Neurorestoration Induced by the HDAC Inhibitor Sodium Valproate in the Lactacystin Model of Parkinson’s is Associated with Histone Acetylation and Upregulation of Neurotrophic Factors

Short Running Title: Valproate Neurorestoration in Parkinsonian Rats

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Abstract

Background and Purpose: Histone hypoacetylation is associated with Parkinson’s disease, due possibly to an imbalance between the activities of enzymes responsible for histone (de)acetylation; correction of which may be neuroprotective/neurorestorative. This hypothesis was tested using the anti-epileptic drug sodium valproate, a known histone deacetylase inhibitor, utilising a delayed-start study design in the lactacystin rat model of Parkinson’s disease.

Experimental Approach: 10µg of the irreversible proteasome inhibitor lactacystin was stereotaxically injected into the left substantia nigra of Sprague-Dawley rats which subsequently received valproate for 28 days starting 7 days after lactacystin lesioning. Animals were assessed by a combined phenotyping approach, of motor behavioural testing, clinically comparable longitudinal structural MRI, and post-mortem assessment of the integrity of the rat brain nigrostriatal system to track changes in this model of Parkinson’s disease and quantify neuroprotection/restoration. Subsequent cellular and molecular analyses were performed to elucidate the mechanisms underlying valproate’s effects.

Key Results: Despite producing a distinct pattern of structural re-modelling both in the healthy and lactacystin lesioned brain, delayed start valproate administration induced dose-dependent neuroprotection/restoration against lactacystin neurotoxicity, characterised by motor deficit alleviation, attenuation of morphological brain changes detected using MRI, and protection and restoration of dopaminergic neurons in the substantia nigra. Moreover molecular analyses revealed that valproate alleviated histone hypoacetylation induced by lactacystin and induced up-regulation of brain neurotrophic/neuroprotective factors.

Conclusions and Implications: These results suggest that histone acetylation and up-regulation of neurotrophic and neuroprotective factors associated with valproate treatment culminate in a neuroprotective and neurorestorative phenotype in this validated animal model of Parkinson’s disease. Given the structural re-modelling in the brain induced by valproate these results do not suggest that valproate is a viable candidate for the disease but provide proof-of-principle that histone deacetylase inhibitors could hold therapeutic potential as disease modifying agents in Parkinson’s disease.

Abbreviations

\(\alpha\)Syn \(\alpha\)-synuclein
6-OHDA 6-hydroxydopamine
AcH3-Lys9 histone protein H3 acetylated on lysine 9
BDNF brain derived neurotrophic factor
GDNF glial derived neurotrophic factor
HATs histone acetyltransferases
HDACIs histone deacetylase inhibitors
HDACs histone deacetylases
HSP70 heat shock protein 70
i.p. intraperitoneal
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR magnetic resonance
PD Parkinson’s disease
qRT-PCR quantitative real time polymerase chain reaction
SNpc substantia nigra pars compacta
TBM tensor based morphometry
TH tyrosine hydroxylase
Introduction
Parkinson’s disease (PD) is the most prevalent movement disorder, with cardinal symptoms of rigidity, tremor and bradykinesia, primarily resulting from degeneration of the dopaminergic nigrostriatal pathway (Jenner et al., 2006). The pathological hallmark of the degenerating neurons in PD are intracytoplasmic protein inclusions known as Lewy bodies and Lewy neurites, composed predominantly of a synaptic protein called α-synuclein (αSyn), which are thought to be at least partly responsible for the dopaminergic neuronal cell death observed (Dexter et al., 2013; Spillantini et al., 1997).

In recent years epigenetic mechanisms such as DNA methylation and histone remodelling have become implicated in PD pathogenesis (Ammal Kaidery et al., 2013). Increased expression or the presence of mutated forms of αSyn sequesters DNA methyltransferase 1 (DNMT1) to the cytoplasm in PD and the promoter region of the αSyn gene, SNCA, is hypomethylated in both transgenic mice and in sporadic PD (Desplats et al., 2011). Similarly it has been observed that nuclear αSyn ‘masks’ histone proteins preventing their acetylation, resulting in histone hypoacetylation and subsequent apoptosis (Kontopoulos et al., 2006). In the healthy brain a carefully controlled balance exists between the activities of the two enzymes that control histone acetylation status: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs facilitate the acetylation of histone N-terminal tail lysine residues, causing relaxation of chromatin structure and transcription factor access to DNA. Equally, HDACs reverse this process by removing acetyl groups from histone N-terminal tail lysine residues causing condensation of chromatin structure and transcriptional repression. We and others postulate that this deregulation of the balance of histone acetylation and deacetylation could be rectified with the use of HDAC inhibitors (HDACIs), reducing the neurodegeneration observed as a result of HAT/HDAC misbalance (Chuang et al., 2009; Dietz et al., 2010; Hahnen et al., 2008; Harrison et al., 2013; Kazantsev et al., 2008).

Sodium valproate (2-propylpentanoic acid), first marketed over 45 years ago for the treatment of epilepsy (Löscher, 2002), is now a commonly prescribed mood stabiliser and anti-convulsant used to control generalised and partial seizures (Perucca, 2002). Subsequently, valproate was discovered to relieve HDAC-dependent transcriptional repression and cause histone hyperacetylation both in vitro and in vivo (Gottlicher et al., 2001; Phiel et al., 2001) by acting as a pan-inhibitor of HDAC classes I and IIa: inhibiting HDAC 1, 2, 3, 4, 5, 7 and 8 with varying potencies (Gurvich et al., 2004). This property of valproate has been shown to reduce neuroinflammation leading to neuroprotection in cell culture models of neuroimmune cell activation and dopaminergic cell death (Chen et al., 2006; Kidd et al., 2010; Monti et al., 2007; Monti et al., 2009; Peng et al., 2005; Wu et al., 2008). Furthermore, pre-clinical in vivo studies highlight the neuroprotective potential of valproate in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model (Kidd et al., 2011), and the rotenone (Monti et al., 2010) and 6-hydroxyp Dopamine (6-OHDA) (Monti et al., 2012) rat models of PD when given as a pre-treatment. Given its longevity of clinical use, these data suggest valproate is a candidate drug for clinical repositioning in the treatment of PD. Critically however, in prior pre-clinical neuroprotection studies, valproate was administered as a pre-treatment prior to administering the neurotoxin responsible for modelling Parkinsonian nigral degeneration. These studies were also in animal models which induce cell death through mechanisms other than altered protein deposition, the neuropathological hallmark of PD. Hence, this design, whilst effective for proof-of-concept studies lacks any clinical translatability or relevance. Likewise the molecular mechanism linking inhibition of HDACs by valproate leading to neuroprotection remains unclear. Current evidence from pre-clinical studies is therefore insufficient to facilitate translation of these basic findings to the clinic.
In this study, for the first time we have addressed these issues by investigating the neuroprotective effects of delayed-start valproate treatment, initiated one week after lesioning the rat nigrostriatal system, using the proteasome inhibitor, lactacystin. Importantly, this model recapitulates the formation of neurotoxic αSyn protein inclusions, which are known to affect epigenetic mechanisms, within the substantia nigra to induce dopaminergic cell death (Pienaar et al., 2015; Vernon et al., 2010a; Vernon et al., 2011). Similarly this model is associated with a specific reproducible pattern of neuroanatomical changes in the brain overlapping that of late stage PD (Duncan et al., 2013; Vernon et al., 2010b). For the first time we also use a combined phenotyping approach, combining motor behavioural testing, clinically comparable longitudinal structural MRI and post-mortem assessment of the integrity of the rat brain nigrostriatal system to track changes in this model of PD and detect neuroprotection. Subsequent cellular and molecular analyses were also performed to elucidate the mechanisms underlying valproate’s neuroprotective effects: including quantification of histone acetylation and expression levels of a number of different neurotrophic factors, apoptotic regulators and genes of interest to PD, previously shown to change upon treatment with HDACIs (Monti et al., 2009).
Materials and Methods

