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Supplementary Materials for

Chronic administration of an HDAC inhibitor treats both neurological and systemic Niemann-Pick type C disease in a mouse model

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The PDF file includes:

Supplementary Materials

Extended Materials and Methods

Materials

All fine chemicals including HPBCD powder (H107) and PEG400 were obtained from Sigma (St Louis, MO, USA) unless otherwise indicated. Vorinostat was from Selleck Chemicals (Houston, TX, USA). DMEM and trypsin were from Life Technologies (New York, NY, USA). FBS was procured from ATCC. Oligonucleotides for qPCR were purchased from Invitrogen (Carlsbad, CA, USA).

Production of *Npc1^{nmf164}* mutant mice

Npc1^{nmf164} is a BALB/*c* strain derived from the *Npc1^{nmf164} in* C57BL/6J (*11*) which contains an ethyl-nitroso urea-induced point mutation in the *Npc1* gene. The mutation is a single nucleotide change (A to G at cDNA bp 3163) resulting in an aspartate to glycine change at position 1005 (D1005G). This results in slower diseases progression due to a partial loss in NPC1 function. The mutation was transferred from C57BL/6J to the BALB/c strain by Robert P. Erickson, University of Arizona Health Sciences Center, Tucson, AZ, USA and is available at 'The Jackson Laboratories', Bar Harbor, Maine, USA. Homozygous mutants (*Npc1^{nmf164}*) along with wild-type littermates (*Npc1$^{+/+}$*), were generated by crossing heterozygous mutant (*Npc1$^{+/nmf164}$*) males and females, in-house. For genotyping, restriction digestion method published earlier (*11*) was used with slight modification. A DNA fragment of 469bp was PCR amplified using forward primer 5'-CTTAATATGAATGGCTTGAGTCTGT-3' and reverse primer 5'-TAAGGAGTTTCTTTAAACC GTGTC-3' and subsequently digested with BstEII.

Npc1$^{+/+}$ is not digested, *Npc1*$^{+/nmf164}$ yields three bands of sizes 469, 285 and 184bp and *Npc1*^{nmf164} yields two bands of sizes 285 and 184bp.

Preparation of drug and injection to mice

Vorinostat (50mg/Kg)-Vorinostat was first dissolved in DMSO (100mg/ml) and then diluted with 9 volume of Polyethylene Glycol 400 (PEG). This drug solution was named as 'solution A'. Solution A was diluted with equal volume of water where the final concentration of each component was as follows; Vorinostat, 5mg/ml; DMSO, 5% and PEG, 45%. Mice were given a once weekly intraperitoneal (i.p) injections starting at 21 days. Vorinostat (100mg/Kg)- Vorinostat was first dissolved in DMSO (200mg/ml) and then mixed with 9 volume of PEG. Rest of the methods and injections plan were as described for vorinostat (50mg/Kg). In the study, vorinostat was used at 50mg/Kg unless mentioned otherwise. 2 x HPBCD (4000mg/Kg, double the dose used in TCF)- 40% HPBCD solution prepared in water. Mice were given a once weekly i.p injections starting at 7 days. HPBCD (2000mg/Kg) - 20% HPBCD solution prepared in water. Mice were given a once weekly i.p injections starting at 7 days. Triple combination formulation (TCF) (Vorinostat, 50mg/Kg + HPBCD, 2000mg/Kg + PEG, 45% + DMSO, 5%)- To prepare the formulation, the 'solution A' was first prepared as described above and equal volume of 40% HPBCD solution was slowly layered on top of it. The solution was gently mixed for 10 min at RT on rocker set at low speed. The final concentration of each component in the formulation was as follows; Vorinostat, 5mg/ml; HPBCD, 20%. PEG, 45% and DMSO, 5%. Mice were given two i.p doses of HPBCD (2000mg/Kg) at 7 and 15 days. Starting from 21 days, mice were given a once weekly i.p injection of TCF. Vehicle control (5% DMSO and 45% PEG) - It was made by mixing 1 volume of DMSO with 9 volume of PEG and then diluted with equal volume of water.

Mice were given a once weekly i.p injection starting from 21 days. HPBCD (2000mg/Kg) with vehicle (DMSO, 5% + PEG, 45%). It was made by mixing 1 volume of DMSO with 9 volume of PEG and then diluted with equal volume of 40% HPBCD. Mice were given two i.p doses of HPBCD (2000mg/Kg) at 7 and 15 days. Starting from 21 days, mice were given a once weekly i.p injection of HPBCD with the vehicle. All drug solutions were stored at -80°C. Fresh vials of frozen stock were thawed for injection on different days. The injection volume across the treatment group was 10ml/Kg body weight. For marker analysis, mice were sacrificed at 100 days and organs were harvested. For survival studies, injections were continued until the animals lost ≥30% of maximum weight at which they were sacrificed. Age at sacrifice served as days of survival.

