taiwan) was diluted 1:3000 in TBST containing 1% BSA, while primary β-Actin primary mouse monoclonal antibody (Abcam, Cambridge, MA, USA) was diluted at 1:30,000. The membrane was washed in TBST containing 0.1% Tween 20 four times for about 10 min each. The secondary antibody used was goat anti-mouse IgG (H&L) (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) and was diluted in TBST (with 1% BSA) at 1:20,000 dilution. The western blots were visualized using the Amersham ECL Prime Western blotting detection reagent (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

2.4. Measurement of BH4 in RN46A cells using competitive ELISA

RN46A cells were grown in 6-well culture plates for 72 h, then extracted by trypsinization and lysed as described above in section 2.3. The cell lysate was then used for ELISA. Competitive ELISA was carried out using the Rat tetrahydrobiopterin ELISA Kit (EIAAB Science Co Ltd, Wuhan, China). ELISA plates precoated with BH4-specific monoclonal antibody were used to incubate the samples (cell lysates) and test standards along with fixed amount of biotin-labelled BH4. Following this, excess sample and reagents were washed off the plate, and avidin conjugated to horseradish peroxidase (HRP) was added to each well and incubated again. The 3,3′,5,5′-Tetramethylbenzidine substrate was then added to each well followed by incubation for a further 10 min. The enzyme–substrate reaction was terminated by adding a strong acid and the product was measured spectrophotometrically at 450 nm. The concentration of BH4 in each sample was then calculated by comparing the optical density to a standard curve.

2.5. Methylation analysis of Spr promoter by direct bisulfite sequencing

For DNA extraction, the cells were first trypsinized and centrifuged at 3000*g* for 5 min. The pellet was washed with PBS and centrifuged at 13,000*g* for 2 min. The supernatant was removed and DNA was extracted from the cell pellet using the Wizard™ Genomic DNA extraction kit (Promega Corporation, Madison, WI, USA). Bisulfite conversion of genomic DNA was carried out using the EZ DNA Methylation-Gold™ Kit, (Zymo Research, CA, USA) according to the manufacturer's instructions. For PCR amplification of bisulfite-converted DNA, primers were designed such that they did not contain any CpG dinucleotide for unbiased amplification. This was done using the MethPrimer program (Li and Dahiya, 2002). Primer sequences and amplicon sizes are listed in Supplementary Table S2. PCR was performed in a 50 μL reaction volume containing 1X high fidelity PCR buffer (Invitrogen), 200 μM dNTP mix, 3 mM $MgSO_4$, 0.3 μM each of forward and reverse primers, 2 μL of bisulfite converted DNA, and 1U of Platinum® Taq (Invitrogen) DNA Polymerase High Fidelity (5U/μL). PCR cycling conditions involved an initial denaturation at 94 °C for 1 min followed by a touch-down PCR, 94 °C for 15 s, with annealing temperatures ranging from 65 to 50 °C at (1 °C per cycle) for 15 s (15 cycles) and extension at 68 °C for 30 s. This was followed by another 20 cycles of 94 °C for 15 s, annealing at 50 °C for 15 s and extension at 68 °C for 30 s and a final extension at 68 °C for 5 min. PCR products were purified and methylation patterns detected by direct Sanger sequencing on an AB13730 Genetic analyzer using the BigDye™ Terminator Version 3.1. Sequences were analysed using Geneious software version 5.5.3 (Biomatters, Auckland, New Zealand).

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the Histone ChIP Kit (Diagenode, Liège, Belgium) following the manufacturer's instructions. Briefly, RN46A cells were grown in T75 flasks in 24 mL DMEM/F12 medium for 72 h. The cells were first fixed and chromatin was sheared by sonication (Omni-Rupter 4000, Omni International, GA, USA) followed by centrifugation. The resulting supernatant contained the sheared chromatin and was used for ChIP. ChIP was performed using antibodies for the following histone marks: H3K9/K14ac, H3K4me3 and H4K8ac. Rabbit anti-IgG antibody was used as a negative control. A "no antibody" control was also included for ChIP. Following immunoprecipitation, DNA was purified by standard phenol/chloroform purification method after reversing the crosslinking. All antibodies used in ChIP are listed in Supplementary Table S3.

