



Published in final edited form as:

*Neuropharmacology*. 2017 October ; 125: 189–196. doi:10.1016/j.neuropharm.2017.07.020.

## Valproate Increases Dopamine Transporter Expression through Histone Acetylation and Enhanced Promoter Binding of Nurr1

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### Abstract

The dopamine transporter (DAT) is the key regulator of dopaminergic transmission and is a target of several xenobiotics, including pesticides and pharmacological agents. Previously, we identified a prominent role for histone deacetylases in the regulation of DAT expression. Here, we utilized a rat dopaminergic cell line (N27) to probe the responsiveness of DAT mRNA expression to inhibitors of histone acetylation. Inhibition of histone deacetylases (HDACs) by valproate, butyrate and Trichostatin A led to a 3 to 10-fold increase in DAT mRNA expression, a 50% increase in protein levels, which were accompanied by increased H3 acetylation levels. To confirm the mechanism of valproate-mediated increase in DAT mRNA, chromatin immunoprecipitation (ChIP) assays were used and demonstrated a significant increase in enrichment of acetylation of histone 3 on lysines 9 and 14 (H3K9/K14ac) in the DAT promoter. Expression of Nurr1 and Pitx3, key regulators of DAT expression, were increased following valproate treatment and Nurr1 binding was enriched in the DAT promoter. Together, these results indicate that histone acetylation and subsequent enhancement of transcription factor binding are plausible mechanisms for DAT regulation by valproate and, perhaps, by other xenobiotics.

### Keywords

Dopamine transporter; epigenetics; HDAC; valproate; histone deacetylase; Pitx3; Nurr1

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The authors declare that they have no competing financial interests or other conflicts of interest.

## 1. Introduction

Mammalian histone deacetylase (HDAC) inhibitors are classified based upon sequence homology, structural differences and expression patterns (Morris and Monteggia, 2013). Class I HDACs (1, 2, 3 and 8) are generally located within the nucleus, whereas Class IIa (HDACs 4, 5, 7 and 9), Class IIb (HDACs 6 and 10) and Class IV (HDAC 11) HDACs are located in the cytoplasm or both (Fischer et al., 2010; Haberland et al., 2009). Although cancer treatment has been the most intensely studied application for HDAC inhibitors, the high expression of many HDAC isoforms within the brain (Broide et al., 2007) suggests that they may be promising targets in the treatment of neurological diseases or disorders. Indeed, drugs possessing HDAC inhibitory properties are currently used to treat bipolar disorder and epilepsy (Casey et al., 2003; Monti et al., 2009; Peterson and Naunton, 2005).

The class I HDAC inhibitor valproate has been implicated in the reduction of Schizophrenia-like behaviors and demonstrated neuroprotective effects in models of neurodegeneration (Kidd and Schneider, 2011; Tremolizzo et al., 2005), which has been linked to potential effects on the dopamine system. Dopaminergic transmission is essential to carry out brain functions such as locomotion, memory, motivated behaviors and cognition (Giros and Caron, 1993; Palmiter, 2008). Disruption in dopaminergic signaling is associated with many neurological disorders such as Parkinson's disease, Attention-deficit Hyperactivity disorder, Bipolar disorder and Schizophrenia (Damier et al., 1999; Howes and Kapur, 2009; Volkow et al., 2007). The dopamine transporter (DAT) is a transmembrane protein responsible for modulating dopaminergic transmission through selective uptake of the neurotransmitter dopamine (DA). Studies on the regulation of the DAT gene expression identified core promoter features and putative transcription factor binding motifs (Kouzmenko et al., 1997; Sacchetti et al., 1999), including that of the orphan nuclear receptor nuclear receptor related 1 protein (Nr4a2; Nurr1) and paired-like homeobox 3 protein (Pitx3) (Martinat et al., 2006; Riddle and Pollock, 2003; Smits and Smidt, 2006). In addition to transcription factor binding, the DAT promoter lacks a conserved TATA box, suggesting the potential for regulation by histone acetylation, similar to other TATA-less genes (Choi and Kim, 2008).

Work from our laboratory and others has demonstrated an increase of human DAT gene expression following treatment with the histone deacetylase (HDAC) inhibitor valproate *in vitro* (Wang et al., 2007). However, little is known about the epigenetic modifications and transcription factor binding patterns that may mediate the change in DAT gene expression following treatment with valproate. Histone acetylation is a post-translational modification of chromatin resulting in changes in DNA repair, DNA replication and gene transcription (Groth et al., 2007; Shahbazian and Grunstein, 2007). In this study, we further explore the role of histone acetylation in DAT gene regulation and identify a potential role for the nuclear transcription factor Nurr1 as a mediator of valproate-induced increases in DAT mRNA expression.

## 2. Materials and Methods

### 2.1. Chemicals

Cell culture supplies, including Roswell Park Memorial Institute media (RPMI 1640) with L-glutamine, penicillin (100 units/ml)/streptomycin (100 µg/ml), fetal bovine serum (FBS), phosphate-buffered saline (PBS) 1× without calcium and magnesium, trypsin EDTA (0.05% trypsin, 0.53mM EDTA in Hank's Balanced Salt Solution- without sodium bicarbonate, calcium, and magnesium) 1×, were purchased from Mediatech, Inc. (Herndon, VA). Valproic acid sodium salt (VPA), sodium butyrate (NaB) and Trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO). AlamarBlue reagent was purchased from Life Technologies (Grand Island, NY).

### 2.2. Cell Culture

The immortalized dopaminergic cell line 1RB<sub>3</sub>AN<sub>27</sub> (N27) has been extensively characterized for dopaminergic gene expression and was a generous gift from Dr. Anumantha Kanthasamy (Iowa State University). N27 cells were generated from embryonic day 12 rat mesencephalic tissue and transfected with plasmid vectors carrying T-antigen genes from SV40 for immortalization (Prasad et al., 1994). These cells stably express dopaminergic markers, including tyrosine hydroxylase (TH), NURR1 and DAT (Clarkson et al., 1998). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 IU/mL penicillin, 50µg/mL streptomycin and were maintained at 37°C with 5% CO<sub>2</sub>.

