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Protective Effects of Valproic Acid on the Nigrostriatal Dopamine System in an MPTP Mouse Model of Parkinson's Disease

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Abstract

The use of animal models (including the 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) mouse model) to mimic dopaminergic (DAergic) cell loss and striatal DA depletion, as seen in Parkinson's disease (PD), has implicated a multitude of factors that might be associated with DAergic cell death in PD including excitotoxicity, inflammation, and oxidative stress. All of these factors have been shown to be reduced by administration of histone deacetylase (HDAC) inhibitors (HDACis) resulting in some degree of neuroprotection in various models of neurodegenerative disease including in Huntington's disease and amyotrophic lateral sclerosis. However, there is limited information of effects of HDACis in PD models. We have previously shown HDACis to be partially protective against 1-methyl-4-phenylpyridinium (MPP^+) mediated cell loss *in vitro*. The present study was conducted to extend these findings to an *in vivo* PD model. The HDACi valproic acid (VPA) was co-administered with MPTP for 5 days to male FVBn mice and continued for an additional 2 weeks, throughout the period of active neurodegeneration associated with MPTP-mediated DAergic cell loss. VPA was able to partially prevent striatal dopamine depletion and almost completely protect against substantia nigra DAergic cell loss. These results suggest that VPA may be a potential disease modifying therapy for PD.

Keywords

HDAC inhibitor; dopamine; neuroprotection; valproate

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc) and the loss of dopamine (DA) terminals in the striatum. Although effective symptomatic therapies exist for PD, there is no current treatment that has been shown to unequivocally slow or stop the progression of the disease. As such, new disease modifying therapies are needed to address the inexorable progression of PD.

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The 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) mouse model of PD has been a useful screening tool for identification of compounds that may potentially interfere with substantia nigra (SN) DA cell death and consequent striatal DA depletion. There are different MPTP mouse models that utilize different toxin administration protocols, result in different modes of cell death and respond differently to putative neuroprotective agents (Anderson *et al.*, 2006). The sub-acute MPTP mouse model has been associated with primarily apoptotic cell death (Tatton and Kish, 1997), presumably as a result of impaired activity of complex I in the mitochondria leading to oxidative stress and free radical generation (Singer et al., 1987, Langston, 1996, Speciale, 2002). Additionally, glial cells (both astrocytes and microglia) become activated in the SN (and striatum) further contributing to the toxic milieu in the nigrostriatal system (Hirsch *et al.*, 2003). This increase in inflammatory cytokines and potentially excitotoxic compounds may perpetuate the initial insult from MPTP and exacerbate DAergic cell loss. Drugs that are able to inhibit one or more of these mechanisms of toxicity may be able to confer protection against MPTP-mediated damage.

Valproic acid (VPA) has been used clinically in the treatment of epilepsy and bipolar disorder for over 30 years (Bowden and McElroy, 1995). Its exact mechanisms of action are not completely understood but VPA is thought to modulate levels of the inhibitory neurotransmitter γ-amino butyric acid (GABA) as well as limit glycogen synthase kinase-3β (GSK3-β) activation (reviewed by (Gurvich and Klein, 2002). More recently, VPA has been shown to inhibit HDACs (Phiel *et al.*, 2001). As an HDACi, VPA is effective in limiting the excitotoxic response to glutamate in primary cortical cultures (Leng *et al.*, 2008) and induces apoptosis in normal microglial cultures (Chen *et al.*, 2006) while suppressing lipopolysaccharide mediated activation of microglia in mixed cultures (Peng *et al.*, 2005) suggesting it could possibly limit microgliosis *in vivo*. VPA has also been shown to increase transcription of anti-apoptotic proteins and free radical scavengers (Ryu et al., 2003, Kim et al., 2007). In addition, we have previously shown that HDACis including VPA can partially protect DAergic cells from 1-methyl-4-phenylpyridinium (MPP^+, the active metabolite of MPTP) toxicity *in vitro* (Kidd and Schneider, 2010). The ability of VPA to act through a variety of potential protective mechanisms likely contributes to its ability to be protective in multiple neurodegeneration models.

The present study was conducted to extend previous *in vitro* findings and assess the potential of the HDACi, VPA, to protect against DAergic cell loss and loss of striatal DA in a mouse MPTP model of Parkinsonism.