2.6.1. ChIP-qPCR

The immunoprecipitated DNA was analysed by qPCR using primer sets for rat *Spr*, *Actb* (which represented a positive control locus for the histone marks studied) and a region at the 3′-end of *Spr* (representing a negative control locus). ChIP-qPCR analysis was carried out with the Roche LightCycler® 480 (Roche Applied Science) using the "percentage of input" method. qPCR was carried out in a 10 μL reaction volume containing 2.5 μL immunoprecipitated DNA, 1 μL of 10X Roche buffer with Mg^{2+}, 1 μL 2 mM dNTPs, 0.5 μL of 10 μM primers, 0.15 μL of Taq polymerase (U/μL), and 0.35 μL Syto 9 green fluorescent nucleic acid stain (Invitrogen, Eugene, OR, USA) and deionized water. Reactions were performed in duplicate. No template control (NTC) and no-antibody controls along with the input DNA samples were used for every primer pair in qPCR. All the genes amplified by qPCR were normalized for their amplification efficiency by standard curves using serial dilutions of input DNA. Before calculation of the percentage of input for each sample, the Cq of the input was first adjusted to 100% as only 1% of the starting chromatin was used as input. To adjust the input, a dilution factor of 100 (representing 6.644 cycles, which is $\log_2$ of 100) was subtracted from the Cq values of the input. Following this the percentage of input was calculated by $100 \times E^{(Cq \text{ of adjusted input} - Cq \text{ of immunoprecipitated DNA})}$, where E represents amplification efficiency of each gene.

Primers were designed across a 600 base pair (600 bp) region at the promoter region of genes using Geneious software version 5.5.3 (Biomatters, Auckland, New Zealand). Primer sequences used for ChIP-qPCR are listed in Supplementary Table S2.

2.7. Statistical analysis

Statistical analysis of qPCR data was carried out using SigmaPlot™ version 12.5 (Systat Software, Inc. San Jose, CA, USA) data analysis tool for one-way analysis of variance (ANOVA). Multiple testing correction was done using Holm-Sidak multiple comparison method. Data is expressed as mean ± standard error of mean (SE) for at least three independent experiments. Fold difference in expression of *Spr* or other genes between drug-treated and untreated cells were assessed employing a significance level of $p < 0.05$.