Experimental Animals
All animal procedures were carried out in accordance with the Home Office Animals (Scientific Procedures) Act, UK, 1986, and were previously approved by the Imperial College Animal Welfare and Ethical Review Board. Presentation of data complies with the ARRIVE guidelines (Kilkenny et al., 2010). Male Sprague-Dawley rats (250±10g, Charles River, UK) were housed in groups of two or three at 21±1°C on a 12 hour light dark cycle with the relative humidity maintained at 55±10%. Standard rat chow and drinking water were available ad libitum throughout the duration of the study, and were supplemented with standard rat wet diet for seven days post-surgery.

Animal Treatment Groups
Five animal treatment groups (Fig. 1) underwent serial evaluation of brain structure by MRI and motor behavioural testing. The baseline assessment was prior to lactacystin-lesioning of the substantia nigra pars compacta (SNpc) with follow up assessments at 1, 3 and 5 weeks post-surgery. Animals were injected daily for 28 days with either saline or valproate (200mg/kg or 400mg/kg i.p.) initiated 7 days post-surgery. After the final in vivo assessments, animals were sacrificed and brain tissue harvested for subsequent analysis. An additional group of animals was also stereotaxically lesioned with lactacystin however sacrificed and brain tissue harvested for subsequent analysis, 7 days post-surgery.

Stereotaxic Lesioning of the SNpc with Lactacystin
The left SNpc was stereotaxically lesioned using the irreversible proteasome inhibitor, lactacystin (BML-PI104, Enzo Life Sciences, Exeter, UK), as previously published (Vernon et al., 2010a). Briefly, isoflurane (05260-05, IsoFlo®, Abbott Laboratories, Maidenhead, UK) anaesthetised animals were then positioned in a stereotaxic frame (Kopf Instruments, Tujunga, USA) in the horizontal skull position with the incisor bar positioned 3.3mm below the interaural line. Intramuscular administration of buprenorphine (QN02AE01, Alstoe Animal Health, York, UK) provided analgesia whilst bupivacaine (PL-20910/0008, Taro Pharmaceuticals, NY, USA) was utilised to produce local anaesthesia in the scalp prior to making a midline incision to expose the skull where bregma was identified (Paxinos et al., 2009). A small burr hole was made in the skull above the SNpc and 10µg of lactacystin (2.5µg/µl in sterile (0.9%) saline, 4µl in total) was stereotaxically administered via a Hamilton syringe to the SNpc: anterio-posterior, -5.2mm, medio-lateral, +2.5mm and ventral to dura, -7.6mm (Paxinos et al., 2009). The lactacystin was injected at a rate of 1µl/min, the needle was left in situ for 3mins before being retracted. Post-surgery fluid replacement (Glucosaline, 5ml of 0.18% NaCl, 4% Glucose, given i.p. prior to surgery) was administered and animals were left to recover in a heated recovery chamber.

Behavioural Testing

Vertical Cylinder Test
To assess asymmetry in forelimb motor function the vertical cylinder test was employed similar to that previously published (Schallert et al., 2000). Briefly, animals were placed into a Perspex cylinder (200mm diameter by 300mm height) and rearing behaviour was recorded with a video camera for either ten complete rears or 3mins. Forelimb movements were examined using frame by frame analysis of video recordings. The percentage of contralateral forelimb use was calculated as:

\[ N = \left( \frac{\text{no. of uses of contralateral forelimb} + \frac{1}{2} \text{no. of uses of both forelimbs simultaneously}}{\text{no. of uses of ipsilateral forelimb} + \text{contralateral forelimb} + \text{both forelimbs simultaneously}} \right) \times 100 \]
**Amphetamine Induced Rotation Test**

Rotational asymmetry was assessed using the amphetamine induced rotation test similar to that previously published (Ungerstedt et al., 1970). Briefly, animals were administered 5mg/kg amphetamine (i.p., D-amphetamine sulphate (A0922, Sigma, Poole, UK) in sterile saline at 5mg and placed in a clear circular test arena of dimensions 400mm diameter by 360mm height (Circling Bowl, Harvard Apparatus, MA, USA) for 30mins to acclimatise. After this time behaviour was recorded for 30mins and the numbers of contraversive and ipsiversive rotations were counted in bins of 5 minutes. The net number of ipsiversive rotations per 5 minute bin was calculated as:

\[ N = (\text{no. of ipsiversive rotations}) - (\text{no. of contraversive rotations}) \]

**Magnetic Resonance Imaging**

T2-weighted (T2W) magnetic resonance (MR) images were acquired similar to that previously described (Vernon et al., 2010a; Vernon et al., 2011) using a 4.7 Tesla DirectDrive horizontal small bore MRI scanner (Varian, Palo Alto, CA, USA) and a separate 72mm quadrature birdcage head RF coil (M2M Imaging, OH, USA) linked to a LINUS-based control console running VnmrJ acquisition software (v2.3, Varian). Briefly, animals were anaesthetised by inhalation of isoflurane vaporised into O₂ in an anaesthetic chamber. The animal was then positioned into an MRI compatible polytetrafluoroethylene (PTFE) stereotaxic head holder and bed (M2M Imaging, OH, USA) and maintained under anaesthesia using isoflurane. Depth of anaesthesia was monitored using a respiratory balloon (SA Instruments, Stoney Brook, NY, USA) placed under the animal’s chest and body temperature was monitored and maintained using a rectal probe and heated fan (SA Instruments), respectively. T2W images were acquired using a multi-echo, multi-slice spin-echo pulse sequence (MEMS), with the following scan parameters: FOV=35mm×35mm; matrix=192×192; TR=5155.2ms; TE=10, 20, 30, 40, 50, 60, 70, 80, 90, 100ms; 4 averages, scan duration 1hr 5mins 59secs. Fifty contiguous 500µm thick coronal slices with an in plane resolution of 256×256µm were acquired such that the entire brain of each animal was covered. Once scanning was completed, animals were removed from the magnet bore to a separate holding room and placed in a heated recovery chamber. Following full recovery from anaesthesia, animals were then returned to their home cages.

**MR Image Analysis**

Post-acquisition, images corresponding to the ten TE times used were summed using the “Z Project” function in ImageJ (v1.4, National Institutes of Health, Bethesda, MD, USA) to give the fifty T2W images representing fifty contiguous 500µm thick coronal slices of the rat brain.