Neurobehavioral assessment of mice

A modified version of the previously described method (*49*) was used for assessing the neurobehavioral functions in mice. Six different parameters (Fig. 3A) associated with neurobehavioral functions of mice were assessed. Each mouse was assessed individually in an observation box (length, 31.8 cm, width, 19.8 cm and height, 10.5 cm) with a grid floor. A mouse was assessed for tremor (0 and 2), body position (0, 1 and 2), gait (0, 1 and 2), grooming (0, 1 and 2), limb tone (0,1 and 2), and weight loss (0, 1, 2 and 3). More specific descriptions of the assessments along with the equivalent human symptoms are provided in the figure 3A. For each symptom except weight, a mouse received a score 0 if no symptom was observed and score 2 when the most severe impairment in the function was seen. A mouse was given score 0 for weight loss below 5%, 1 for 5-10%, 2 for >10 and up to 20%, and 3 for >20 up to 30%. A cumulative score of 0-3 correlate with no neurobehavioral impairment and a score of 13 is the

most impaired neurobehavioral function. Operator-independence scoring was also tested by two independent blinded operators on 6 *Npc* (*Npc1*^{nmf164}) and 4 healthy control (*Npc1*$^{+/nmf164}$) mice.

Mouse fibroblasts culture and drug treatment

Ear pinna was cleaned with 70% alcohol and 2-3 small pieces (3×3mm) were chopped and placed in 70% alcohol for 2 min and transferred to DMEM. Tissues were cut into small pieces, 2 ml of 0.25% trypsin were added, vigorously vortexed for 2 min and incubated at 37 °C with vortexing every 10 min. Trypsin was inactivated by adding 2ml of culture media (DMEM+10%FBS). Cells were collected by spin (1000rpm for 5min) and were grown in DMEM+10% FBS in the presence penicillin (50U/ml) and streptomycin (50µg/ml). For treatment with vorinostat, fibroblasts (4×10^4) were plated in 24 well plate containing glass slide. *Npc1*^{nmf164} fibroblasts were treated with 5µM vorinostat for 48 hrs. Cells incubated with 0.025% DMSO served as vehicle control. Cells were fixed with 4% paraformaldehyde followed by incubation with filipin (100µg/ml) to stain cholesterol. Slides were mounted using Vectashield (Vector laboratories) and processed for fluorescence microscopy.

RNA extraction and quantitative PCR

Formalin fixed paraffin embedded brain and liver were sectioned (sagittal in the case of brain,4-5µm) and total RNA was isolated using RNeasy FFPE kit (Qiagen, Germantown, MD, USA) which included treatment with DNAse. Total RNA from frozen spleen was isolated using RNeasy Plus Universal kit from Qiagen. The quantity of RNA was determined using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative PCR (qPCR) was performed using Power SYBR Green RNA-to-C_T 1-Step Kit and an ABI Prism 7500 Fast real-time PCR system (Applied Biosystems, Grand Island, USA). The reaction was set in 20µl using 100 nM primers and 5-100 ng total RNA as a template in duplicate wells. The thermal cycling parameters were as follows: step 1, 48 ºC for 30 min; step 2, 95 ºC for 10 min; step 3, 95 ºC for 15 sec; step 4, 60 ºC for 15 sec. Step 3-4 was repeated for 40 cycles followed by melt curve analysis. The nucleotide sequences of the primers for *Npc1* transcripts were, forward, 5'-GGTCTTACTCGGAGCCACTC-3; reverse, 5'-GAGCCGTTCTCTCTCTGTCC-3', for calbindin1 were, forward, 5'-GGAGCTATCACCGGAAATGA-3'; reverse, 5'-CTGTGGGTAAGACGTGAGCC-3', for *Gba* (glucocerebrosidase) were, forward, 5'-GCTTTGTCCCCACCTACTCA-3'; reverse, 5'-GATGGAGAAGTCACAACTGGC-3', for *Galc* (galactosylceramidase) were, forward, 5'-CGAGCGATAATCTCTGGGAG-3'; reverse, 5'-CCTGACATCTTTGCATTCCA-3' and for *Glb1* (galactosidase, beta1) were, forward, 5'-ACTGCTGCAACTGCTGGG-3'; reverse, 5'-AATGGCTGTCCATCCTTGAG-3'. Other primers were as described elsewhere (*33*). Specific amplification was validated by analysis of template titration, melt curves, and agarose gel electrophoresis. The mRNA levels were normalized to the housekeeping gene, *Gapdh* (*Glyceraldehyde 3-phosphate dehydrogenase*). The Relative quantification of gene expression was done by comparative C_T method using average C_T values of untreated age-matched healthy control ($Npc1^{+/nmf164}$) mice as a reference.

Organ harvest

Mice were sacrificed by asphyxiation using CO_2. Harvested organs were cut into two halves (sagittal half in case of the brain), one-half was immersed fixed in 10% neutral buffered

formalin (~4% formaldehyde) for 24 hrs at RT whereas the other half was flash frozen in the liquid nitrogen and subsequently stored at -80 °C. The fixed organs were subsequently stored in 70% alcohol at RT until transfer to paraffin.