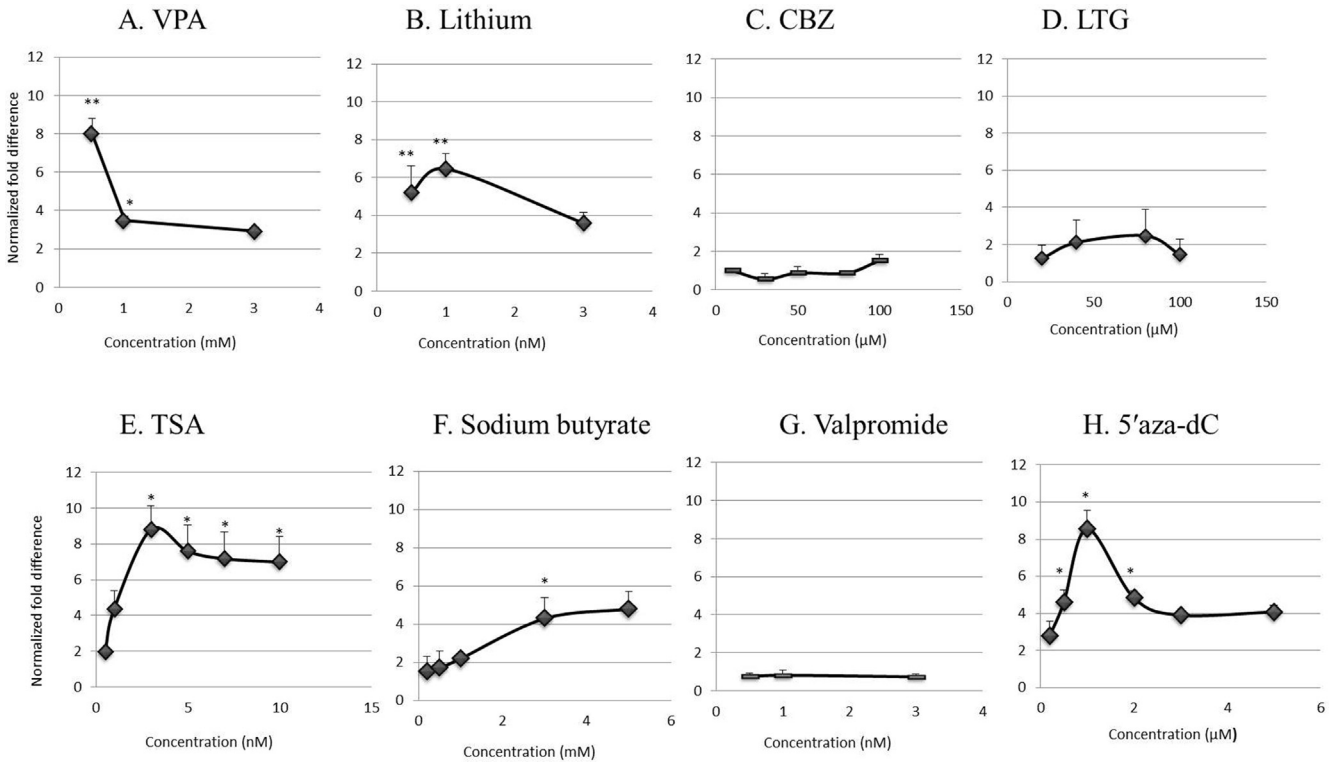

Fig. 1. *Spr* expression in response to multiple compounds in RN46A cells. *Spr* expression was measured in response to 72 h exposure to (A) 0.5–3 mM VPA; (B) 0.5–3 mM lithium; (C) 10–100 μM CBZ; (D) 20–100 μM LTG; (E) 0.5–10 nM TSA; (F) 0.2–5 mM sodium butyrate; (G) 0.5–3 mM VPD; (H) 0.2–5 μM 5′-aza-dC. Data is presented as fold-change after normalization to untreated control cells, using the mean of 3–5 independent experiments (error bars represent SE). Statistical analysis was carried out with raw data prior to this normalization. Each dose was compared to the untreated control cells across the five independent experiments using one-way repeated measures analysis of variance (ANOVA). $^{**}p < 0.005$; $^{*}p < 0.05$.

ChIP-qPCR data, western blot densitometry assay and ELISA were statistically analysed by two-tailed paired Student's t-test ($p < 0.05$) using Microsoft Office Excel version 2007. Data are expressed as the mean of three independent experiments ± SE.

3. Results

3.1. VPA increases Spr mRNA expression and protein levels in cultured RN46A cells

We carried out dose–response experiments in RN46A cells, examining the effect of VPA (0.5–3 mM) on *Spr* expression at 72 h using concentrations that spanned the known human therapeutic plasma concentrations (Chiu et al., 2013; Devulder, 2006; Johannessen et al., 2003; Lusznat et al., 1988) and extending to the maximum concentration that was not cytotoxic to the cells (established as explained in Section 2.1). A significant increase in *Spr* mRNA expression was observed for VPA (0.5–1 mM) with maximal expression at 0.5 mM (~8-fold, $p < 0.005$) (Fig. 1 A). To ascertain whether this effect of VPA on *Spr* expression was specific to this serotonergic cell line, or occurred more widely in other types of neural cells, we extended this work into two other rat neural cell lines. These were the glioma cell line C6 and the hippocampal cell line H19-7. *Spr* upregulation was also seen in the C6 cells, albeit at a higher concentration of 2 mM (Supplementary Figure S1). VPA at the same doses had little or no effect in the other neuronal cell line, H19-7. The fact that VPA did not cause any upregulation of *Spr* in the hippocampal cell line suggests specificity for certain neuronal cells.

3.2. Effect of mood stabilizers lithium, carbamazepine and lamotrigine on Spr expression

We then exposed RN46A cells to other commonly prescribed mood stabilizer drugs including the widely used drug lithium (Fig. 1B). We observed that lithium treatment for 72 h resulted in a similarly significant induction of *Spr* expression at therapeutic doses (0.5–3 mM). Maximum induction was observed at 1 mM (6-fold, $p < 0.001$).

Other drugs tested were CBZ and LTG, which are both antiepileptic drugs used as mood stabilizers. Dose-response experiments using LTG (20–100 μM) and CBZ (10–100 μM) did not result in any significant change in *Spr* mRNA expression (Fig. 1C and D).

3.3. Spr is upregulated by TSA and 5′aza-deoxycytidine but not valpromide

Given the strong upregulation of *Spr* caused by VPA, which is a known HDACI, two other HDACIs, TSA and sodium butyrate, were also tested for their effects on *Spr* expression in RN46A cells. The dose range chosen for each drug (TSA, 0.5–10 nM; sodium butyrate, 0.2–5 mM) was empirically determined, and extended up to the maximum *in vitro* tolerated dose for these drugs in RN46A cells. TSA caused significantly increased *Spr* expression at most concentrations studied, with 3 nM showing the highest increase. Sodium butyrate, on the other hand, showed some suggestion of upregulation particularly at 1 mM, but this was much less marked than observed for TSA (Fig. 1E and F).