For regional volumetric analysis of MR images, structures were delineated manually using ImageJ software by a single rater blinded to animal treatment. Six brain regions (whole brain, lateral ventricles, corpus striatum, hippocampus, midbrain and cerebellum) were delineated based on anatomical landmarks previously published (Vernon et al., 2010a; Vernon et al., 2011) and with reference to the rat brain stereotaxic map (Paxinos et al., 2009) (Supplementary Fig. 1). Volumes were then calculated by multiplying the sum of the areas for a given structure on all slices by the slice thickness (500µm). Brain volumes were then expressed as percentage change from baseline.

To perform an unbiased whole-brain analysis of apparent volumetric differences between groups, an automated image-processing pipeline for Tensor Based Morphometry (TBM) was used (Crum et al., 2013a). Briefly, a single, well-positioned good-quality control animal (surgically naïve, saline treated) scan was chosen as an initial reference for rigid (6
degrees of freedom) and rigid + scaling (9 degrees of freedom) registration using a robust population approach (Crum et al., 2013b). Then, a template image was constructed as the mean of the registered control group (surgically naïve, saline treated) scans and used as the reference in subsequent processing. High-dimensional non-rigid registration was applied to each scan to warp it onto the control template, and thereby obtain maps of apparent local volume difference for each scan, encoded as the Jacobian determinant at each voxel (Crum et al., 2013a). Non-parametric t-tests at each voxel were used to detect differences in the Jacobian determinants between each study group and the control group and thereby infer differences in volume across groups (Bullmore et al., 1999). Significance values were corrected for multiple comparisons using the False Discovery Rate with q=0.05 (Genovese et al., 2002).

**Tissue Collection and Preparation**

At the end of the study period, animals were sacrificed, decapitated and the brain removed from the skull. Using a rodent brain matrix, each brain was cut coronally at the level of the infundibular stem to produce forebrain and hindbrain blocks. Frontal brain tissue was dissected out, snap frozen on dry ice and stored at -80°C for protein and mRNA extraction. The hindbrain was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 72hrs before being cryoprotected in 30% sucrose in PBS until the tissue was observed to have sunk. Blocks were then snap frozen in isopentane pre-chilled on dry ice and stored at -80°C for subsequent sectioning.

**Immunohistochemistry**

30µm thick coronal sections were collected throughout the extent of the SNpc onto SuperFrost® Plus slides (HECH2409/1, VWR international, Lutterworth, UK) using a cryostat (Bright Instruments, Huntingdon, UK). Slides were then stored at -80°C until analysis. Immunohistochemistry for tyrosine hydroxide (TH), the rate limiting enzyme in monoamine synthesis was utilised as the cellular marker for dopaminergic neurons in the SNpc, and cresyl violet was used as a counterstain for the Nissl body of all neurons. For this the Avidin-Biotin Complex (ABC)/peroxidase method of immunohistochemistry with a cresyl violet counterstain was performed similar to that previously published by our group (Pienaar et al., 2015). Briefly, endogenous peroxidase activity was blocked by incubation in 0.3% H\textsubscript{2}O\textsubscript{2} in methanol for 45mins before sections were rehydrated in a descending series of alcohol washes followed by PBS containing 0.1% TritonX-100 (PBS-T). Non-specific binding was blocked by incubation with 20% normal goat serum in PBS-T for 1hr at room temperature before incubating with the primary antibody (Rabbit Polyclonal Anti-Tyrosine Hydroxylase, AB152, Millipore, MA, USA) at 1:1000 in PBS-T for 24hrs at room temperature. Sections were then washed in PBS-T and incubated in the secondary antibody (Biotinylated Goat Anti-Rabbit Secondary Antibody, BA-1000, VectorLabs, Peterborough, UK) at 1:200 in PBS-T for 1hr at room temperature. Sections were then washed again and incubated in Avidin-Biotin Complex (Vectastain Elite ABC Kit, PK-6200, VectorLabs) for a further 1hr at room temperature before being washed thoroughly in Tris-Buffered Saline (TBS) (pH 8.4), and staining visualised with 3, 3’-diaminobenzidine (DAB). Sections were then washed in H\textsubscript{2}O before being counterstained using cresyl violet (0.1% in dH\textsubscript{2}O) for 2mins and mounted.

**Stereological Cell Quantification**

Stereological quantification of the number of TH positive (TH+) and Nissl positive (Nissl+) cells were made in the entire SNpc. For this, a computer based stereology software system (ImagePro, MediaCybernetics, PA, USA) attached to a Nikon Eclipse E8—microscope
(Nikon Instruments, Surrey, UK) and JVC (London, UK) 3CCD camera was used. The optical fractionator method of stereology was used as previously published (West et al., 1991). Briefly, for each section the SNpc was delineated manually with relation to previously published boundaries (Carman et al., 1991), to create an area of interest (AOI). The software system then created counting frames (140 x 160µm) which fell within the AOI using the uniform random sampling method. The total area of the counting frames relative to the area of the AOI gives the area sampling fraction (asf). The height of the optical dissector, which was measured by taking an average of 3 random points across the section using a Heidenhain microcator (Hedenhain, Traunreut, Germany), relative to the section thickness gives the height sampling fraction (hsf). The section sampling fraction (ssf) was 1/6 as every 6th section throughout the SNpc was analysed. To avoid edge effects, when counting TH+ and Nissl+ cells within the counting frames, “acceptance” and “forbidden” lines were used. Total cell estimates were calculated as follows, where n equal the number of positive cells counted:

\[ N = n \left( \frac{1}{ssf} \right) \left( \frac{1}{asf} \right) \left( \frac{1}{hsf} \right) \]

**Protein and mRNA Extraction and Quantification**

30mg of frontal brain tissue was homogenised in QIAzol® Lysis Reagent (79306, Qiagen, Crawley, UK) using a tissue homogeniser (Ultra-Turrax T18, IKA, Staufen, Germany). mRNA and protein was extracted from homogenised brain tissue using the RNeasy® Plus Universal Mini Kit (73404, Qiagen) as per the manufacturer’s instructions. Isolated RNA was quantified spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) and RNA purity verified by an average A₂₆₀/₂₈₀ ratio of 1.99 (range 1.97-2.01). The quantity of isolated protein was determined using the 96-well variant of the Bradford Assay (B6916, Sigma): colour change determined using a 96-well plate reader (VersaMax Microplate Reader, Molecular Devices, CA, USA) at A₅₉₅. mRNA and protein were stored at -80°C and -20°C respectively until further analysis.