Indirct Immunofluorescence assay

Paraffin-embedded tissue sections (4–5 µm) were dewaxed in xylene and alcohol. Calbindin antigen retrieval was done by pre-incubating deparaffinized samples with 0.05% proteinase K (Dako, Germany) in 50mM Tris-HCl (pH 7.5) for 8 min at RT. Cathepsin S, NPC1, and Iba1 was retrieved by boiling the sections in acidic condition for 30 min. Blocking was done either with 2% goat serum (for calbindin, NPC1 and Iba1) or 2% rabbit serum (for cathepsin S) for 30 min at RT. Sections were incubated with anti-calbindin (1:1000, C9848, Sigma), anti-cathepsin S (20µg/ml, M-19, Santa Cruz Biotechnology), anti-NPC1 (custom made against human NPC1 protein, 20µg/ml), anti-Iba1 (1:500, 019-19741, Wako Chemicals) overnight at 4 °C. The appropriate FITC or TRITC-conjugated secondary IgG (MP Biomedicals, Solon, OH, USA) antibodies were used at 1:200 dilution. Sections were subsequently washed with PBS containing DAPI (0.5µg/ml). Sections after mounting using Vectashield (Vector laboratories) processed for fluorescence microscopy.

Western blotting

Rabbit polyclonal antibodies to human NPC1 protein was custom made (Thermo Scientific, Rockford, IL, USA) against the peptide ^{1272}CATEERYKGTERERLLNF^{1289} corresponding to the C-terminus of the protein. Antibodies were purified using peptide affinity column. To detect NPC1 protein in fibroblasts, cell lysate was prepared in RIPA buffer and

proteins were resolved by 8% SD-PAGE. For analysis in the brain, sagittal section of frozen tissue was homogenized and the lysate was prepared using Mem-PER^{TM} Plus Membrane Protein Extraction Kit (Thermo Fischer Scientific) as per the manufacturer's instructions. The soluble cell lysate was resolved by 8% reducing SDS-PAGE. After blotting, membrane was treated with 62.5mM Tris-Cl, pH 6.8 containing 2% SDS and 100mM beta-mercaptoethanol at 50°C for 30 min. The blot was extensively washed before proceeding to blocking. Monoclonal anti-alpha-tubulin antibodies (Millipore, CA, USA) was used as loading control. To check the acetylation of histones**,** *Npc1*^{nmf164} mice (6-7 weeks) were injected with different drugs with the doses used for survival studies as described above through i.p route. Mice were sacrificed 1hpi by asphyxiation using CO_2. Harvested organs were immediately snap-frozen in liquid nitrogen. Brain and liver pieces were homogenized in Dounce homogenizer and the total histones were extracted using 'EpiQuik Total Histone extraction kit' (Epigentek, NY, USA) as per manufacturer's instructions. An equal amount of histones (15µg/lane) were resolved by 18% SDS-PAGE. Antibodies used to test acetylation of histones were anti-histone H3 (Lys14) and anti-histone H4 (Lys5/8/12/16) from Millipore (CA, USA). For loading control, an equal amount of histones were resolved on a parallel gel, stained with Coomassie Brilliant Blue and identified based on their molecular weights. Appropriate HRP-conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA). Quantification was done using ImageJ software (NIH, MD, USA).

Fluorescence microscopy

Tissue sections after immunofluorescence assay and filipin-stained fibroblasts were visualized with 40× oil-immersion objective lens (NA 1.35). Filipin stain was visualized using DAPI filter. Digital image collection were performed using an Olympus IX inverted fluorescence

microscope and a Photometrix cooled CCD camera (CH350/LCCD) driven by DeltaVision software from Applied Precision (Seattle, WA, USA). DeltaVision software (softWoRx) was used to deconvolve these images. Images are single optical sections. Images were analyzed using 'softWoRx' or 'ImageJ' software (NIH, MD, USA).

Nissl and H&E staining

Paraffin-embedded brain sections (4–5 μm) were dewaxed in xylene and alcohol. Sections were stained with acidified 0.1 cresyl violet solution for 7 min followed by differentiation in 95% ethanol for 5 min. After further dehydration in 95% alcohol for 5 min, sections were cleared in xylene and mounted in cytoseal XYL (Thermo Scientific, Kalamazoo, USA). Tissue sections were processed for H&E staining according to standard methods. Images were visualized with DPIan Apo 40×/1.00 oil immersion objective lens (Nikon) and captured on a Nikon Olympus microscope, using a Nikon digital DS-Fi1-U2 camera controlled by NIS-Elements F3.0 Nikon software (all from Nikon Instruments INC, Tokyo, Japan).

Plasma markers for liver and kidney toxicity

Healthy *Npc1*$^{+/nmf164}$ mice were treated with TCF similar to *Npc1*^{nmf164} mice for 7-10 months and plasma markers of liver and kidney toxicity were custom analyzed by Charles River Laboratories (Wilmington, MA, USA).