Valpromide (VPD) is an amide derivative of VPA which lacks HDAC inhibitory activity (Eyal et al., 2005; Phiel et al., 2001). In our experiments VPD was used at a dose range similar to that of VPA

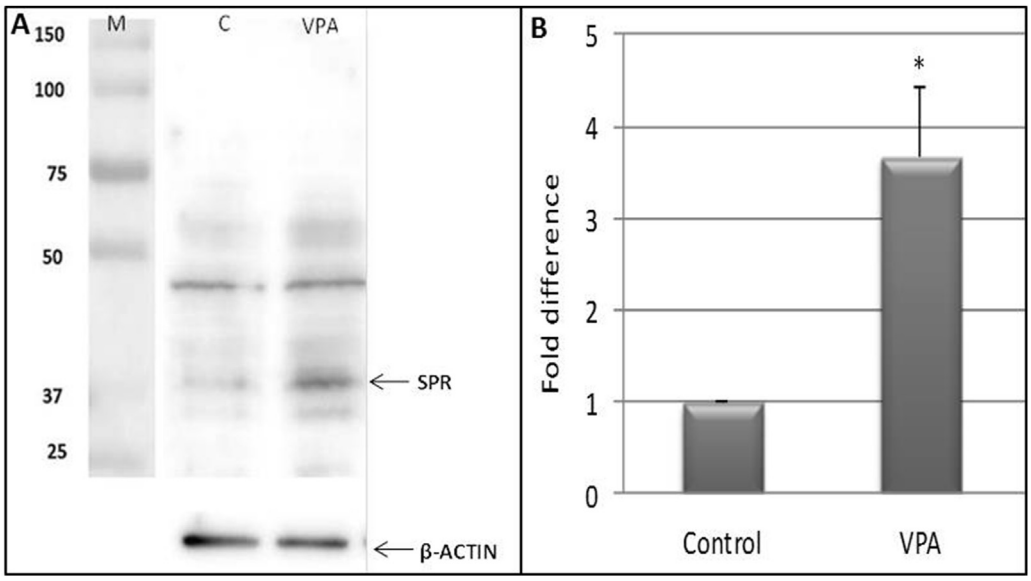

Fig. 2. Western blot of SPR protein after VPA treatment in RN46A cells. (A). Representative image of a western blot of RN46A cells treated with 0.5 mM VPA for 72 h or left untreated for 72 h. Proteins were extracted and run on SDS-PAGE under reducing conditions. An anti-SPR antibody was used to detect SPR protein. β-Actin was used as a loading control. M – protein size marker (numbers indicate sizes in kDa), C – untreated cells, VPA – cells treated with 0.5 mM VPA. (B). Densitometric analysis for quantification of bands showing fold-change normalized to untreated cells. Data represent mean of three independent experiments. Error bars represent SE. Statistical analysis was done with paired Student's t-test using raw data prior to normalization to the untreated control cells. *$p < 0.05$.

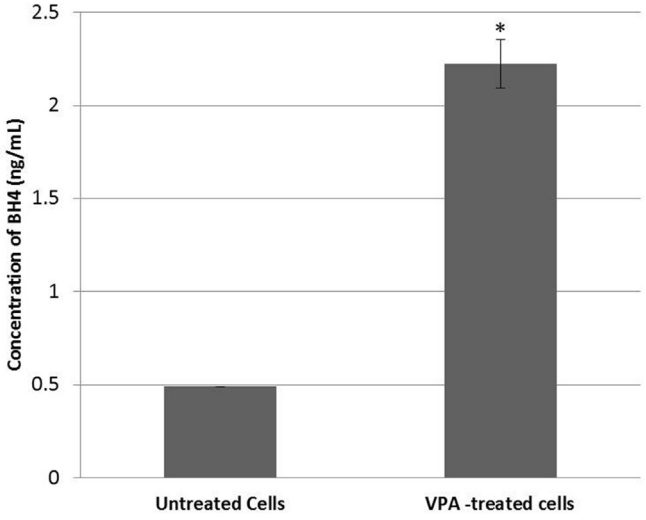

Fig. 3. Competitive ELISA for the BH4 immunoreactivity in RN46A cells treated with 0.5 mM VPA vs untreated cells. Data represent mean of three independent experiments. Error bars represent SE. *$p < 0.05$ (paired t-test).