**Western Blot Analysis**

Laemmli sample buffer (S3401, Sigma) was added to 10µg of extracted protein sample and denatured by incubating at 95°C for 15mins. Samples were loaded onto a 1mm thick hand-cast 15% Tris-Glycine gel and proteins were separated by electrophoresis (65mA for 40mins). Proteins were transferred onto methanol soaked PVDF membrane with a pore size of 0.45µm using semi-dry transfer (20V for 45mins). Membranes were then equilibrated in TBS containing 0.2% Tween-20 (TBS-T), before being blocked in 5% non-fat milk in TBS-T for 1hr at room temperature. Membrane was washed in TBS-T again before being incubated in primary antibodies against histone protein H3 acetylated on lysine 9 (rabbit anti-AcH3-Lys9, H9286, Sigma, 1:10,000) and mouse anti-β-actin antibody (1:20,000, Ab6276, Abcam, Cambridge, UK) for 1hr at RT. Membranes were then washed again and incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (either Goat anti-Rabbit [1:10,000] and Horse anti-Mouse [1:10,000] for AcH3-Lys9 or β-actin respectively, both Vector Laboratories) for 1hr at RT. Membranes were washed again in TBS-T and developed using chemiluminescence (170-5060, Clarity Western ECL Substrate, Bio-Rad, Hemel Hempstead UK). Bands were quantified using densitometry analysis software (ImageJ, v1.4).

**Quantitative Real Time Polymerase Chain Reaction**

For cDNA synthesis, 500ng of total RNA from each sample was reverse transcribed according to the manufacturer's instructions using the QuantiTect® reverse transcription kit (205310, Qiagen) with integrated removal of genomic DNA contamination. The reactions were stored at -20°C until further use. Real-time reverse transcriptase quantitative
polymerase chain reaction (RT-qPCR) experiments were performed using a Mx3000™ real-time PCR system with MxPro software (v4.10, Stratagene, La Jolla, CA, USA) and the Brilliant® II QPCR master mix with low ROX (600806, Agilent technologies UK Ltd, Edinburgh, UK). For each gene of interest in each sample, 20µl reactions were set up in triplicate, and run in duplex with a novel reference gene (XPNPEP1 [X-prolyl aminopeptidase (aminopeptidase P) 1] (Durrenberger et al., 2012)), with each reaction containing 10µl of 2× Brilliant® II QPCR master mix, 7 µl of RNase-free water, 1 µl template cDNA, 2µl (1µl gene of interest + 1µl reference gene) of 10× PrimeTime™ qPCR assays (Integrated DNA technology, Coralville, IA, USA, Supplementary Table 1). Reactions were carried out with the following cycling protocol: 95°C for 10 min, then 60 cycles with a 3-step program (95°C for 30s, 55°C for 30s and 72°C for 30s). Fluorescence data collection was performed during the annealing step. A negative control containing no cDNA template was also run in each plate. Similarly an inter-plate calibrator, created by pooling control cDNA samples, was also run in each plate. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method normalising to the expression of the novel reference gene (Durrenberger et al., 2012) and the appropriate control group.

Statistical Analysis
All data is presented as mean ± standard error of mean (SEM). Two-way (repeated measures) analysis of variance (ANOVA) with Bonferroni post-tests were used for analysis of vertical cylinder test, amphetamine induced rotation test and MRI manual segmentation analysis datasets. An unpaired t-test was used to compare the baseline and week 1 forelimb use of lactacystin-lesioned animals in vertical cylinder test data. Paired t-tests were used to compare stereological cell counts in the ipsilateral and contralateral hemispheres of animal brains. A one-way ANOVA with Bonferroni post-tests were used to compare cell loss percentages calculated from stereological cell counts. A two-way ANOVA with Bonferroni post-tests were used to compare qRT-PCR data. A one-way ANOVA with Bonferroni post-tests were used to compare Western blot data. All statistical tests were performed using GraphPad Prism (v5.0 for Windows, GraphPad Software, San Diego, CA, USA).
**Results**

**Valproate Attenuates Behavioural Motor Deficits Caused by Lactacystin**

The vertical cylinder test and amphetamine induced rotations were utilised to assess for motor asymmetry in the animals’ movement due to unilateral injection of lactacystin into the SNpc.

**Vertical Cylinder Test**

At baseline there was equal use of both the left and right forelimbs in all animals (Fig. 2A, 51.69±0.89% contralateral forelimb use). However, one week after surgery there was a significant reduction in the contralateral forelimb use of lactacystin-lesioned animals compared with non-lesioned animals (week 1, mean forelimb use of lactacystin-lesioned animals, 37.99±1.53%, vs. mean forelimb use of non-lesioned animals, 53.69±1.38%, p<0.05 in all comparisons). Contralateral forelimb use of saline treated animals continued to decline with time (week 1, 39.59±4.11%; week 3, 36.01±3.37%; week 5, 35.50±4.08%, p<0.01 compared with control animals at each time point). However, the continued decline in motor deficits due to lactacystin toxicity from week 1 was halted in animals treated with the lower dose of valproate (200mg/kg) (week 5, valproate, 44.39±6.94% vs. saline treated animals, 35.50±4.08%). Whereas in animals treated with the highest dose of valproate (400mg/kg) the continued decline in motor deficits due to lactacystin toxicity from week 1 was totally reversed with the animals failing to show any deficit after 28 days of treatment (week 5, valproate, 52.63±2.66% vs. saline treated animals, 35.50±4.08%, p<0.01).

**Amphetamine Induced Rotations**

Ipsilateral rotations following an amphetamine challenge were observed at one week in all lactacystin-lesioned animals (Fig. 2B, 20.44±3.35 net ipsiversive rotations per 5 minutes). Saline treated lactacystin-lesioned animals performed increasing numbers of rotations over the further two time points examined (area under curve produced by plotting number of rotations vs. time, Fig. 2C, week 1, 612.01±121.59; week 3, 1039.33±247.75; week 5, 1641±477.50). However animals treated with the lowest dose of valproate performed fewer rotations than saline treated animals after 28 days of valproate treatment, whereas animals treated with the highest dose of valproate (400mg/kg) perform fewer rotations than the saline treated group after both 14 and 28 days valproate treatment (week 5, valproate, 585.83±123.47 vs. saline treated animals, 1641±477.50, p<0.05).

**MRI Reveals Dose-dependent Attenuation of Lactacystin Induced Volumetric Changes by Valproate**

*In vivo* rat brain MR images were acquired longitudinally to follow the neuropathological progression of the lactacystin PD model as previously described (Vernon et al., 2010a; Vernon et al., 2011). A combination of manual segmentation analysis and automated, unbiased TBM were used to quantify neuropathological changes observed as a result of lactacystin-lesioning and determine the effects of valproate treatment upon this progression.

**Manual Segmentation Analysis**

No significant difference was observed in baseline brain volume between groups (data not shown) however to account for individual variations in brain size, data is presented as percentage change from baseline. In line with rat growth and consistent with MRI finding in rats of comparable age to those used in this study, there was a steady increase in whole brain volume in surgically naïve saline treated animals over the 5 week study (Fig. 3A, week 1, 2.09±0.86%; week 3, 4.64±1.33%; week 5, 6.60±1.29% increase from baseline). Lactacystin-lesioned animals treated with saline and lesioned animals treated with the lower dose of valproate however both exhibit a greater increase in brain volume compared to control animals (9.07±2.44% and 9.33±0.69% increase from baseline at week 5 respectively vs. 6.60±1.29% increase in control animals). Whereas animal groups treated with valproate at its
higher dose, both lactacystin-lesioned and surgically naïve, exhibit a discernible shallower increase in whole brain volume over the 5 weeks of treatment (3.76±1.19% and 3.07±1.34% increase from baseline at week 5 respectively vs. 6.60±1.29% increase in control animals).