### 2.3. HDAC Inhibitor Treatment

One day prior to treatment, cells were seeded  $2 \times 10^5$  cells per well into 6 well plates. For HDAC inhibitor treatment, 25mM stock solutions of valproate and butyrate were made fresh in RPMI 1640 medium, diluted accordingly and added to the cells. A 1µM stock solution of Trichostatin A was prepared in 95% ethanol and diluted in RPMI media before adding to the cells. The final concentration of ethanol was less than 0.1% for all experiments. Following 24 hr of exposure, cells were harvested for RNA, protein and/or chromatin isolation.

### 2.4. Cell Viability Assay

N27 cells seeded at  $6 \times 10^3$  cells per well of a 96 well plate were treated the following day with various concentrations of HDAC inhibitors. Twenty four hours later the cell medium was replaced with 10% AlamarBlue (Life Technologies) reagent in 1× RPMI media and incubated for 2 hours at 37°C. The fluorescent signal was quantified with Spectromax M5 microplate reader at 570nm excitation and 585nm emission.

### 2.5. Immunofluorescence

Cells were seeded at 25,000 cells/well in 8 well chamber slides. Following treatment, cells were fixed with 10% formalin and then washed with PBS. Cells were then permeabilized using 0.1% Triton X-100 for 10 min at RT. Following this, cells were blocked with 2% BSA for 1 hour and incubated overnight with 1:1000 1:1000 DAT antibody (cat MAB369, Millipore). Cells were then incubated for 1 hour with rabbit anti-rat Alexa Fluor 488 secondary antibody. Images were taken using a Leica DMi8 SPE II confocal microscope

using a 40× oil lens (N.A. 1.15) and fluorescence intensity quantified using ImageJ. Specificity was determined by omission or primary or secondary antibodies. In ImageJ, a minimum threshold was set for all imaging, and fluorescence intensity was measured over the same area from multiple images (3 images) from multiple chamber slide wells (4 wells), approximately the same number of cells were measured for all wells.

## 2.6. RNA Isolation and cDNA Synthesis

RNA was isolated from cells using the Qiagen RNeasy Kit (Qiagen) as described previously (Hossain and Richardson, 2011). RNA concentration was determined using NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). One µg of total RNA was used for cDNA synthesis with the First Strand Synthesis Kit (Invitrogen) as per manufacturer instructions.

## 2.7. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR reactions were prepared in 25 µl total volume using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA); 1:15 diluted cDNA and 1µM of forward and reverse primers as described previously (Green et al., 2015). Primers were designed using the National Center for Biotechnology Information PrimerBLAST application (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>; Figure 1). PCR reactions were performed on a ViiA7™ Real-Time PCR system (Applied Biosystems, Carlsbad, CA) at the appropriate annealing temperature for each primer set. Relative gene expression was determined using the SYBR Green technology (Applied Biosystems, Carlsbad, CA). All samples were run in duplicate; β-actin and TATA Binding Protein (TBP) were used as normalization controls for each gene and gave similar results. All primer sets yielded a single PCR product of expected size by agarose gel electrophoresis (data not shown). Relative values were calculated using  $2^{-Ct}$  method as described by Livak and Schmittgen (Livak and Schmittgen, 2001).

## 2.8. Western Immunoblotting

Nuclear extracts were isolated from N27 cells treated with 1mM valproate using NE-PER™ Kit (Thermo Scientific) as per manufacturer instructions. Protein homogenates were supplemented with 0.1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentrations were determined using the Pierce™ bicinchoninic acid (BCA) assay kit (Thermo Scientific) and 50 µg of protein sample was loaded per lane on a 4–12% Bis-Tris Polyacrylamide Gel (Invitrogen). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in 7.5% non-fat milk in 0.1% Tween 20 and Tris buffered saline (TTBS) for 1 hour at room temperature. The membranes were incubated at 4°C overnight with rabbit anti-H3Ac primary antibody (1:2000; cat# 06-599 Millipore), followed by a 1 hour room temperature incubation with anti-rabbit HRP-conjugated secondary antibody. The bound antibody was detected by SuperSignal® West Dura Extended Duration Substrate Kit (Thermo Scientific) and imaged using Alpha Innotech Fluorochem imaging system. The membrane was stripped using Pierce Stripping Buffer (Thermo Scientific) and re-probed with rabbit anti-Histone 3 antibody to confirm equal protein loading.

## 2.9. Chromatin Immunoprecipitation (ChIP) Assay

N27 dopaminergic cells were plated  $3 \times 10^6$  cells per 150cm<sup>2</sup> flask one day prior to cell treatment and treated with 1 mM valproate for 24 hours. Cells were crosslinked using a final concentration of 1% formaldehyde for 10 minutes. The crosslinking reaction was stopped by adding 10× glycine, to a final concentration of 125 mM, for 5 minutes then washed 3 times in cold PBS supplemented with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cells were scraped into cold PBS and centrifuged at 2000rpm for 5 minutes at 4°C. Pellets were resuspended in cold cell lysis buffer (10mM Tris pH 8, 10mM NaCl, 3mM MgCl<sub>2</sub>, 0.5% NP40) supplemented with protease inhibitors for 15 minutes. Nuclei were pelleted at 2000rpm for 5 minutes at 4°C and resuspended in nuclear lysis buffer (1% SDS, 5mM EDTA, 50mM Tris pH8).