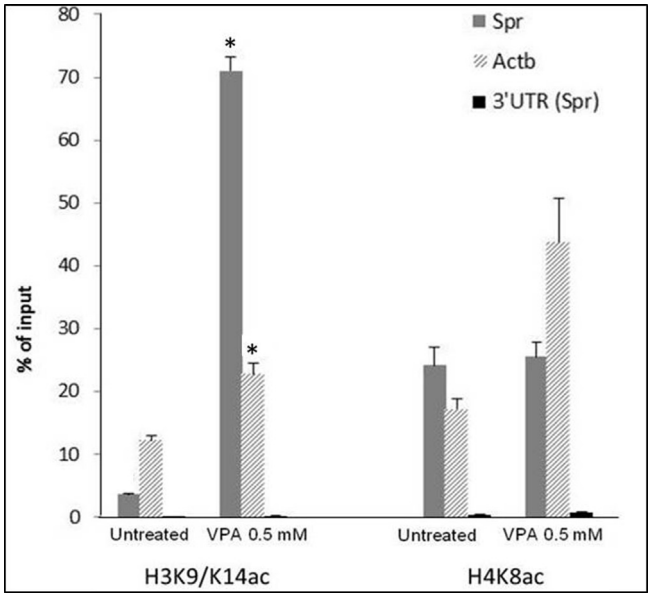

Fig. 4. ChIP-qPCR analysis of *Spr* in RN46A cells exposed to 0.5 mM VPA for 72 h. ChIP was performed using antibodies to acetylated histone marks H3K9/K14ac and H4K8ac. Results are presented as enrichment levels normalized to percentage of input DNA. Rabbit anti-IgG antibody was used as a negative control antibody in ChIP and the enrichment for IgG at all the loci was <0.04 (data not shown). *Actb* and 3′-UTR of *Spr* represent positive and negative control loci, respectively, for the two histone marks studied. Data represent mean of three independent experiments. Error bars indicate SE and * $p < 0.01$ (two-tailed t-test).

(0.5–3 mM), and it was apparent that VPD at these doses did not have any significant effect on *Spr* expression (Fig. 1G).

In addition, dose–response analysis using the DNA methylation inhibitor 5′aza-deoxycytidine also showed a significant increase in *Spr* expression with maximal increase at 1 μM (Fig. 1H).

3.4. VPA increases Spr protein expression in RN46A cells

To determine whether the gene expression change correlated with increased Spr protein in RN46A cells, we carried out western blot analysis. As shown in Fig. 2A and B, a significant difference was observed between untreated cells and cells exposed to VPA for 72 h.

3.5. VPA increases levels of BH4 in RN46A cells

A competitive ELISA was used to measure intracellular BH4 levels in RN46A cells treated with 0.5 mM VPA for 72 h, compared to untreated cells. As shown in Fig. 3, a significant increase in intracellular BH4 concentration (from about 0.5 ng/mL to over 2 ng/mL) was observed in cells treated with VPA ($p < 0.01$).

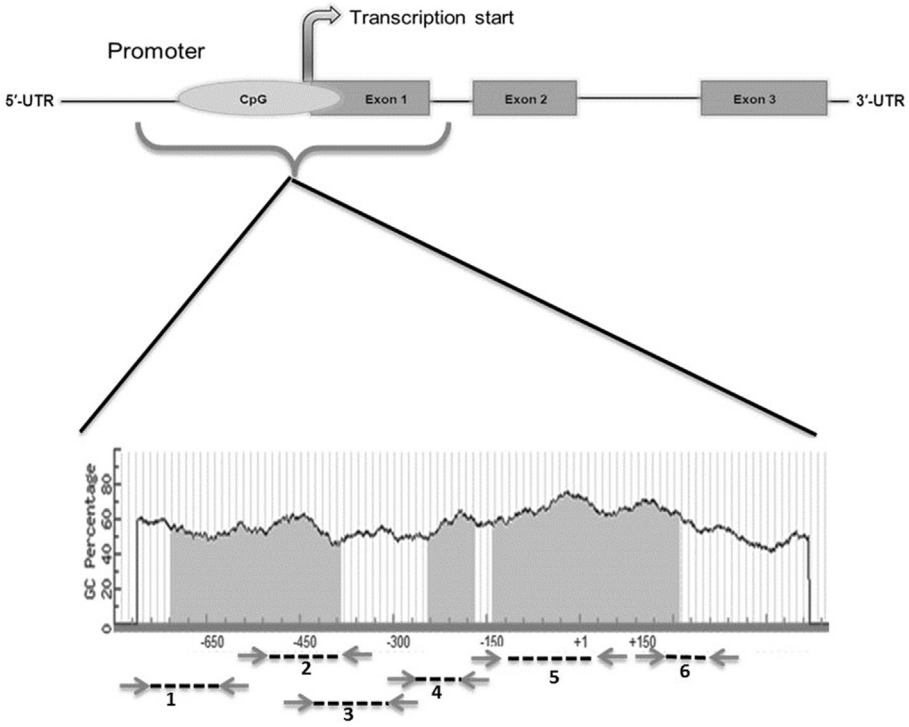

Fig. 5. Location of bisulfite sequencing amplicons. Modified output of MethPrimer program showing the Rat *Spr* proximal promoter region and the location of the PCR primer sets. (1–6 indicates the six amplicons studied). Coordinates shown are in relation to the translation initiation site (+1). Arrows indicate the different primer pairs. CpG rich regions (GC content of 68%) are shaded. Genomic coordinates: rn5_Spr refGene_NM_019181 range: chr4:181493032–181494323.