In surgically naïve animals, the volume of the lateral ventricles increases comparably in both hemispheres over the 5 week study (Fig. 3B). However, lactacystin-lesioned animals subsequently treated with saline, exhibit a greater increase in ventricular volume, a change which was more pronounced in the lesioned hemisphere. In contrast, lactacystin-lesioned animals treated with valproate (both 200mg/kg and 400mg/kg) do not exhibit these increases in ventricular volume over time (left ventricle at week 5, 200mg/kg and 400mg/kg valproate treated animals, 40.81±28.20% and 60.05±14.17% increase from baseline respectively compared with saline treated animals, 192.93±61.54% increase from baseline, both p<0.05).

One week after lesioning surgery, prior to starting vehicle/drug treatment, the ipsilateral midbrain volume of all lactacystin-lesioned animal groups was significantly lower than the volume of both surgically naïve groups (Fig. 3C, lactacystin-lesioned saline, 200mg/kg and 400mg/kg valproate treated animals, 7.07±3.05%, 4.51±2.42% and 4.74±0.83% decrease from baseline respectively vs. 10.88±4.73% and 13.80±3.08% increase in non-lesioned saline treated and valproate treated groups respectively, p<0.05 in all comparisons). The ipsilateral midbrain volume in lactacystin-lesioned saline and low dose (200mg/kg) valproate treated animals continued to decline over the further two time points examined, remaining significantly different from the volume change in surgically naïve groups at both week 3 and week 5 (p<0.001 in all comparisons). In contrast, lactacystin-lesioned animals treated with the higher dose (400mg/kg) of valproate display a reversal of the decrease in ipsilateral midbrain volume seen at week 1 in the following two time points: reaching a significant difference from saline and low dose (200mg/kg) treated animals at week 5 (400mg/kg valproate treated animals, 3.70±2.36% increase from baseline vs. saline and 200mg/kg valproate treated animals, 16.77±5.94% and 15.90±2.36% decrease from baseline respectively, p<0.01 in both comparisons). Identical albeit more subtle changes are similarly observed contralateral hemisphere of the midbrain.

No significant differences are observed in corpus striatum volume in either the ipsilateral or contralateral hemispheres. However a number of trends in the ipsilateral hemisphere exist subtly mimicking those changes observed in the midbrain (Supplementary Fig. 2). Similarly no discernible changes in volume of the either ipsilateral or contralateral hippocampus, or the cerebellum were observed (Supplementary Fig. 2).

**Tensor Based Morphometry Analysis**

To confirm and extend our manual segmentation observations, we applied TBM analysis to MR images acquired from all groups 5 weeks post-surgery (Fig. 4). After correcting for global differences in brain volume (9dof registration) to search for relative differences in brain volume, several distinct anatomical patterns were observed across treatment groups (all data shown are corrected for multiple-comparison over voxels using the False Discovery Rate with q<0.05).

Surgically naïve animals treated with valproate, showed bilateral clusters of contracted voxels in the globus pallidus, internal capsule, third ventricle, ventromedial thalamic nuclei, perirhinal cortex, amygdala and brainstem. Conversely, clusters of expanded voxels were detected in the hippocampus (dorsal and ventral regions), dorsal entorhinal cortex, external capsule and the 3rd and 4th cerebellar lobules.

In lactacystin-lesioned animals treated with saline, we observed widespread significant contraction of cortical voxels in the ipsilateral (lesioned) hemisphere of the brain. These included the cingulate, motor, somatosensory and parietal cortical sub-fields. Sub-cortically, widespread clusters of contracted voxels were observed in the ipsilateral striatum, globus pallidus, thalamus, ventral midbrain and brainstem nuclei. Some of these clusters
extended across the midline into the contralateral (non-lesioned) hemisphere. No significantly contracted voxels were observed in the hippocampus. Clusters of significantly expanded voxels were also observed in the ipsilateral hemisphere. Primarily this reflected an increase in cerebrospinal fluid signal accompanying deformation of the ventral midbrain. Significantly expanded voxels were however also seen in the cerebellar white matter and dorsolateral entorhinal cortex.

In lactacystin-lesioned animals treated with low (200 mg/kg) and high doses (400 mg/kg) of valproate a more complex pattern of anatomical changes were detected. Valproate treatment dose-dependently reversed cortical atrophy in the cingulate, motor, sensorimotor and parietal cortices in the ipsilateral (lesioned) hemisphere. Sub-cortically, valproate treatment also dose-dependently reversed atrophy of the ventromedial thalamus, ventral midbrain and expansion of CSF space. These effects were more marked at the higher dose tested. Drug-specific effects of valproate were however also present, which make interpretation of the results difficult. Indeed, low dose valproate treatment resulted in bilateral atrophy in the ventromedial thalamus, globus pallidus, internal capsule and third ventricle. These patterns are consistent with the effects of valproate in non-lesioned animals. These patterns were however, paradoxically not present in lesioned animals given a high dose of valproate.

**Valproate Treatment Causes Dose-dependent Protection/Restoration of Dopaminergic Neurons in the SNpc in Lactacystin Lesioned Animals**

Post-study, animals were culled and hind brain tissue collected for immunohistochemical staining and stereological quantification of dopaminergic neurons (tyrosine hydroxylase positive (TH+)) in the SNpc (Fig. 5 and 6). Non-lesioned animals treated with either saline or valproate (400 mg/kg) did not show any interhemispheric loss of TH+ neurons in the SNpc. Animals lesioned with lactacystin and culled 7 days post-surgery, the time point when valproate administration was initiated, however exhibit a marked interhemispheric loss of TH+ neurons due to the intranigral injection of lactacystin (left SNpc, 14027±538 TH+ cells, −45.17±10.96% difference, p<0.05). The loss of TH+ neurons continued to increase with time, rising to −64.24±11.34% in the lactacystin-lesioned animals treated with saline for 28 days, demonstrating further neurodegeneration after week 1 (left SNpc, 4257±1364 vs. right SNpc, 12328±580 TH+ cells, % difference, −64.24±11.34, p<0.01). In contrast valproate administration in lactacystin-lesioned animals resulted in a dose-dependent neuroprotective/restorative effect. When administered at its higher dose (400 mg/kg) for 28 days afforded near complete protection/restoration of SNpc TH+ neurons against the toxic effects of lactacystin (left SNpc, 9729±1347 vs. right SNpc, 11913±578 TH+ cells, % difference −19.08±10.17%, p>0.05). Similarly, administration of the lower dose (200 mg/kg) of valproate resulted in only a partial protection of the TH+ neurons against lactacystin toxicity (left SNpc, 7659±1228 vs. right SNpc, 12739±862 TH+ cells, % difference −40.26±9.87%, p<0.05). All changes observed in SNpc TH+ cell number were reproduced in the numbers of Nissl+ cell number, indicative of TH+ neuronal cell death rather than loss of the TH enzyme expression in dying neurons.