Chromatin was sheared for 12 cycles (15s on/60s off) to an average length of 300–700 bp using a sonic dismembrator (Fisher Scientific). Insoluble material was pelleted at 14,000rpm for 10 min at 4°C. The protein concentration of each chromatin sample was measured using the BCA assay kit. Sixty µg of chromatin was used for each immunoprecipitation (IP) reaction. IP reactions were performed with 4 µg of each antibody H3K9K14ac (cat # 9441, Cell Signaling), Nurr1 (cat # sc-991, Santa Cruz), Pitx3 (cat # 19307, Santa Cruz), Normal Rabbit IgG (cat# 12-370, Millipore) overnight at 4°C. Magnetic beads (Cell Signaling) were added to each IP reaction and rotated for 3 hours at 4°C. The beads were sequentially washed in low salt buffer (0.1 % SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8, 500mM NaCl), LiCl buffer (0.25M LiCl, 1% NP40, 1% Na deoxycholate, 1mM EDTA, 10mM Tris pH 8) and TE buffer (10mM Tris pH 8, 1mM EDTA), respectively. Chromatin was eluted from the beads using elution buffer (1 % SDS, 0.1M NaHCO<sub>3</sub>) and rotated at 65° C for 30 minutes. Crosslinking was reversed by adding 5M NaCl to each eluate and incubated at 65° C for 5 hours. DNA was treated with RNase A, Proteinase K and purified using Qiagen PCR Purification kit (Qiagen). qPCR was performed to determine relative enrichment of proteins in DAT promoter regions (See Fig. 1 for ChIP primer sequences).

## 2.10. Statistical Analyses

All data were analyzed using GraphPad Prism 5.0 Software (GraphPad Software, San Diego, CA). All experiments were performed at least three times on three different days. Individual experiments were conducted in duplicate or triplicate and averaged. Data are presented as mean ± SEM and analyzed by One-way ANOVA, Two-way ANOVA or Student's *t*-test, where appropriate. Statistical significance was determined at level of  $p < 0.05$ .

## 3. Results

### 3.1. Dose dependent increase in DAT mRNA expression following HDAC inhibitor treatment

Previously, we and others demonstrated that valproate increases DAT mRNA expression in human SK-N-AS neuroblastoma cells (Wang et al., 2007). We performed a dose response using valproate in rat mesencephalic cells to determine the highest dose, which also resulted in increased DAT expression. Although more sensitive to the dose used previously in SK-N-AS cells, peak expression was reached in N27 cells at 1mM with a 5-fold increase ( $F=$

43.53,  $df=5$ ,  $p<0.0001$ ) in DAT mRNA (Fig. 2A top figure). To determine whether other HDAC inhibitors had a similar effect, we used additional HDAC inhibitors that targeted histone proteins of different classes. Sodium butyrate, also a Class I HDAC inhibitor, showed up to 4-fold increase ( $F=16.51$ ,  $df=5$ ,  $p<0.0001$ ) in DAT mRNA expression profile following the same concentrations as valproate (Fig 2A middle figure). The more potent Class II HDAC inhibitor, Trichostatin A, showed 10-fold increase ( $F=9.96$ ,  $df=5$ ,  $p=0.001$ ) in DAT expression at 600nM (Fig. 2A bottom figure).

### 3.2. Reduced cell viability with increasing concentrations of butyrate and Trichostatin A

To determine optimal concentrations that induced DAT mRNA without compromising cell viability, an AlamarBlue assay was performed. There was no reduction in cell viability ( $F=1.61$ ,  $df=5$ ,  $p=0.223$ ) in any of the concentrations tested for valproate (Fig 2B top figure). Cell viability was significantly reduced with increasing concentrations of both butyrate ( $F=9.64$ ,  $df=5$ ,  $p=0.0003$ ) and Trichostatin A ( $F=8.47$ ,  $df=5$ ,  $p=0.0006$ ) (Fig. 2B middle and bottom figures, respectively). Because of this toxicity, valproate was the only compound used for remaining studies.

### 3.3. Increased DAT protein following 24 hour valproate treatment

In order to assess whether increased DAT mRNA corresponded to changes in DAT protein, we performed and quantified immunofluorescent staining of DAT in N27 cells following 24 hour exposure to 1 mM valproate. Data indicate a 50 % increase ( $t=10.23$ ,  $df=6$ ,  $p<0.0001$ ) in DAT protein levels (Figure 3).

### 3.4. Increased acetylated histone 3 protein in N27 cells following valproate treatment

Dose response and cell viability assays demonstrated 1mM valproate to be the optimal concentration. To determine whether histone proteins were altered following valproate treatment, western immunoblotting was performed on N27 cell nuclear lysates. Results confirmed over 10-fold increase ( $F=65940$ ,  $df=2$ ,  $p$  value  $<0.0001$ ) in acetylated histone 3 protein compared to untreated cells (Fig. 4A).

### 3.5. Histone protein enrichment in DAT promoter following valproate treatment in N27 cells

The known regulatory region of DAT includes an 18-kb promoter (Zhou et al., 2014) and the core promoter, spanning between  $-236$  and  $+44$  relative to the transcription start site, has been previously demonstrated to drive gene expression of DAT (Sacchetti et al., 1999). The lack of a conserved TATA and CAAT box suggests the presence of other regulatory elements, which may contribute to regulation of gene expression. The responsiveness of DAT mRNA induction following treatment of SK-N-AS neuroblastoma cells to histone deacetylase inhibitors (Green et al., 2015) further suggests a potential role for an epigenetic mechanism. To determine the role of histone acetylation and transcription factors in the role of DAT regulation, we performed chromatin immunoprecipitation (ChIP). Regions I-V represent 200bp fragments of the rat DAT promoter ( $-810$  to  $+218$ ; Fig. 1B). Co-immunoprecipitation of DAT with H3K9/K14ac showed increased enrichment within regions I ( $t=3.471$ ,  $df=4$ ,  $p=0.0256$ ) and IV ( $t=5.031$ ,  $df=3$ ,  $p=0.0151$ ) of the DAT promoter following 24-hour valproate treatment (Fig. 4B).

### 3.6. Transcription factor mRNA expression and enrichment in DAT

The transcription factors Nurr1 and Pitx3 are essential for the development and maintenance of midbrain dopaminergic neurons (Lee et al., 2010). Binding of Nurr1 in the DAT promoter has been shown to enhance DAT expression (Sacchetti et al., 2001). Previous studies established Pitx3 as an essential regulator of Nurr1-mediated transcription in midbrain dopaminergic cells (Yi et al., 2014). The mRNA expression of Nurr1 ( $t=4.912$ ,  $df=2$ ,  $p=0.039$ ) and Pitx3 ( $t=6.587$ ,  $df=2$ ,  $p=0.0223$ ) increase 4 and 6-fold, respectively, following 1mM valproate treatment of N27 cells (Figs. 5A and 6A). Immunoprecipitation of Nurr1 showed significant enrichment in region 1 ( $t=4.344$ ,  $df=3$ ,  $p=0.0225$ ) of the DAT promoter (Fig. 5B). There were no significant changes in Pitx3 enrichment (Fig. 6B).