2. Experimental Procedures

2.1. Animals and drug administration

All procedures used in this study were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee, and studies were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male FVBn mice 8–10 weeks of age (Charles River Labs, Wilmington, MA) were housed 3–5 per cage with *ad libitum* access to food and water for one week prior to injection with VPA (Sigma-Aldrich, St. Louis, MO). The dosing paradigm for VPA (400mg/kg i.p.) was selected based on existing studies in which hyperacetylation was shown to occur in the rodent brain at this dose (Rouaux et al., 2007, Lv et al., 2011). Either saline or VPA was administered 30 min prior to administration of MPTP-HCl (Sequoia Research Products Ltd, Pangbourne, UK). MPTP was administered twice daily (25 mg/kg, s.c., 4 hours apart) for 5 consecutive days. Animals continued to receive VPA or saline once daily for 2 weeks after the final MPTP injection. Three hours after the final VPA or saline injection, mice were euthanized by decapitation and striatal tissue was rapidly removed and frozen for

catecholamine analysis. Frontal cortex was removed and frozen for western blot analysis. The remaining tissue was post-fixed in 4% parafomaldehyde for 72hrs.

2.2. High pressure liquid chromatographic (HPLC) analysis of tissue catecholamines

Striatal samples were sonicated in 0.4M percholoric acid and centrifuged at 15000 rpm for 5 minutes at 4°C. Supernatant was removed for analysis by HPLC as previously described (Anderson *et al.*, 2008), using isoproterenol (Sigma-Aldrich) as an internal standard. Samples were analyzed on a C18 column with a 20 μL injection loop using MDTM mobile phase (ESA Inc., Chemlsford, MS) on a Coulochem III system with an electrochemical detector (ESA Inc.). Peak heights were compared with internal standard values to determine the concentration DA and its metabolites (EZchrome V3.1, Agilent Technologies, Santa Clara, CA).

2.3. Immunohistochemistry and unbiased stereology

Fixed tissue blocks were immersed in 30% sucrose as a cryoprotectant and sectioned frozen on a sliding microtome (30μm section thickness) through the rostro-caudal extent of the substantia nigra pars compacta (SNc). Every third section was processed for tyrosine hydroxylase (TH) immunohistochemistry (rabbit anti-TH, 1:1000, Pel-freez, Rodgers, AZ) and adjacent sections were stained with cresyl violet as previously described (Anderson *et al.*, 2008). Cells (both TH^+ and cresyl violet stained ($Nissl^+$)) were counted using unbiased stereology (SteroInvestigator, MBFbioscience, Williston, VT) on an Olympus BX-60 microscope equipped with a Ludl motorized stage. The region of interest (i.e., SNc) was outlined under low magnification (4×) and a grid measuring 195μm × 85μm was randomly placed over the region. Cells were then counted at high power (100×) using a counting frame measuring $40\mu m^2$. A cell was counted only if a nucleus was clearly identifiable and the cell was completely within the counting frame. This process was repeated for each section in the series for a given animal and a total of 10 total sections/animal were analyzed. $Nissl^+$ cells were counted by overlaying the region of interest outline from the adjacent TH^+ section and sampling was accomplished using the same parameters described above.

2.4. Immunofluorescence

Mice were injected with VPA or saline for 1 week as described above. Sections were washed in PBS, blocked in 5% non-fat milk containing 0.3% triton x-100 for 1hr and incubated in primary antibody (rabbit anti-AcH3, 1:1,000in block, Millepore, Billerica, MA), overnight at 4°C. Primary antibody was removed, sections were rinsed, and secondary antibody (488 DyLight goat anti rabbit, 1:500, Jackson ImmunoResearch Labs Inc., West Grove, PA) was added and sections were incubated in the dark for 2 hours. Sections were then washed multiple times, mounted and cover-slipped using Aqua-perm mounting media (Thermo Fisher, Waltham, MA) and stored at 4°C until imaged. Images were collected on a Zeiss Axioplan 2 confocal microscope.