Analysis of vorinostat in mice

Npc1$^{+/nmf164}$ mice (age 6-7 weeks) were injected with either vorinostat (50mg/Kg in 45% PEG and 5% DMSO) in 45% PEG or TCF through i.p route. For analysis in plasma, mice were

sacrificed 1hpi by asphyxiation using CO_2. Total blood was collected through a cardiac puncture in the presence of 100µl heparin and transferred to K_2EDTA microtainer tubes (VWR International, Chicago, IL, USA). Blood was immediately spun at 1500g at 4°C for 15 min. Plasma was transferred to a separate tube, immediately flash-frozen in liquid nitrogen and stored at -80°C until analyzed.

For pharmacokinetic experiment in brain, *Npc1*$^{+/nmf164}$ mice (age 6-8 weeks) were injected with vorinostat (50mg/Kg in 45% PEG and 5% DMSO) or TCF (Vorinostat, 50mg/Kg + HPBCD, 2000mg/Kg + PEG, 45% + DMSO, 5%) through intraperitoneal route. At 0.5, 1, 2 and 4 hr post injection, mice were asphyxiated with CO_2, blood was drawn by cardiac puncture and organs were perfused with 20 ml ice-cold PBS through the ventricle. Harvested brain was cut into two equal half (sagittal) and flash frozen in liquid nitrogen. Brain (one-half) was homogenized using a Precelly bead homogenizer system utilizing ceramic CK 14 beads. 2ng of deuterated internal standard (d_5-Vorinostat, Toronto Research Chemicals, Ontario, Canada) was added to plasma (50 µl) and brain homogenate prior to liquid extraction. To each, 1 ml of cold acetonitrile was added to precipitate the protein before collecting the supernatant and drying using a vacuum concentrator system. Prior to HPLC/MS/MS analysis, each sample was reconstituted in 100 µL of 50% water/50% acetonitrile. An Agilent 1200 Rapid Resolution liquid chromatography (HPLC) system coupled to an Agilent 6460 series QQQ mass spectrometer (MS/MS) was used to analyze Vorinostat in each sample. An Agilent Zorbax XBD-C18 2.1 mm x 50 mm, 3.5 µm column (Agilent Technologies, Santa Clara, CA) was used for HPLC separation. The buffers were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was as follows: time 0 minutes, 5% B; time 1 minute, 5% B; time 10 minutes, 95% B; time 11 minutes, 95 % B; time 12 minutes, 5% B; time 15 minutes, 5% B.

Retention time for Vorinostat /d_5- Vorinostat was 6.7 minutes. Multiple reaction monitoring was used for MS/MS analysis. The data were acquired in positive electrospray ionization (ESI) mode by monitoring the following transitions for Vorinostat: 265→232 with collision energy of 5 V, 265→172 with collision energy of 5 V, and 265→55 with collision energy of 40 V. For d_5-Vorinostat, data were acquired by monitoring the following transitions: 270→237 with collision energy of 5 V, 270→172 with collision energy of 5 V, and 270→55 with collision energy of 40V. The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 275°C, sheath gas flow rate of 7 L/minute, capillary voltage of 4000 V, and nozzle voltage of 1000 V. All data were acquired and analyzed using Agilent MassHunter software (version B.06). In the final drug calculation in mice plasma, the contribution of heparin to total volume was subtracted before plotting the numbers. Analysis of Vorinostat in both plasma and brain was performed at Metabolite Profiling Facility, Bindley Bioscience Center, Purdue University, IN, USA.

Fig. S1

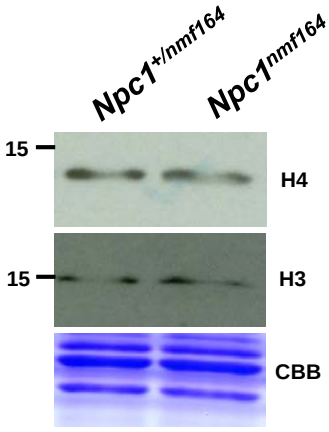

Fig. S1. **Baseline acetylation of histones in mouse brain tissue.** Baseline acetylation of histones 3 and 4 (H3 and H4) in brains of *Npc1*$^{+/nmf164}$ (healthy) and *Npc1*^{nmf164} (*Npc*) mice detected in western blots. Coomassie stained gel (CBB; blue) confirmed equal loading of samples. Molecular weight markers as shown in kDa.