3.6. VPA increases acetylation at H3K9/K14ac histone mark in the Spr promoter region

Because of the known HDAC inhibition properties of VPA, we tested whether upregulation of *Spr* correlated with increased histone acetylation at *Spr* promoter, using chromatin immunoprecipitation (ChIP). VPA treatment resulted in significantly increased acetylation at the H3K9/K14ac histone mark in the *Spr* promoter (Fig. 4). Although the basal levels of H3K9/K14ac were higher for the *Actb* promoter than *Spr,* VPA treatment caused only a minor increase in acetylation at this mark relative to *Spr*. Differences in the levels of H4K8ac before and after VPA treatment were not significant. The negative control region, which was an amplicon from the 3′-UTR of *Spr,* showed comparatively very little enrichment for both the histone marks studied. The non-specific antibody used as negative control for the immunoprecipitation also showed almost no enrichment for the histone marks, indicating that minimal background signals were generated during the ChIP procedure (Fig. 4). The histone mark H3K4me3 was not detectable by qPCR for any of the loci analysed in this study.

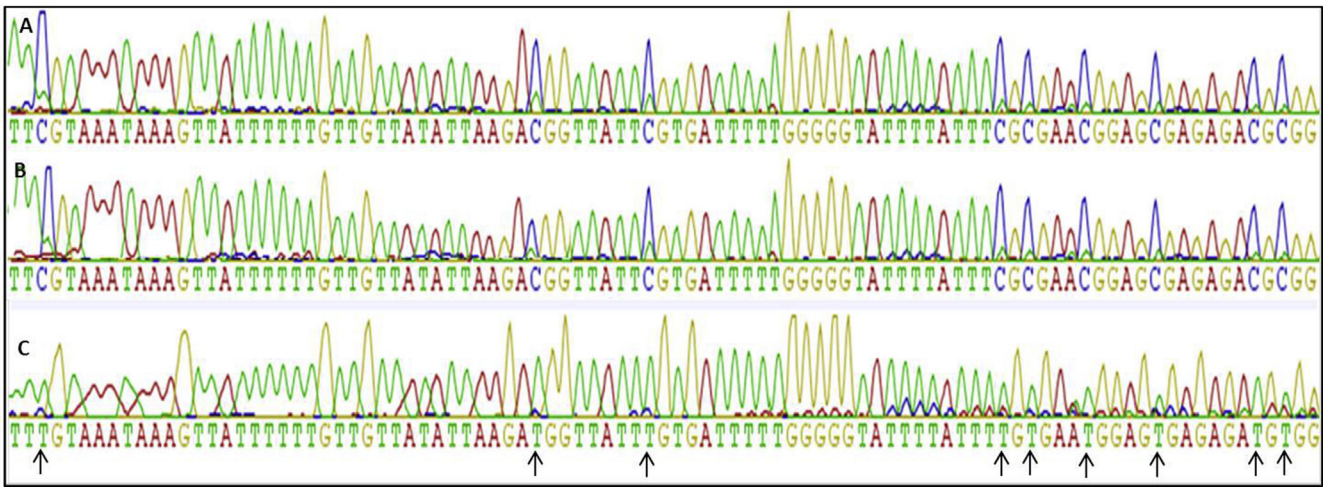

Fig. 6. Effect of VPA and 5′aza-dC on methylation at the *Spr* promoter region. Alignment of a part of the sequenced Spr promoter region from RN46A cells before (A) and after 72 h treatment with 0.5 mM VPA (B) and 1 μM 5′aza-dC (C). Note the C-T change of the CpG dinucleotide (arrows) only after treatment with 5′aza-dC (C), indicating demethylation at these cytosine residues.

3.7. Methylation status of Spr promoter in RN46A cell line

Because of the HDAC inhibitory activity of VPA and relationships between histone modification and genomic methylation (Jones et al., 1998; Lorincz et al., 2001; Nan et al., 1998; Nguyen et al., 2001), we examined CpG methylation in the *Spr* promoter. Analysis of the *Spr* promoter region suggested a 362 bp CpG island upstream of the translation start site with over 68% GC content, containing 36 CpGs (UCSC Genome Browser Rat, March 2012 (RGSC 5.0/rn5)). However, the MethPrimer program used to design the bisulfite-PCR primers identified a larger ~800 bp CpG island region spanning three CG-rich regions (Fig. 5). Primers were thus designed to cover ~1100 bp including ~600 bp upstream and ~500 bp downstream of the transcription start site, and extending into the first intron of *Spr*. The region included 70 individual CpG dinucleotides.