2.5. Western Blot

Tissue samples were homogenized in hypotonic lysis buffer (10mM Hepes, 1.5mM $MgCl_2$ and 10mM KCl, pH 7.9 with 1x HALT protease inhibitor, Thermo Fisher Scientific) using 5mm stainless steel beads and using a Qialyser (50 Hz for 3 min, Qiagen Inc., Germantown, MD). Homogenized samples were transferred to fresh tubes and 1M HCl was added to a final concentration of 0.2M HCl. Following 30min incubation on ice samples were centrifuged at 15000rpm for 10min and the resulting supernatant was used for western blot analysis. Ten micrograms of protein were loaded onto 4–12% bis-tris gels (Invitrogen Inc., Carlsbad, CA) and proteins were separated by electrophoresis in MOPS SDS running buffer (Invitrogen Inc.) for 1 hour at 200V. Proteins were transferred to 0.2μm nitrocellulose using

semi-dry transfer (15V for 15min/membrane). Membranes were equilibrated in Tris buffered saline containing 0.1% Tween-20 (T-TBS) and then blocked for 1hr in 5% non-fat milk in T-TBS. Primary antibody (rabbit anti-acetyl H3 Lys 9 (1:10,000) or rabbit anti-β–actin (1:2,000) (Imgenex, San Diego, CA) was added for 1hr. Membranes were washed in T-TBS and exposed to secondary HRP-conjugated goat anti-rabbit (1:40,000 and 1:20,000 respectively, Thermo Fisher Scientific) in T-TBS for 1 hr. After washing in T-TBS to remove residual antibody, blots were developed using chemluminesence (Thermo Fisher Scientific) and were quantified using densitometry software (MCID Basic V 7.2).

2.6. Statistical analysis

All data are presented as mean ± standard error. Statistical significance was determined (defined as $p < 0.05$) using a one way analysis of variance with either Bonferonni or Dunnett post-hoc comparisons where appropriate (unless otherwise specified).

3. Results

3.1. Valproate treatment promotes histone hyperacetylation in the brain

Systemic administration of VPA resulted in increased levels of acetylated histone 3 lysine 9 (AcH3 Lys 9) in the brain. In addition to inducing hyperacetylation in the SNc (Figure 1 A and B), VPA administration significantly increased the ratio of acetylated H3 Lys 9 to β-actin in the frontal cortex of control mice as well as animals exposed to MPTP (127.1 ± 7.9 and 120.1 ± 2.8 % respectively, $F_{(3,18)}$=13.14; P<0.001, q=4.702, 3.393 p<0.01 vs. control, Figure 1 C and D). .

3.2. Valproate partially protects the nigrostriatal system from the effects of MPTP

Treatment with MPTP significantly affected striatal DA levels (Kruskal-Wallis statistic = 31.07, P<0.0001) and DA turnover ($F_{(3,38)}$=21.34, P<0.001). Sub-acute administration of MPTP resulted in a 77.0 ± 1.3% reduction in striatal DA levels (p<0.01, Figure 2A). Treatment with VPA resulted in significantly increased striatal DA levels compared to saline-treated control mice (10.21 ± 0.36 and 8.77 ± .25 μg/g wet tissue respectively; p< 0.001). Mice that received VPA and MPTP also had significantly higher striatal DA levels than animals that received MPTP and saline (2.24 ± 0.07 and 1.94 ± 0.11 μg/g respectively; p< 0.001). Dopamine turnover (DOPAC: DA) was increased 185.7 ±14.2 % following MPTP exposure (t= 6.17, p<0.01, Figure 2B). There was no difference in DA turnover between mice receiving MPTP or MPTP/VPA (0.23 ± 0.02 and 0.23 ± 0.01 respectively).

Treatment with MPTP significantly affected both DAergic ($F_{(3,20)}$= 12.55, P<0.001) and total cell ($F_{(3,20)}$= 14.7, P<0.001) numbers in the SNc. Specifically, the number of SNc DAergic neurons was reduced by 31.9 ± 2.3% (t= 5.56, p<0.001) following treatment with MPTP (Figure 3B), slightly more than the estimated loss of SNc neurons (20.3 ± 1.6%, t=5.95, p<0.001) obtained from counts of $Nissl^+$ sections (Figure 3C). There were significantly more TH^+ neurons in the SNc in animals treated with VPA and MPTP than in MPTP/saline treated animals (88.1 ± 4.4 and 68.1 ± 2.3 % of saline/saline TH^+ neurons, respectively, t= 3.48 p< 0.05) There was also a significant increase in the number $Nissl^+$ cells in the SNc of VPA/MPTP mice compared to saline/MPTP animals (94.6 ± 2.6 and 79.7 ± 1.6 % of saline/ saline $Nissl^+$ cells respectively, t=4.36 p< 0.01).