Fig. S2

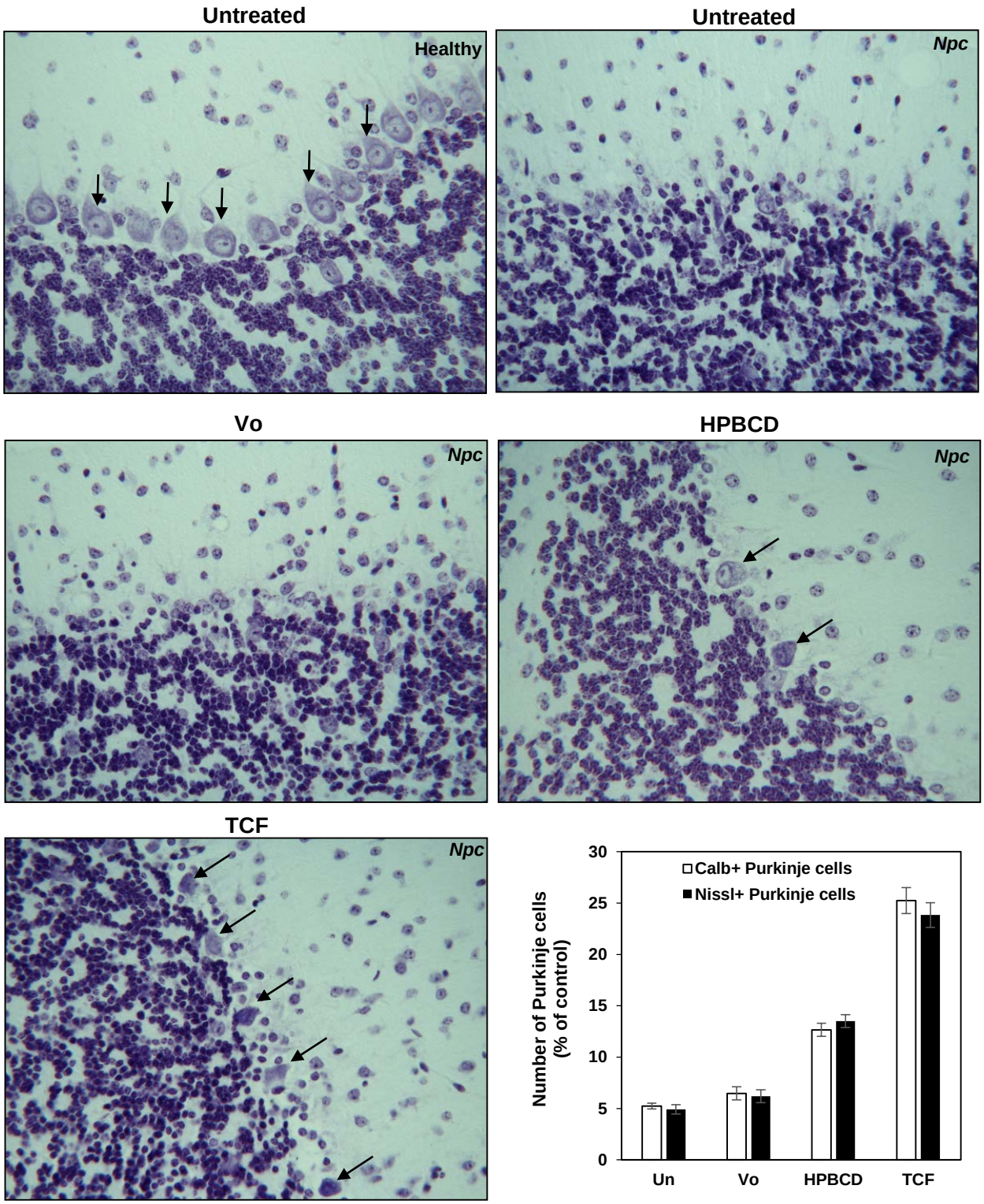
Untreated
Healthy
Untreated
Npc
Vo
Npc
HPBCD
Npc
TCF
Npc
Number of Purkinje cells (% of control)
Calb+ Purkinje cells
Nissl+ Purkinje cells
30
25
20
15
10
5
0
Un
Vo
HPBCD
TCF

Fig. S2. Nissl staining of mouse brain sections. Brain sections from heterozygous *Npc1*$^{+/nmf164}$ (Healthy) or *Npc1*^{nmf164}(*Npc*) mice treated as indicated were stained with Cresyl Violet acetate also known as Nissl stain. Numerous Purkinje cells (indicated by arrows) are evident in healthy controls. Several Purkinje cells were detected in TCF treated mice. Micrographs are representative images of *IX* lobule of the cerebellum. For quantitative analyses, 4 mice in each group were utilized to yield 8 sections (2 per mouse). The number of Nissl positive Purkinje cells were counted, and in the bar graph represented as a fraction of Purkinje cells seen in healthy control mice (*Npc1*$^{+/nmf164}$; set at 100%). Data for calbindin positive Purkinje cells were taken from Figure 2. Analysis of Purkinje cells by labelling with anti-calbindin antibodies and Nissl stain yielded similar numbers. Therefore, reduction in the number of Purkinje cells is attributed to the loss of Purkinje cells and not to reduced calbindin expression. Vo, vorinostat; HPBCD, 2-Hydroxypropyl beta cylcodextrin; TCF, triple combination formulation. Calb+, calbindin positive.