## 4. Discussion

Here, we report that HDAC inhibitors are effective dose-dependent inducers of DAT gene expression *in vitro*. These findings confirmed previous studies showing valproate treatment increased DAT mRNA (Bence et al., 2012; Wang et al., 2007), and extended them by defining a molecular mechanism responsible for this effect. Previous studies found global changes in gene expression following HDAC inhibitor treatment and gene regulation through acetylation of other non-histone proteins (Bantscheff et al., 2011; Fass et al., 2013; Marinova et al., 2009; Perisic et al., 2010). The direct epigenetic modifications that occur on the DAT gene following HDAC inhibitor treatment have not yet been identified. Using HDAC inhibitors as model compounds to manipulate DAT gene expression, we identified a potential role of histone acetylation and enhanced Nurr1 transcription factor binding in the transcriptional regulation of the DAT following valproate treatment.

Valproate (valproic acid) was first identified as an anti-epileptic drug in the 1960's and is used today with broader therapeutic applications (Nalivaeva et al., 2009). A known mechanism of action of valproate is inhibition of Class I and IIa HDACs (Dokmanovic et al., 2007). Previous studies with valproate in human neuroblastoma cells showed over 4-fold increase in DAT mRNA with concentrations at 5mM (Wang et al., 2007), whereas we observed that doses over 2mM produced cell death. Similar results were obtained following treatment with the Class I/IIa inhibitor sodium butyrate and the more potent Class I/II inhibitor Trichostatin A. Because reduced cell viability was observed with increasing concentrations of butyrate and Trichostatin A, we focused on valproate for the remainder of the mechanistic studies. Valproate and sodium butyrate target the same HDAC classes within a similar  $IC_{50}$  range (Gottlicher et al., 2001; Marks et al., 2001; Rodriguez et al., 2006). Therefore, the cytotoxicity observed with sodium butyrate suggests that alternate targets other than HDACs may mediate this effect.

In addition to inhibiting HDACs, valproate has a number of targets that could mediate its effects on DAT expression (Novy et al., 2011). To verify histone acetylation was being targeted, we evaluated the most well studied and modified histone, H3 (Xie et al., 2012). Western immunoblotting showed a significant increase in H3 acetylation following valproate treatment. In addition to the common H3 acetylated histone marker, H3K9/K14ac is generally associated with open chromatin structure and increased gene expression (Roy et al., 2014). The hyperacetylated regions I and IV within the DAT promoter may be more

promising in identifying additional transcription factors and other markers involved in the regulation of DAT. Although valproate has been observed to induce DNA demethylation in some promoters (Manev and Uz, 2002; Tremolizzo et al., 2002), others have not observed this effect (Balasubramanian et al., 2015). Further, the DAT promoter is highly unmethylated (over 95%) in rat primary neurons (He et al., 2011) and we found no significant effect of DNA methyltransferase inhibitors (RG108 and 5-aza-2-deoxycytidine) on DAT expression in SK-N-AS cells (Green et al., 2015) or in N27 cells (data not shown). Therefore, it is unlikely that DNA demethylation by valproate contributed to the effects observed in our experiments reported here.

The orphan nuclear receptor Nurr1 and the paired-like homeobox gene Pitx3 are among the transcription factors involved in the development and maintenance of dopaminergic neurons (Riddle and Pollock, 2003; Smits and Smidt, 2006). Previous studies reported that Nurr1 and Pitx3 target genes involved in dopaminergic metabolism and regulation (Baffi et al., 1999; Hwang et al., 2009; Zetterstrom et al., 1997). Studies evaluating loss of Pitx3 demonstrate significant reduction in DAT mRNA expression and protein levels (Hwang et al., 2003; Smidt et al., 2004). Nurr1 knockout experiments show no expression of DAT and other dopaminergic genes suggesting a more dominant role of Nurr1 in DAT regulation (Jankovic et al., 2005; Smits et al., 2003). Valproate treatment significantly increased DAT mRNA expression in N27 rat mesencephalic cells, which was accompanied by significant increases in Nurr1 and Pitx3 mRNA expression. This is consistent with recent studies in which Nurr1 expression was increased following valproate treatment in a variety of cell types (Almutawaa et al., 2014; Yoshikawa et al., 2013). Although long assumed to regulate the DAT through different mechanistic pathways (Chung et al., 2005), growing evidence suggests that several transcription factors generally work together to maintain and regulate DAT expression in dopamine neurons (Martinat et al., 2006; Wallen et al., 1999).

He and co-workers used ChIP-qPCR to demonstrate enrichment of Nurr1 within the promoter of the DAT and increased DAT gene expression in neural progenitor cells from the ventral mesencephalon following depolarization with KCl (He et al., 2011). In that study, sites I and V of the rat DAT promoter were demonstrated to be enriched for Nurr1 following depolarization. Here, we determined that region I of the DAT promoter showed significant enrichment of Nurr1, whereas no change in binding was observed for region V. This difference may relate to the fact that He and co-workers did not observe increased H3 acetylation in any of the promoter regions following depolarization, but rather increased H3K4 methylation indicative of a different mechanism of relaxing chromatin and allowing enhanced binding of transcription factors.

In contrast to Nurr1 and in spite of significantly increased Pitx3 mRNA following valproate treatment, no significant enrichment for Pitx3 was observed in any of the DAT promoter regions. This is relatively consistent with a previous study, which has demonstrated that Nurr1 target genes were upregulated following HDAC inhibitor treatment in the absence of Pitx3 (Jacobs et al., 2009). It should be noted that although Nurr1 has been thought to directly bind to the DAT promoter through the NGFI-B responsive element (NBRE) (Murphy et al., 1996). Some studies suggest that, although Nurr1 may be immunoprecipitated at the DAT, it does not bind at the conserved NBRE site (Sacchetti et al., 2001) and that a Nurr1-



interacting protein may be responsible for the effects on DAT expression (Luo et al., 2008). More recently, *Foxa2* has been demonstrated to serve an important role in dopamine neuron development and increase DAT expression by driving an activator complex with Nurr1 that results in relaxed chromatin structure and increased H3 acetylation (Yi et al., 2014), similar to that observed here following valproate treatment. Further investigation will be required to directly assess the role of direct or indirect Nurr1 binding to the promoter region of the DAT following treatment with valproate.