**Valproate Dose-dependently Attenuates Lactacystin Induced Reduction in Histone Acetylation**

Upon removal of brain tissue at the end of the study the frontal brain was snap frozen for subsequent quantification histone acetylation through quantification of histone protein H3 acetylated on lysine 9 (AcH3-Lys9) using Western blot analysis (Fig. 7). Significantly less AcH3-Lys9 was observed in both hemispheres lactacystin-lesioned animals treated with saline compared with non-lesioned animals (left hemisphere, non-lesioned animals,
This effect was dose-dependently attenuated in both hemispheres of animals treated with valproate, reaching significance from saline treated animals in the ipsilateral hemisphere (ipsilateral hemisphere, saline treated animals, 0.02±0.03 vs. animals treated with 400mg/kg valproate, 0.84±0.08, p<0.05). No difference in the amount of AcH3-Lys9 in either hemisphere was observed in non-lesioned animals treated with valproate compared with control.

**Valproate Upregulates Expression of Neurotrophic Growth Factors and Neuroprotective Proteins**

In non-lesioned animals, administration of valproate induced a marked upregulation of brain derived neurotrophic factor (BDNF) (Fig. 8A, ipsilateral valproate treated, 2.14±0.36 times greater than saline treated controls, p<0.001; contralateral valproate treated, 1.94±0.36 times greater than saline treated controls, p<0.01). Valproate did not alter the expression of the other genes examined in these surgically naïve animals. However, in lactacystin-lesioned animals valproate significantly and dose-dependently up regulated the expression of BDNF, glial derived neurotrophic factor (GDNF) and the anti-apoptotic factor Bcl2, in the frontal brain hemisphere contralateral to the lesion. Most notably GDNF expression was greater in the contralateral hemisphere of valproate compared with saline treated animals (Fig. 8B, high dose (400mg/kg) treated animals, 5.15±1.48 times greater than saline treated controls; low dose (200mg/kg) treated animals 4.32±1.34 times greater than saline treated control, p<0.001 in both comparisons). Similarly, expression of BDNF and Bcl2 was elevated in a dose-dependent manner in the frontal brain hemisphere contralateral to the lesion, however this only reached significance at the higher dose of valproate (BDNF expression, 4.08±0.88 times greater than saline treated control, p<0.01; BCL2 expression, 3.09±0.32 times greater than saline treated control, p<0.05). Similar trends of an up regulation of gene expression of native αSyn and heat shock protein 70 (HSP70) upon treatment with valproate were observed but these did not reach significance.
Discussion
Valproate dose-dependently afforded neuroprotection and neurorestoration of nigrostriatal dopaminergic neurons in the lactacystin animal model of PD, as evidenced by an attenuation of motor behavioural deficits, longitudinal MRI brain volume changes and quantification of the dopaminergic neurons within the SNpc. Molecular analyses indicate that valproate’s neuroprotective effects may be mediated through epigenetic changes via inhibition of histone deacetylation, chromatin remodelling and upregulation of neurotrophic and neuroprotective genes, culminating in the observed neuroprotective and neurorestorative effect.

MR imaging studies using toxin-based models of PD have largely been focused on alterations in $T_2$ water $^1$H relaxation time and $^1$H MR spectroscopy, whilst potential morphological changes have been overlooked. Recently however, a method of using MRI to non-invasively monitor the morphological progression and nigrostriatal neuropathology in this proteasome inhibitor rat model of PD has been established (Vernon et al., 2010a; Vernon et al., 2011). To the best of our knowledge, the current study is the first to use this method in conjunction with a candidate neuroprotective drug to longitudinally assess its efficacy in an animal model of PD. Manual segmentation analysis was performed to examine the temporal progression of morphological changes in selected brain regions. In line with previously published data following nigrostriatal neuropathology induced as a result of SNpc lactacystin (Vernon et al., 2010a; Vernon et al., 2011) we observed a marked reduction in the volume of the ipsilateral midbrain in the weeks following lactacystin-lesioning. Similarly we observe an increase in the volume of the lateral ventricles; more pronounced in the ipsilateral as opposed to the contralateral hemisphere. Both of these changes were dose-dependently attenuated by delayed start valproate treatment. Automated, unbiased TBM analysis (Crum et al., 2013b; Vernon et al., 2011) confirms and extends the manual segmentation data. Indeed, TBM highlights that chronic valproate treatment by itself induces a specific pattern of structural remodelling (expansion and contraction) in the healthy brain. This is interesting in light of recent observations for other psychotropic drugs, including Lithium, a mood stabiliser (Vernon et al., 2013; Vernon et al., 2012). Similarly TBM data confirms lactacystin-lesioning by itself is associated with a specific reproducible pattern of neuroanatomical changes in the brain, detectable by MRI (Vernon et al., 2011), modelling features of advanced PD, and also atypical forms of PD, such as Progressive Supranuclear Palsy and Multiple System Atrophy (Duncan et al., 2013; Vernon et al., 2010b). This both enhances the validity of the lactacystin-model and confirms that our analysis pipeline is robust and reproducible enough to detect the same patterns in data generated and acquired in different academic centres. These data suggest a complex pattern of drug x disease interactions drive the anatomical effects observed herein. On the one hand, TBM identifies a clear neuroprotective effect of valproate, namely prevention of brain atrophy, entirely consistent with manual segmentation results. On the other hand, valproate itself induces both brain atrophy and growth in topographically distinct brain regions, reinforcing the importance of examining drug effects in both normal and diseased animals. Nevertheless, our unbiased, automated approach reinforces our manual observations. That is, we are able to detect a dose-dependent neuroprotective effect of valproate on lactacystin-induced brain atrophy, which was associated with both cellular and functional neuroprotection and neurorestoration. The combination of longitudinal in vivo MRI and automated TBM, a clinically comparable technology, therefore has great potential for pre-clinical assessment of drugs with disease-modifying potential in pre-clinical models of PD, and other neurological disorders, which may speed the translation of basic findings to the clinic.