Fig. S3

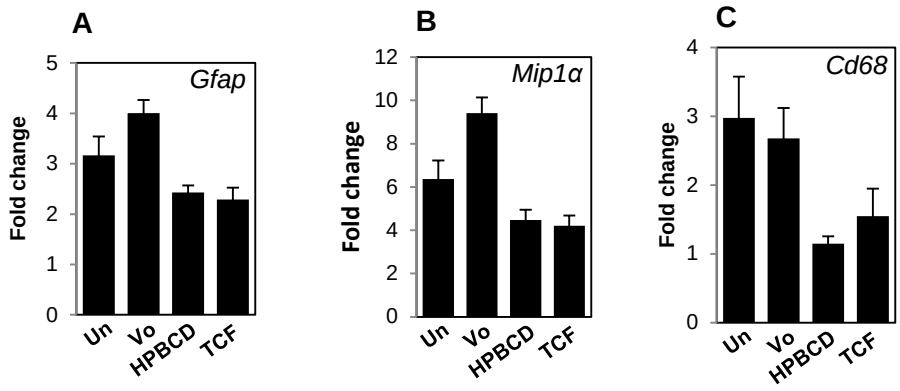

Fig. S3. **Quantitative analyses of brain inflammation. (A-C)** Quantitative PCR showing of inflammatory markers *Gfap*, *Mip1a* and *Cd68* at 100 days in the brains of *Npc1*^{nmf164} mice, treated as indicated. The fold change is expressed relative to untreated healthy control (*Npc1*$^{+/nmf164}$, set at 1) mice. Each group consisted of 4-5 mice. Un, untreated.