Altered DAT levels adversely affect dopaminergic transmission and have been associated with several neurological disorders. Valproate has been shown to affect dopamine-related behaviors in several *in vivo* model systems (Tremolizzo et al., 2005; van Enkhuizen et al., 2013). Indeed, a previous study using an *in vivo* knock down model of the DAT showed that neurobehavioral alterations were attenuated following valproate treatment (Ralph-Williams et al., 2003), which might be the result of increasing DAT expression. More recently, valproate has been shown to be neuroprotective in animal models of Parkinson's disease (Carriere et al., 2014; Kidd and Schneider, 2011). Although several potential mechanisms for this neuroprotection are possible, the ability of valproate to increase Nurr1 expression and binding to promoter regions of catecholamine genes, including the DAT, tyrosine hydroxylase and the vesicular monoamine transporter 2 (Smits et al., 2003) may result in maintenance of these phenotypic markers of dopamine neurons during neurodegeneration. Moreover, Nurr1 has recently been demonstrated to play an important role in dampening neuroinflammation through its action in microglia (Fan et al., 2009; Saijo et al., 2009), which would be expected to be protective in Parkinson's disease. Additional studies will be required to determine the relative roles of these potential mechanisms in the neuroprotective effects of valproate.

## 5. Conclusions

The work presented in this manuscript is novel because we provide a direct mechanism by which valproate increases DAT expression that links the disparate individual observations identified above. Our results indicate that valproate treatment results in enhanced histone acetylation at the DAT promoter in cultured rat N27 cells. In addition to these findings, increased expression of the transcription factor Nurr1 and enhanced binding of Nurr1, or proteins associated with Nurr1, to the DAT promoter appears to be primarily responsible for the pharmacological induction of DAT by valproate. Taken together, these data provide a potential mechanism by which valproate exerts its effect on dopamine-related behaviors and for some of its neuroprotective effects in models of Parkinson's disease.

## Acknowledgments

This work was supported in part by the following NIH Grants: R01ES015991, R01ES021800, R01ES026057, R01GM1104037, P30ES005022, U01NS079249, T32007148 and R21ES013828. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or any other funding source.

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### Highlights

1. Valproate increases DAT mRNA and Protein
2. Valproate increases Nurr1 and Pitx3 mRNA
3. DAT increases result from increased H3K9 and Nurr1 enrichment in the DAT promoter

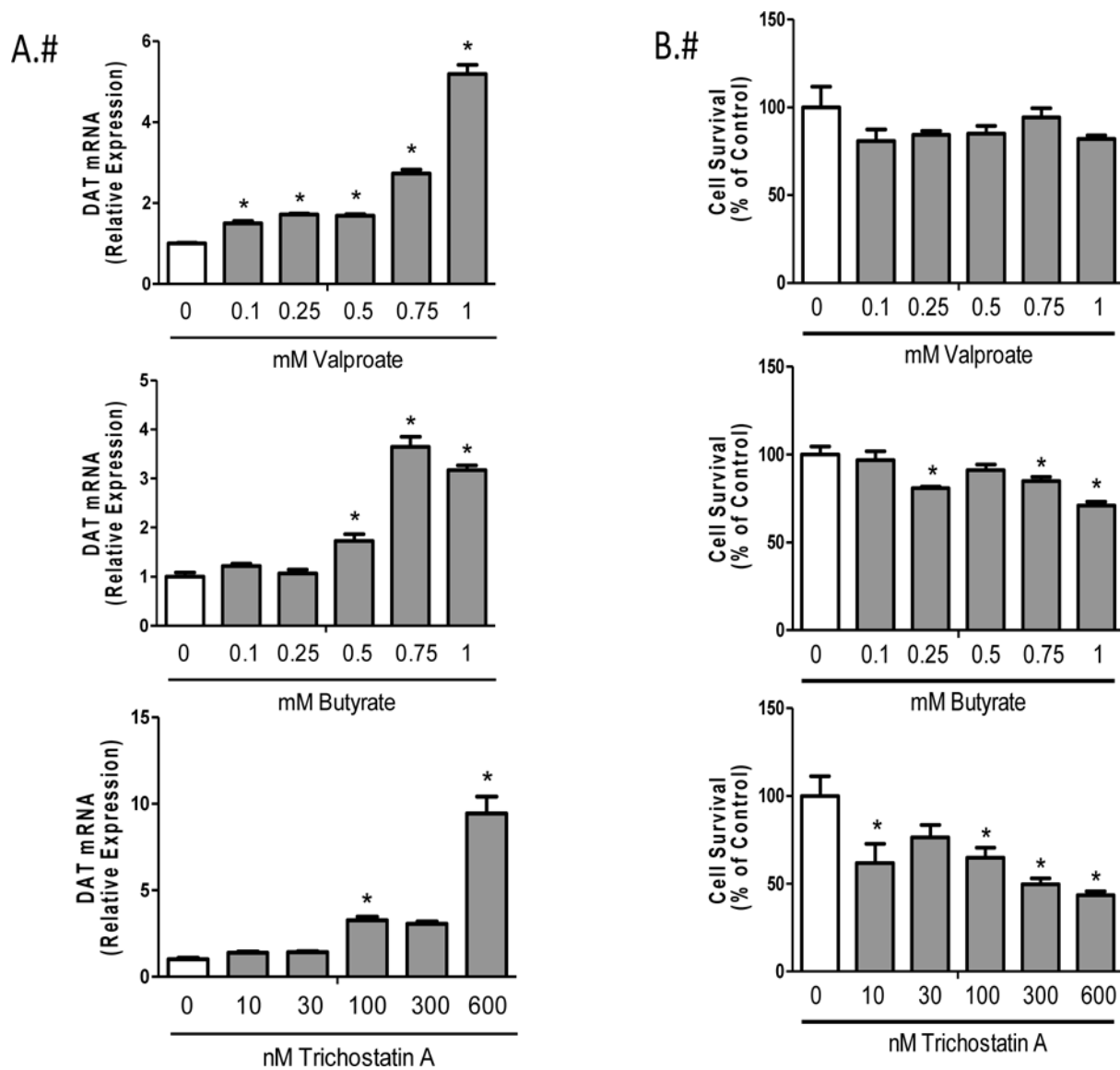
A.