Bisulfite sequencing of the *Spr* promoter showed that it is poorly methylated, as out of 70 CpGs in the region studied only nine were found to be methylated, which were all in a single amplicon (amplicon 1, Fig. 6). Following VPA treatment, no change in the methylation status of these nine CpGs was observed. However, treatment of RN46A cells with 1 μM 5′aza-dC resulted in clear demethylation of the cytosine residues at these CpGs, visualized by C-T changes between untreated and 5′aza-dC-treated cells (Fig. 6C).

4. Discussion

4.1. Choice of cellular model

Dysregulation of the serotonergic system has long been implicated in mood disorders (Cryan and Leonard, 2000; Deneris and Wyler, 2012; Jans et al., 2007). Serotonin in the brain is released from serotonergic neurons originating from the dorsal and median raphe nuclei in the mid brain (Jankowski and Sesack, 2004; Michelsen et al., 2008). For this work we used the RN46A cell line, which was derived from the 13 day embryonic rat medullary raphe nucleus using retroviral transduction (White et al., 1994). Unlike many other neuronal cell lines derived by retroviral transduction which are pluripotent and can differentiate into neuronal or glial cells, RN46A is neuronally restricted with no glial antigens expressed under any conditions (White et al., 1994). Upon differentiation these cells exhibit a complete serotonergic nature, abundantly expressing all the 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C}) as well as the serotonin transporter protein, SLC6A4. In this study undifferentiated RN46A cells were used because differentiation of the cells occurs over a course of eight days (Whittemore and White, 1993) and stably maintaining the differentiated cells for the periods required for drug exposure experiments was found to be difficult. Although undifferentiated RN46A cells do not produce serotonin, they still maintain the essentially serotonergic nature of the cell line and exhibit many of the serotonergic characters including expression of both serotonin transporter and the high-affinity serotonin receptor, 5HT-1A, as well as low levels of tryptophan hydroxylase (Eaton et al., 1995; Whittemore et al., 1995).

4.2. Increased Spr expression

BH4 is an essential cofactor in the synthesis of major neurotransmitters such as noradrenaline and serotonin. In the present study we have focussed on the regulation of the gene for sepiapterin reductase, an essential enzyme for the biosynthesis and intracellular maintenance of BH4. Dose-response experiments showed that VPA (0.5–3 mM) caused a significant upregulation of *Spr* in undifferentiated RN46A cells.

The widely used mood stabilizer drug lithium also upregulated *Spr* at a similar concentration range. It was interesting that both these drugs modulated *Spr* expression at therapeutically relevant concentrations (Fleming and Chetty, 2006; Squassina et al., 2010). Due to the well-known HDACI effects of VPA, we also tested two other HDAC inhibitors for their effects on *Spr*. While sodium butyrate is a class I HDACI structurally similar to VPA, TSA is more potent and broad spectrum, inhibiting both class I and II HDACs and belonging to a structurally different class to VPA (Dokmanovic et al., 2007; Grayson et al., 2010). As a control in these experiments, VPD, which is an amide derivative of VPA lacking HDACI activity (Eyal et al., 2005; Phiel et al., 2001), was also analysed. The HDAC potency of sodium butyrate is comparable to VPA with several reports showing that it is only slightly less (Backliwal et al., 2008; Kuwajima et al., 2007) or more potent than VPA (Gurvich et al., 2004; Singh et al., 2014). In this study it was less effective in *Spr* upregulation than VPA. TSA, on the other hand, is acknowledged as a potent inhibitor of HDAC and resulted in a strong and consistent upregulation of *Spr*. Taken together, the lack of upregulation with VPD in the context of significant upregulation by VPA and other HDAC inhibitors, supports the view that HDAC inhibition might be a mechanism involved in *Spr* regulation.

4.3. Intracellular BH4 levels in RN46A cells

Our study showed that increased *Spr* expression directly correlates with not just Spr protein but also the intracellular BH4 levels in RN46A cells exposed to VPA. Using competitive ELISA, we found that intracellular BH4 levels significantly increased in RN46A cells exposed to VPA compared to control cells. This study provides insights into the regulation of BH4 levels by a different enzyme in the BH4 biosynthesis pathway to GCH1, the rate limiting enzyme of this pathway (Werner et al., 2011). Previous studies have shown Gch1 levels correlate with BH4 levels in various model systems (Tatham et al., 2009; Tegeder et al., 2006). Our qPCR data showed that *Gch1* expression in these cells after VPA exposure was 1.26-fold at $p < 0.05$. Taken together, these results suggest a potentially significant role for Spr in regulating BH4 levels in response to the mood stabilizer VPA, independent of Gch1, in this cell culture model system.