Fig. S4

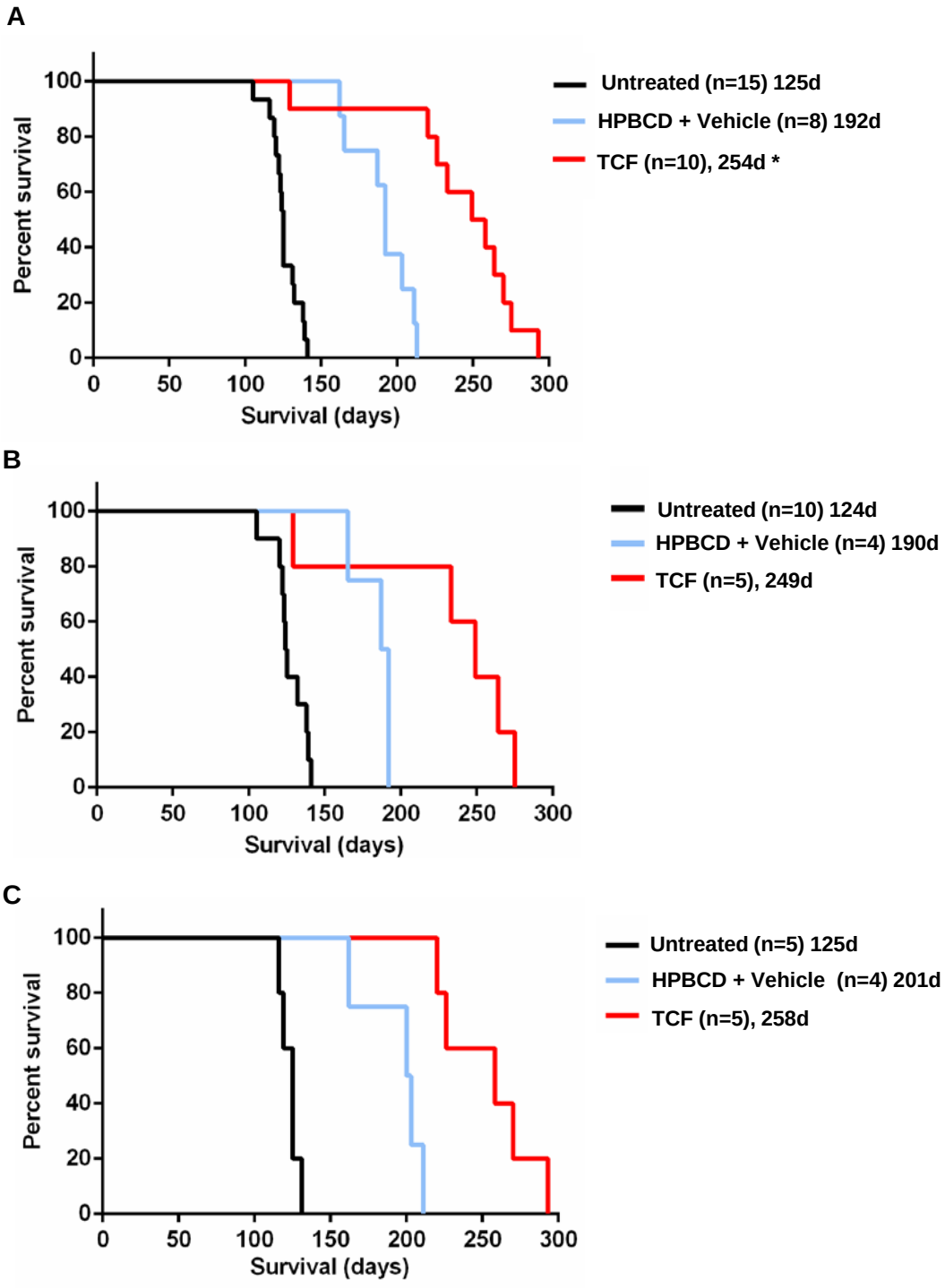

Fig. S4. Comparative analysis of survival between mice treated with TCF and those with HPBCD + vehicle. Kaplan-Meier survival curves of untreated and treated **(A)** *Npc1*^{nmf164} mice (both males and females) **(B)** male *Npc1*^{nmf164} and **(C)** female *Npc1*^{nmf164} mice. As indicated, mice were untreated, treated with TCF, or treated with HPBCD (2000mg/Kg) and vehicle (5% DMSO and 45% PEG) to enable direct comparative benefit of the addition of vorinostat to TCF on survival. Median survival (days) is indicated for each group. n, # mice; d, days. *p<0.001, TCF *vs* HPBCD+Vehicle.

Fig. S5

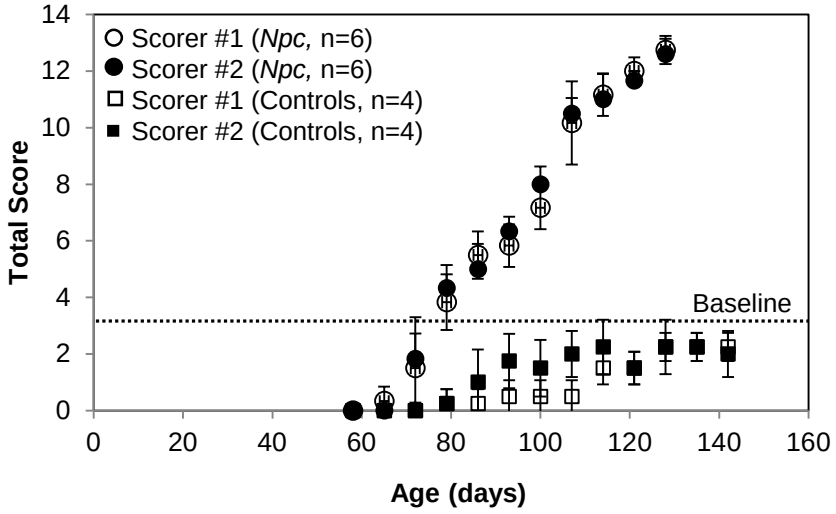

Fig. S5. Comparison of disease severity progression curves by two independent operators. Two operators scorer #1 (open) and scorer # 2 (filled), independently scored parameters used to assess the neurobehavioral symptoms of *Npc1*^{nmf164} (*Npc; circles*) mice and heterozygous *Npc1*$^{+/nmf164}$ mice (control; squares), as described in Materials and Methods. Progression of cumulative scores is shown for ~140 days. Six different symptomatic parameters namely, tremor, body position, gait, grooming, limb tone, weight were assessed on a cumulative score of 0-13. All symptomatic parameters were scored on a scale of 0-2 except weight, which was assessed on scale of 0-3. There was close correspondence between the values obtained by each scorer over the entire time period. Both scorers reported that older control mice showed an increased baseline of 2-3 due to poor grooming (particularly males) and slight impairment in limb tone from days 100-140 days. However, NPC-disease induced severity in these two symptoms occurred considerably earlier and hence these symptomatic domains were retained. Score above 3 (baseline, shown by dotted line) suggested onset of diseased state..