Gene Target	Primer Sequence	Assay
DAT	F: GAG GTT TCC CTA CCT GTG CT	QPCR
	R: GTG AAG CCC ACA CCT TTC AG	
Nurr1	F: TAA AGA AGT GGT TCG CAC GG	QPCR
	R: GTC ATT GCC GGA TTG GAG TC	
Pitx3	F: CGT GCG GGT GTG GTT CAA G	QPCR
	R: TAC ACC TCC TCG TAG GGT GG	
TBP	F: CAC CAC CCC CTT GTA TCC TT	QPCR
	R: CAG CAA ACC GCT TGG GAT TA	
Actin	F: AAG ATC AAG ATC ATT GCT CCT CCT	QPCR
	R: GCT CAG TAA CAG TCC GCC TA	
DAT I	F: TACAGGACCTCAGAGCTGAA	ChIP
	R: CCCTTAGTTCGTGTGGAACG	
DAT II	F: CTTGGAACCAACTGTCCGT	ChIP
	R: TCACTTGGGGACTAGGATGA	
DAT III	F: AACAGTCGTTGGGAGCCCA	ChIP
	R: GGGCCGGCGAGGGGCTTGAC	
DAT IV	F: CTTGCTTTGTCCCTGCGGAG	ChIP
	R: GCGAGGTTGTCAGAAGGAGA	
DAT V	F: CTCTCATGGTCCTGAACAAC	ChIP
	R: ACTGAGGAGGAAAGAGTCC	

B.

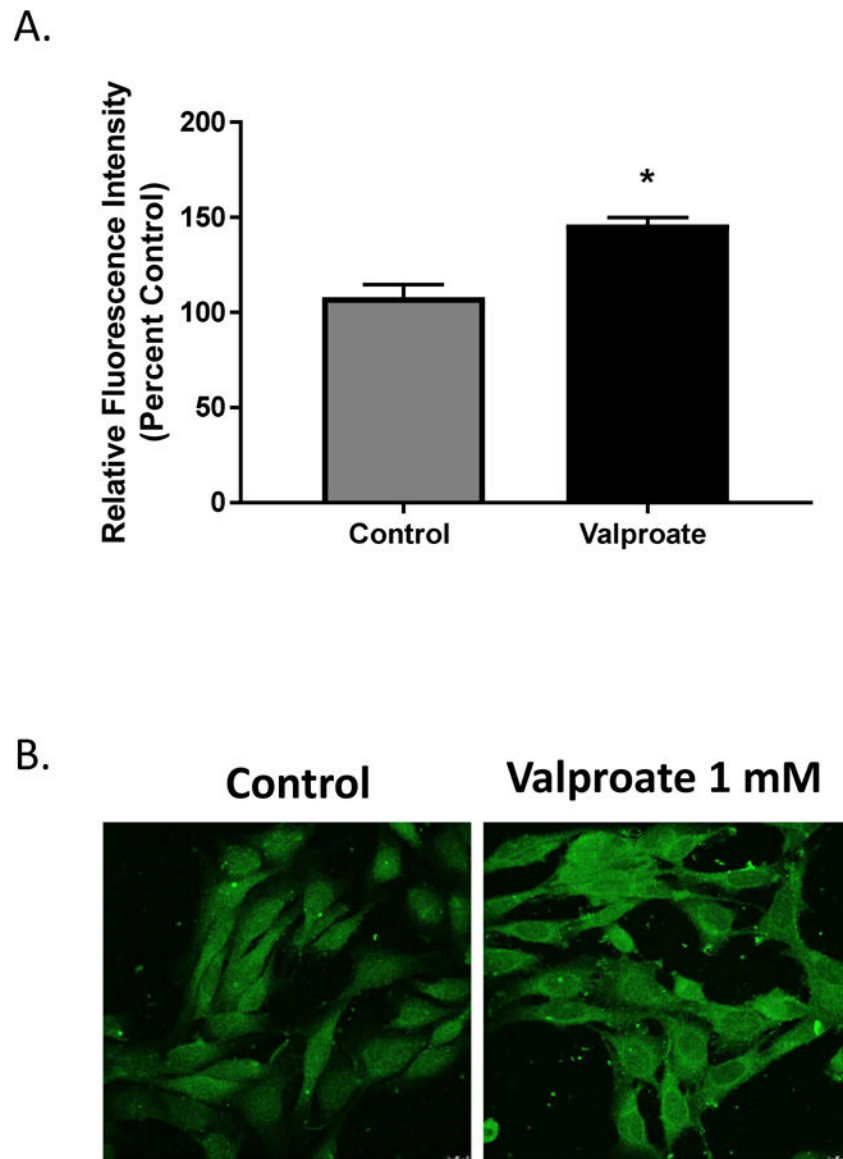
**Figure 1.**

A) Primer sequences for RT-qPCR and ChIP assays B) Schematic view of rat dopamine transporter promoter. The amplicons of each pair of ChIP primers are approximately 200bp each and are Roman numeral labeled accordingly.