4.4. Chromatin modification at Spr promoter

Several recent studies using primary tissues and cell lines have shown that VPA and other HDACIs induce substantial increases in global and gene specific histone acetylation (Larsson et al., 2012; Nightingale et al., 2007; Perisic et al., 2010; Yu et al., 2009). Moreover, clinical studies in bipolar and schizophrenia patients also showed increased acetylation of pan H3 as well as H3K9/K14 histone marks (Gavin et al., 2009; Sharma et al., 2006). However, few studies have analysed effects of VPA on specific histone modifications at promoters of genes relevant to neuropsychiatric disorders (Leng and Chuang, 2006; Tang et al., 2011; Tremolizzo et al., 2002).

We specifically examined histone modifications in the *Spr* promoter region using RN46A cells exposed to VPA. ChIP-qPCR was used to measure the levels of acetylated histone marks H3K9/K14-ac and H4K8-ac, both known to be associated with transcriptionally active chromatin. A single antibody (H3K9/K14ac) was used for analysing the H3K9 and K14ac. These histone marks are known to co-occur at promoters and often analysed together in ChIP analyses (Karmodiya et al., 2012; Roh et al., 2005). In addition, we also attempted to measure a methylated histone mark H3K4me3, which is known to be enriched at the promoter regions of active genes and shown to be modified by HDACIs (Heintzman et al., 2007; Santos-

Rosa et al., 2002). This analysis showed significant enrichment at H3K9/K14ac at the *Spr* promoter, suggesting a role for the HDAC inhibitory activity of VPA in *Spr* upregulation. H4K8ac was not found to be significantly enriched, and H3K4me3 was not detectable in these experiments.

4.5. Lack of involvement of DNA methylation

Because histone acetylation and gene expression can also be modified by cytosine methylation in DNA, we examined the effect of VPA on the underlying pattern of CpG methylation in the *Spr* promoter region. Analysis of the *Spr* promoter region showed it was poorly methylated; only nine out of the 70 CpGs analysed over a ~1100 bp region were found to be methylated. This is not unexpected for gene promoters associated with CpG islands which are generally known to lack methylation (Deaton and Bird, 2011; Weber et al., 2007). The cluster of nine methylated CpGs that was observed ~600 bp from the transcription start site was found to be part of a long terminal repeat region (LTR) present in the rat *Spr* promoter region. This LTR does not appear to be conserved in humans or mouse. Retrotransposons in mammalian DNA are usually heavily methylated (Rowe and Trono, 2011) and this may explain the occurrence of methylation within this part of the *Spr* upstream promoter region.

DNA demethylation by VPA has been observed at other promoters both *in vivo* (Dong et al., 2005; Tremolizzo et al., 2002) and *in vitro* (Manev and Uz, 2002; Mitchell et al., 2005). In our study, VPA exposure did not result in changes to the methylation status for any of the analysed CpGs. Interestingly, 5′aza-dC (which induced significant *Spr* upregulation), did induce demethylation at a cluster of CpGs in the *Spr* promoter region. (Fig. 6). This indicates the potential for methylation changes to play a role in *Spr* induction, but such changes were not evidently part of the mechanism of action of VPA on *Spr*. Several other studies involving exposure of cells to 5′aza-dC or VPA have shown that promoter demethylation is not necessarily observed in association with induction of gene expression by these drugs (Mossman et al., 2010; Soengas et al., 2001; Zhang et al., 2004; Zhu et al., 2001).

5. Conclusions

The present study demonstrated that the mood stabilizer drug VPA significantly upregulated expression of sepiapterin reductase RNA and protein in the raphe nucleus-derived RN46A cell line. This expression change correlated with increased intracellular levels of BH4 in cells exposed to VPA. We also provide evidence for the involvement of the HDAC inhibitory activity of VPA in this upregulation, through alteration of histone acetylation state at the *Spr* promoter. Furthermore, we observed that lithium, but not LTG or CBZ, had similar effects on expression of the *Spr* gene and protein. It was intriguing that both lithium and VPA had similar effects on this gene. Because sepiapterin reductase is involved in the biosynthesis of BH4, a critical co-factor for synthesis of neurotransmitters such as serotonin and dopamine, regulation by VPA and lithium may represent a common, potentially novel, therapeutic target of both these important drugs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2015.06.018.

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