Fig. S6

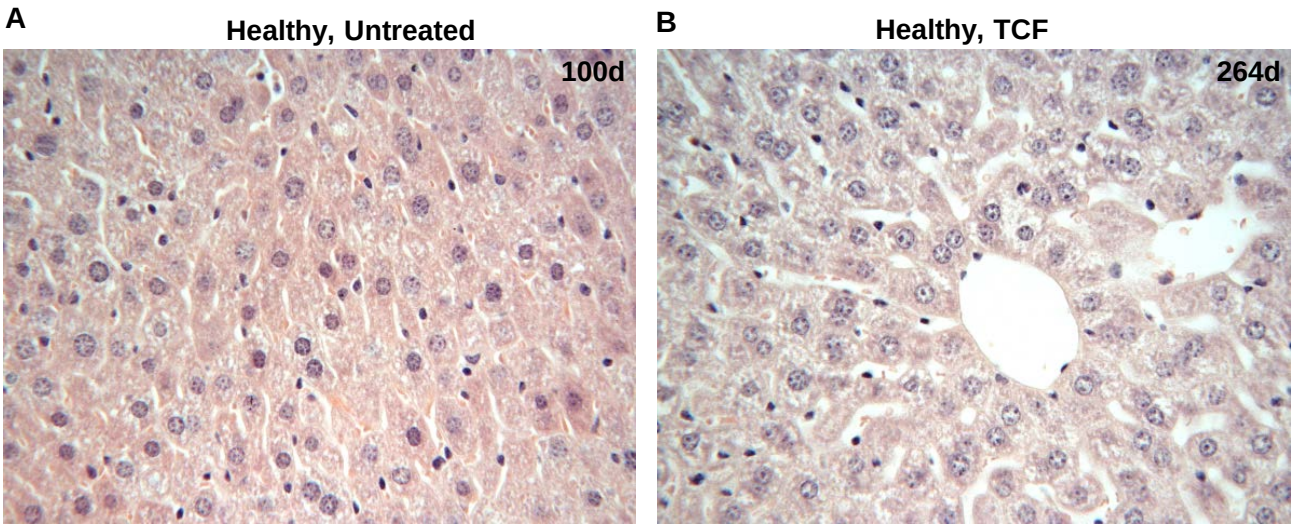

Fig. S6. Histological analysis of liver from mice treated long-term with TCF. Micrographs show H&E stained sections of liver from (A) untreated and (B) TCF-treated healthy (*Npc1*$^{+/nmf164}$) mouse at 100 and 264 days respectively. No signs of tissue lesions, immune cell invasion or hepatocyte structural abnormalities were seen in long-term TCF-treated mice even at 264 days. The images shown are representative from two untreated and four TCF-treated healthy mice. Images were taken with 40x objective lens.

Fig. S7

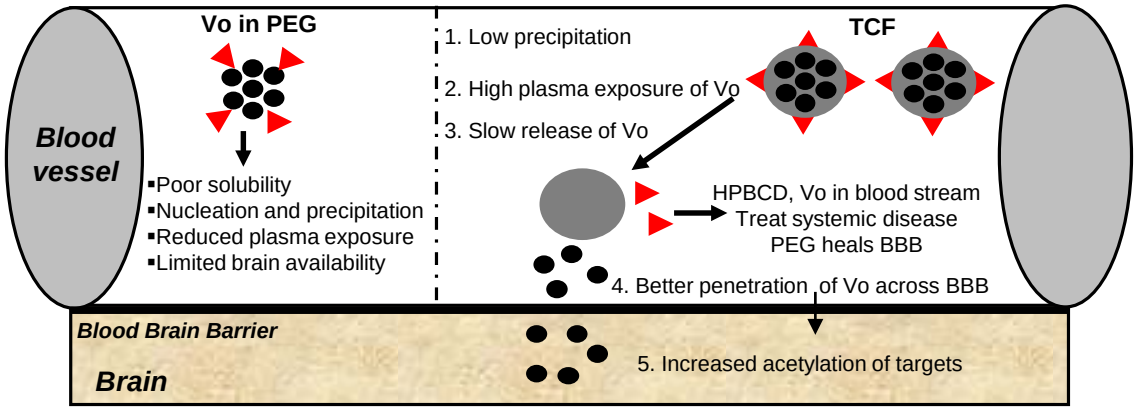

Fig. S7. **Generalized model for TCF in treating cerebral and systemic disease.** Vorinostat (Vo, solubilized in PEG) has poor solubility and therefore reduced plasma concentration which significantly limits its ability to cross the blood brain barrier (BBB). TCF enables slow release of vorinostat from the complex leading to better solubility and higher availability of vorinostat in plasma. Therefore, TCF boost the ability of vorinostat to cross the BBB. Vorinostat in brain act upon the target proteins (histones and others) and induces transcription of genes such as *Npc1*. Vorinostat may also indirectly (with or without the involvement of chaperones) increase levels of mutant NPC1 protein. HPBCD and vorinostat in blood stream treat systemic disease whereas PEG may help in reducing endothelial inflammation.

Table S1

List of parameters used to score the neurobehavioral functions of *Npc* mice

S. no	Parameters	Human equivalent	Score	Description
1	Tremor	Ambulation/ Motor	0	No tremor
			2	Light or strong tremor
2	Body position	Cognition/ Motor	0	Normal body position with straight back
			1	Slightly hunched back
			2	Severely hunched back
3	Gait	Ambulation/ Ataxia	0	Healthy normal gait
			1	Slightly ataxic gait
			2	Severely ataxic or no gait
4	Grooming	Cognition/ Motor	0	Smooth preened hair with sheen
			1	Poorly preened coat, tinge of yellowish fur around the neck
			2	Rough coat with minimal to no preen and sheen
5	Limb tone	Motor	0	Strong grasp with all four limbs, pull away from operator
			1	Grasp but no pull away from operator
			2	No grasp, no pull away from operator
6	Weight	Motor/ Swallow	0	Weight loss 0-5%
			1	Weight loss >5 up to10%
			2	Weight loss >10 up to 20%
			3	Weight loss >20-30%
		Total score	**13**	

Major human NPC symptomatic disease domain their murine counterparts and dynamic range are indicated