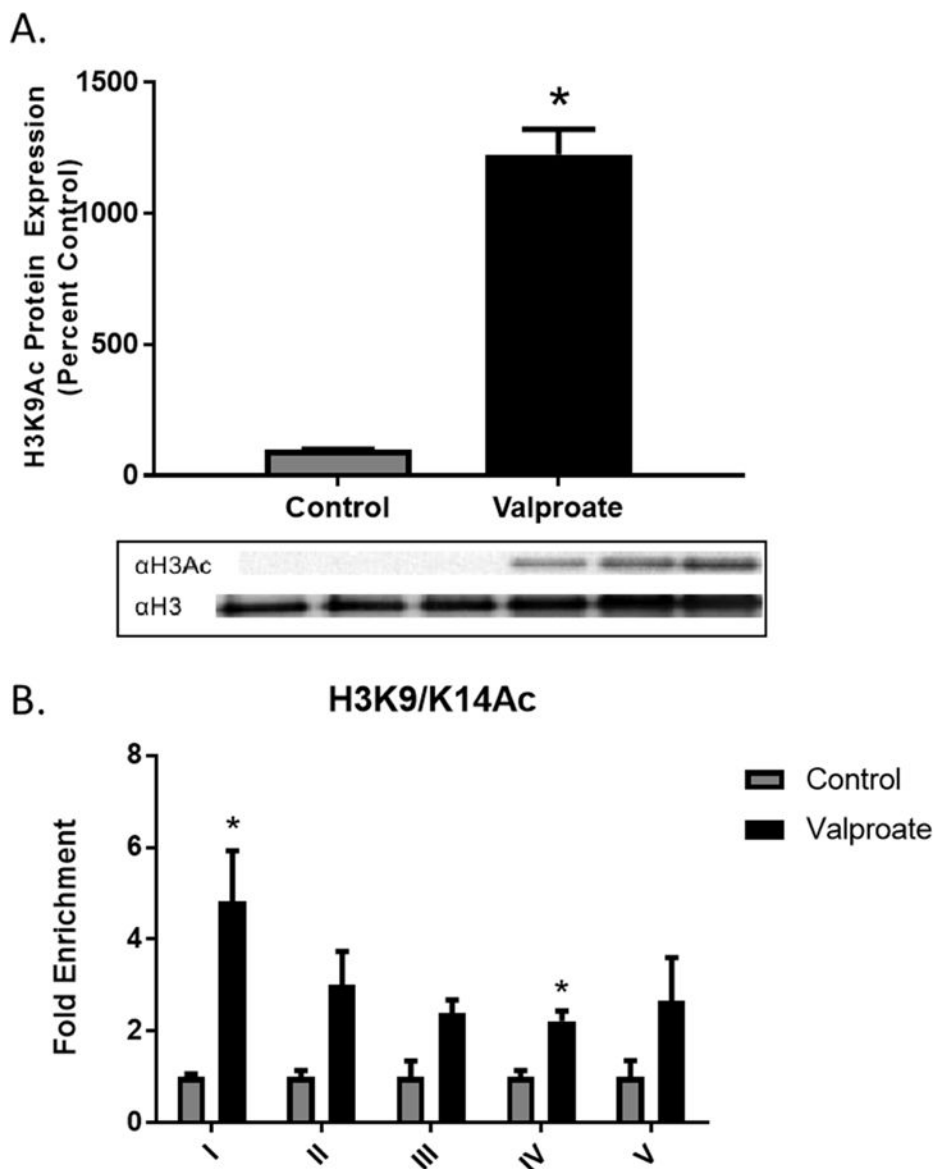
**Figure 2.** HDAC inhibitor dose response in N27 rat mesencephalic cells A) Relative DAT mRNA expression and B) Cell Viability for valproate, butyrate and Trichostatin A. Data represent mean  $\pm$  SEM; N=3-4, \*p 0.05 (One-way ANOVA, Dunnet's post hoc).