The data described here importantly illustrates the neuroprotective effects of valproate when administered post-lesion in the lactacystin rat model of PD are consistent with previous studies in which valproate was administered prior to the neurotoxin in other pre-clinical PD
models. Monti and colleagues (2010) observed that dietary pretreatment resulted in a significant preservation of the SNpc TH+ dopaminergic neurons in rats sub-chronically administered rotenone. They also observed an attenuation of the loss of striatal dopamine and consistent with in vitro findings from their group (Monti et al., 2007) they observed an increase in native αSyn expression in the SNpc and striatum in line with a reduction in mono-ubiquitinated αSyn and its nuclear translocation following valproate administration. αSyn is thought to result in histone hypoacetylation through its ‘masking’ of histone proteins (Kontopoulos et al., 2006). Therefore consistent with the lack of αSyn nuclear translocation, the group also observe an increase in histone acetylation upon valproate treatment. Monti and colleagues (2012) also demonstrated that dietary pretreatment with valproate resulted in protection of the dopaminergic neuronal terminals in the striatum as well as the dopaminergic cell bodies in the SNpc against striatally administered 6-OHDA. Similarly, Kidd and Schneider (2011) demonstrated the neuroprotective effects of valproate, at both the SNpc dopaminergic cell body and striatal dopamine level, when administered systemically prior to the administration of the mitochondrial toxin MPTP to mice. Importantly, in all of these studies valproate was administered prior to the toxin and hence they do not mirror the clinical setting in which a neuroprotective agent will currently be used: by the time the cardinal motor symptoms of PD become evident, 60-70% of neurons in the SNpc and ~80% of striatal dopamine have already been depleted (Riederer et al., 1976). Conversely, in this important study we have for the first time shown valproate to be both neuroprotective and neurorestorative when administered one week after toxin administration in a ‘delayed start design’, when the neurodegenerative process has been initiated and we can already observe clinical behavioural and MRI changes in the animal model. Stereological quantification of dopaminergic neurons in animals culled one week post lactacystin-lesion, i.e. the point at which valproate treatment was started; indicate that the vast majority of nigral degeneration occurs in these first seven days. Importantly however, despite the delayed start in treatment, less neurodegeneration of nigral dopaminergic neurons was observed after four weeks of valproate treatment, indicating that valproate is not only acting neuroprotectively towards dopaminergic nigral neurons but is also acting neurorestoratively. Appropriately, Nissl+ neuronal degeneration was markedly less than that of TH+ cells in animals culled prior to valproate treatment. It could be possible then that valproate in the current study is acting to rescue unhealthy and/or dying neurons that have lost TH expression in the cell body and therefore have reduced function, yet retain their TH+ projections, aiding their functional recovery, translating to performance recovery in behavioural tests such as the vertical cylinder test.

Valproate has also been shown to partially protect against motor deficits in animal models of traumatic brain injury (Dash et al., 2010), spinal cord injury (Lee et al., 2012) and stroke (Kim et al., 2007) yet to our knowledge the present study is the first to examine the ability of valproate to relieve motor symptoms in an animal model of PD. We demonstrate that delayed valproate treatment dose-dependently reverses lactacystin induced reduction in the use of the forelimb contralateral to the lesion, and also attenuates the number of rotations performed upon amphetamine challenge. Interestingly, using a battery of behavioural tests, Castro and colleagues (Castro et al., 2012) also demonstrated that valproate pre-treatment prevented the development of early non-motor symptoms of PD e.g. cognitive and emotional deficits, in animals nasally administered MPTP (Prediger et al., 2011). Authors demonstrated that this was accompanied by a significant preservation of olfactory bulb and striatal dopamine content in MPTP treated animals (Castro et al., 2012). This suggests that valproate is also neuroprotective to other neuronal systems which is particularly important since the neurodegenerative process in PD is not merely confined to the dopaminergic nigrostriatal system (Braak et al., 2003). Our MRI findings are consistent with this: lactacystin induced
atrophy in numerous extra-nigral brain regions such as the cingulate, motor, sensorimotor and parietal cortices are ameliorated by valproate.

The misbalance between HATs and HDACs which are thought to lead to histone hypoacetylation in neurodegeneration was first noted by Rouaux and colleagues (2003). Since then, histone hypoacetylation has become heavily implicated in neurodegenerative diseases such as PD: it being demonstrated that αSyn accumulation actively promotes histone hypoacetylation both in vitro in SH-SY5Y cells and in vivo in drosophila, both overexpressing αSyn (Kontopoulos et al., 2006). Lactacystin dopaminergic neurotoxicity is associated with the aggregation of αSyn to form inclusion bodies in the SNpc, a finding which has been extensively verified since (McNaught et al., 2002; Niu et al., 2009; Vernon et al., 2011; Zhu et al., 2007). αSyn is thought to result in histone hypoacetylation through its ‘masking’ of histone proteins (Kontopoulos et al., 2006). Therefore in line with these previous observations of the effect of αSyn on histone acetylation, in the current study we observed a reduction in histone protein H3 acetylated on lysine 9, in the brains of lactacystin-lesioned animals, used as a surrogate marker for histone deacetylation in the brains of these animals.[1] Importantly, valproate treatment was observed to dose-dependently attenuate this histone hypoacetylation, in parallel with the cellular neuroprotective and restorative effect of the drug shown through stereological cell quantification of SNpc dopaminergic neurons. Valproate is a somewhat promiscuous drug: affecting glutamatergic and GABAergic transmission in the brain, and modulating ionic channels such as the voltage gated Na\(^+\) and T-type Ca\(^{2+}\) channels. However, the dose-dependent attenuation of histone hypoacetylation induced by valproate in line with the extent of neuroprotection/restoration observed is suggestive that valproate’s inhibition of HDACs is at least partly responsible for the effects observed.

Addition of an acetyl group to histone lysine residues neutralises the positive charge of the residue and hence reduces the electrostatic interaction between the lysine in the histone tail and the negatively charged phosphate group on DNA. This disrupts the inter- and intranucleosomal interactions between the histone and DNA and hence relaxes the structure of the chromatin allowing transcription factor access. Inhibition of HDACs and histone acetylation in the brain have therefore been shown to be associated with transcriptional upregulation of numerous factors that are thought to contribute to the neuroprotective/restorative effects observed by valproate (Monti et al., 2009). Similarly we have observed that the neurotrophic factors BDNF and GDNF are significantly upregulated upon valproate treatment, confirming the studies by Wu and colleagues (Wu et al., 2008) who demonstrated that an astrocytic cell line treated with valproate displays a time dependent increase in expression of both BDNF and GDNF; an effect which translated to neuroprotection in midbrain neuronal cultures in medium transfer experiments. Additionally, Wu and colleagues demonstrated that the GDNF promoter associated histone H3 is significantly hyperacetylated when astrocytes are treated with valproate. Importantly, we also showed in this animal study for the first time that gene expression of the anti-apoptotic molecule BCL2 is upregulated upon valproate treatment, and the pro-apoptotic molecule BAD is downregulated. This is in agreement with previously data from Kidd and Schneider (2010) in which dopaminergic cells treated with valproate in vitro displayed a reduction in MPP+ induced activation of caspase-3 indicative of apoptosis inhibition. The findings from the current study therefore suggest that valproate’s mechanism of histone acetylation mediated neuroprotection/restoration is a multifaceted and complex affair, one which may hold therapeutic potential against a complex disorder such as PD.

The doses of valproate administered to rats in this study translate to human equivalent doses of 64 and 32mg/kg/day (400 and 200mg/kg/day rat dose respectively, since rats metabolise valproate quicker) (as calculated using FDA guidelines (FDA, 2005)). These doses are far greater than the usual therapeutic maintenance dose of valproate used for the
treatment of epilepsy in humans (1000-2000mg/day) and hence may result in increased incidence of side effects such as extrapyramidal effects, tremor, sleepiness, cognitive problems etc. if administered to humans (Britain, 2009). Obviously, such side effects would aggravate PD symptomology, hence valproate itself, at the doses examined here, may not be a candidate for repositioning for PD. However, the current study acts as proof-of-principle that delayed start treatment with a HDACI is capable of producing a neuroprotective phenotype in this animal model of PD.