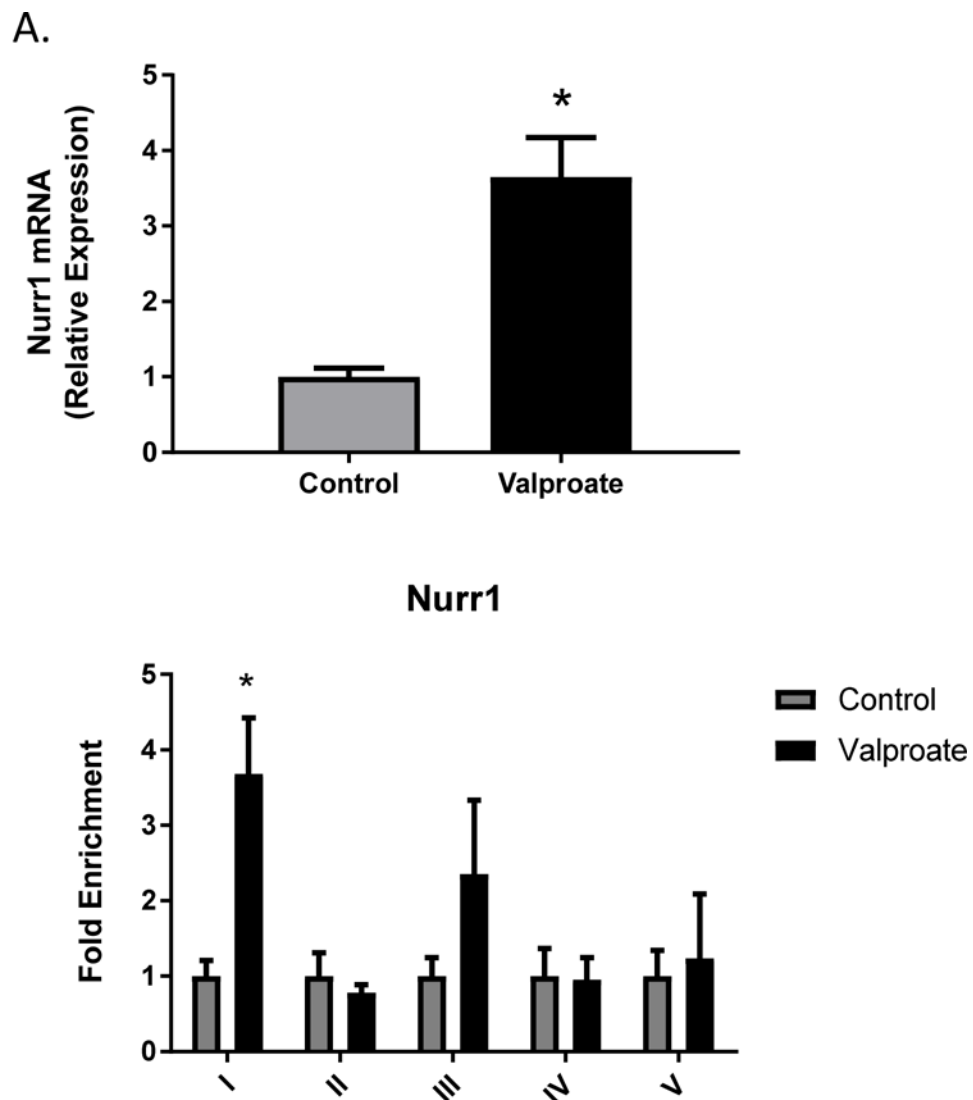
**Figure 3.**

A) Quantification of DAT protein in N27 cells following 24 h exposure to 1mM valproate. Data represented as mean  $\pm$  SEM, \*p 0.05 percent to control, t-test, N=4. B) Representative images of cells taken at 40 $\times$ , scale bar represents 20 microns.

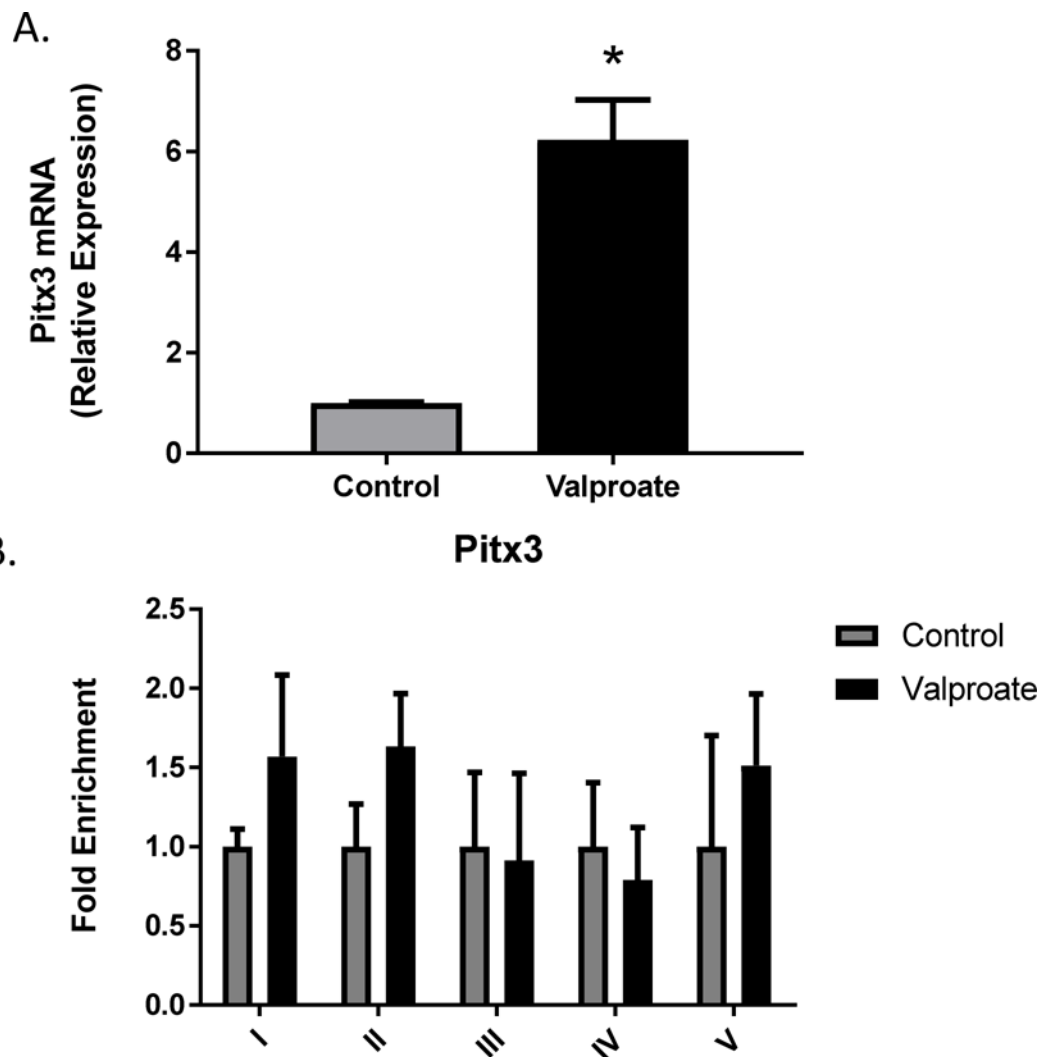


**Figure 4.**

A) Acetylated histone 3 protein in N27 cell lysates following 24 h exposure to 1mM valproate. Data represent mean  $\pm$  SEM, \*p 0.05 compared to control, t-test, N=3. Chromatin Immunoprecipitation (ChIP) of acetylated Histone 3 at lysines 9 and 14 (H3K9/K14ac) in DAT promoter regions. Data were normalized to input, IgG and represented as enrichment relative to control (N=3).



**Figure 5.** Orphan nuclear receptor Nurr1 A) mRNA expression and B) fold enrichment in the DAT promoter following 1mM valproate treatment in N27 cells. Data represented as mean  $\pm$  SEM; N=3–4, \*p 0.05 (t-test). For mRNA expression, data were first normalized to  $\beta$ -actin, then normalized to the control treatment group. For ChIP assay, data were normalized to input, IgG and represented as enrichment relative to control.



**Figure 6.** Pitx3 A) mRNA expression and B) fold enrichment in the DAT promoter binding following 1mM valproate treatment in N27 cells. Data represented as mean  $\pm$  SEM; N=3-4, \*p 0.05 (t-test). For mRNA expression, data were first normalized to  $\beta$ -actin, then normalized to the control treatment group. For ChIP assay, data were normalized to input, IgG and represented as enrichment relative to control.