In conclusion, utilising a clinically relevant drug testing platform this study clearly demonstrates that the HDACI valproate is dose-dependently neuroprotective and neurorestorative in the lactacystin rodent model of PD when administered chronically starting seven days after the toxin administration when behavioural and MRI deficits are already evident and a significant deficit in dopaminergic neurons is observed. These effects of valproate are associated with a reversal of histone hypoacetylation and an upregulation of neuroprotective and neurotrophic factors. This therefore supports the potential neuroprotective benefits of HDAC inhibition in PD and advocates further investigation of which specific HDAC isoforms are responsible for the neuroprotective effects of valproate in lieu of a clinically translatable HDACI treatment strategy for PD.
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Conflicts of Interest Statement
Authors declare that there are no conflicts of interest.
References


Valproate Neurorestoration in Parkinsonian Rats


**Figure Legends**

**Figure 1 - Animal Treatment Groups and Study Design**

*All daily i.p. injections given as 2ml/kg: saline injections given as 2ml/kg empty saline; 400mg/kg valproate injections given as 2ml/kg of 200mg/ml solution of valproate in saline; 200mg/kg valproate injections given as 2ml/kg of 100mg/ml solution of valproate in saline.

#Only groups Lacta(+)VPA(-), Lacta(+)VPA(+) and Lacta(+)VPA(++) intranigrally injected with lactacystin. Control groups remained surgically naïve.

Only groups lesioned with lactacystin were tested using the amphetamine induced rotation test at these time points.

Abbreviations: VCT, vertical cylinder test; AIR, amphetamine induced rotations; MRI, magnetic resonance imaging.

**Figure 2 - Valproate Attenuates Behavioural Motor Deficits Caused by Lactacystin**

(A) Vertical cylinder test outcomes show that once animals begin treatment with valproate at week 1 the lactacystin induced reduction in percentage contralateral forelimb use is reversed in a dose-dependent manner. (B and C) Amphetamine induced rotation test outcomes show that animals treated with valproate perform dose-dependently less rotations at each of the examined time points after starting treatment with valproate at week 1. Statistical significance denoted with either asterisks: (*p<0.05) or letters if p<0.05 for each comparison (see text for degrees of significance) : a, significantly different from group Lacta(-)VPA(-); b, significantly different from group Lacta(-)VPA(++); c, significantly different from group Lacta(+)VPA(-); d, significantly different from group Lacta(+)VPA(+). Data is presented as mean ± SEM. n = 6-7 per group. Abbreviations: Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++), treated with 400mg/kg/day valproate.

**Figure 3 - Manual Segmentation Analysis of MR Images Reveals Dose-dependent Attenuation of Lactacystin Induced Volumetric Changes by Valproate**

Administration of valproate dose-dependently attenuates volumetric changes observed in the (B) lateral ventricles and (C) the midbrain as a result of lactacystin-lesioning, as ascertained through manual segmentation analysis of rat brain MR images. Similarly administration of valproate and/or lactacystin have marked effects on (A) whole brain volume. Statistical significance denoted with letters if p<0.05 for each comparison (see text for degrees of significance): a, significantly different from group Lacta(-)VPA(-); b, significantly different from group Lacta(-)VPA(++); c, significantly different from group Lacta(+)VPA(-); d, significantly different from group Lacta(+)VPA(+). Data is presented as mean ± SEM. n = 6-7 per group. Abbreviations: Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++), treated with 400mg/kg/day valproate. See also Supplementary Fig. 2.

**Figure 4 - Tensor Based Morphometry Validates Findings From Manual Segmentation Analyses of Rat Brain MR Images**

Regions of significant volume difference relative to whole brain for each group compared with Lacta(-)VPA(-) at week 5 are shown. Positive differences indicate where each group has volume increases compared with Lacta(-)VPA(-) and negative differences indicate where each group has volume decreases compared with Lacta(-)VPA(-). Results shown are significant after correction for multiple comparisons across voxels using the False Discovery Rate with q=0.05. n = 6-7 per group. Abbreviations: Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++), treated with 400mg/kg/day valproate.
Figure 5 - Valproate Treatment Causes Dose-dependent Protection/Restoration of Dopaminergic Neurons in the SNpc in Lactacystin Lesioned Animals
Stereologically estimated (A) TH+ and (B) Nissl+ neuron numbers in the SNpc of rats suggest a dose-dependent neuroprotective/restorative effect of valproate in this lactacystin rat model of Parkinson’s disease. This is exemplified by the percentage interhemispheric loss of TH+ (C) and Nissl+ (D) neurons calculated between hemispheres of the SNpc. Statistical significance indicated with asterisks: *p<0.05; **p<0.01, ***p<0.001. Data is presented as mean ± SEM. n = 6-7 per group. Abbreviations: C, contralateral; I, ipsilateral; Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++) treated with 400mg/kg/day valproate.

Figure 6 – Neuroprotection/Restoration in the SNpc of Lactacystin Lesioned Animals
Representative examples of the TH and Nissl stained ipsilateral (A-F) and contralateral (A’-F’) SNpc of rats in each of the five treatment groups and group of animals sacrificed prior to drug treatment. Scale bar equal to 500µm. Abbreviations: Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++) treated with 400mg/kg/day valproate.

Figure 7 - Valproate Attenuates Lactacystin Induced Reduction in Frontal Brain Histone Acetylation
Administration of systemic valproate dose-dependently reverses the reduction in histone H3-lysine 9 acetylation caused by lactacystin. (A) Densitometry analysis of the AcH3-Lys9 band relative to the β-actin band used as a loading control. (B) Representative blot of data presented in (A). Statistical significance indicated with asterisks: *p<0.05; **p<0.01. Data is presented as mean ± SEM. n = 6-7 per group. Abbreviations: C, contralateral; I, ipsilateral; Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++) treated with 400mg/kg/day valproate.

Figure 8 - Valproate Upregulates Expression of Neuroprotective and Neurotrophic Growth Factor mRNA in the Frontal Brain
(A) Administration of systemic valproate alone, in surgically naïve rats upregulates bilateral expression of BDNF. (B) In lactacystin-lesioned animals valproate dose-dependently upregulated unilateral expression of αSyn, BDNF, GDNF, HSP70 and BCL2, as well as reducing the expression of BAD when given at its highest dose. Statistical significance indicated with asterisks and hashes: *p<0.05, **p<0.01, ***p<0.001 compared with the same hemisphere of saline treated group; #p<0.05, ##p<0.01, ###p<0.001 compared with the same hemisphere of Lacta(+)VPA(+). Data is presented as mean ± SEM. n = 6-7 per group. Abbreviations: C, contralateral; I, ipsilateral; Lacta(-), lactacystin-naïve; Lacta(+), lactacystin-lesioned; VPA(-), treated with vehicle; VPA(+), treated with 200mg/kg/day valproate; VPA(++) treated with 400mg/kg/day valproate. See also Supplementary Table 1.
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<td>Vertical Cylinder Test &amp; Amphetamine rotation</td>
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**Figure 1**

- Intanginal injection of lactate or saline
- Daily i.p. injection of either saline or valproate in 2ml/kg
- Baseline, Week 1, Week 3, Week 5
- VCT, VCT & AMT
- Animals sacrificed and brains removed for analysis
Figure 5
Figure 6
Figure 7