4. Discussion

The present results demonstrated that VPA was able to partially protect the nigrostriatal DA system from MPTP toxicity and that systemic treatment with VPA resulted in a significant increase of acetylated histone 3 in the substantia nigra as well as in non-basal ganglia

regions of the mouse brain (i.e., frontal cortex). This hyperacetylation event was associated with greater striatal DA levels (both in normal as well as MPTP-treated mice) as well as increased survival of SNc neurons following MPTP exposure, compared to lesioned control animals. The observed protective effects of VPA appeared to be greater at the level of the cell bodies in the SNc than at the DA terminals in the striatum. However, considering the short time frame of this study (2 weeks following the last toxin administration) it is possible that given a longer post-lesion survival time, a greater increase in striatal DA levels may be observed as SNc neurons re-establish connections to the striatum. It is unlikely that the observed protective effects of VPA in these mice were due to interference with the acute toxicity of MPTP. Valproate does not impair DA transporter function (Chen et al., 2006, Kidd and Schneider, 2010) therefore the uptake of MPP^+ into DAergic cells via the DA transporter should not be affected by VPA. In addition, VPA unlikely inhibited the enzyme responsible for the conversion of MPTP to MPP^+, monoamine oxidase B, at the dose at which it was used in this study (400mg/kg or 2,406μmol/kg). The IC50 for VPA-mediated inhibition of monoamine oxidase B is >10,000 μmol/L (Fisar et al., 2010) and the amount of VPA that enters the brain is only a small fraction of the peripheral dose administered (for review see Loscher, 1999).

Inhibition of HDACs in the brain has been associated with the increased transcription of many factors that may contribute to the protection of DAergic neurons following MPTP exposure, including free radical scavengers (Faraco et al., 2006, Petri et al., 2006), heat-shock proteins (Kim et al., 2007, Marinova et al., 2009, Leng et al., 2010) and anti-apoptotic bcl-2 family members (Faraco et al., 2006, Lv et al., 2011). Oxidative stress is thought to play a significant role in both PD and MPTP-mediated parkinsonism (Jenner, 2003). Given the ability of VPA to increase the activity of superoxide dismutase and catalase resulting in reduced levels of superoxide radicals (Jornada *et al.*, 2011) it may be able to reduce the oxidative burden on SNc neurons allowing them to survive the toxic insult from MPTP. In addition to reducing oxidative stress, the increase in transcript levels of inhibitors of apoptosis (Ryu *et al.*, 2003) and anti-apoptotic bcl-2 family members (Petri et al., 2006, Rouaux et al., 2007) that result from HDAC inhibition may reduce mitochondrial stress caused by MPTP-induced inhibition of complex I, further contributing the survival of DAergic cells. Valproate-mediated hyperacetylation also increases transcription of HSP-70 (Marinova et al., 2009, Leng et al., 2010), induction of which has been shown to protect against excitotoxic stress (Marinova *et al.*, 2009) and MPTP toxicity (Shen *et al.*, 2005). VPA may also reduce inflammatory mediators by triggering apoptosis of microglia cells (Suuronen et al., 2003, Chen et al., 2007). Both astrocytes and microglia are activated in the sub-acute MPTP mouse model and likely contribute to the loss of DA in the nigrostriatal system via excitoxicity and the release of inflammatory cytokines (Hirsch *et al.*, 2003). Inhibiting glial mediated inflammation may protect SNc neurons from MPTP-mediated cell death as long as therapy is continued throughout the time course of neurodegeneration (Aubin et al., 1998, Ferger et al., 1999, Mohanakumar et al., 2000, Du et al., 2001, Wu et al., 2002). Thus, there are multiple possible mechanisms through which VPA-mediated HDAC inhibition could confer protection to SNc neurons against MPTP-mediated toxicity.

The currently observed effects of VPA on the MPTP-damaged DA system might involve other mechanisms in addition to HDAC inhibition. For example, one of the primary means by which VPA is thought to inhibit epileptic activity is via the modulation of the inhibitory neurotransmitter GABA which results in accumulation of GABA in several brain regions including the basal ganglia (Loscher, 1989). MPTP depletes striatonigral GABA levels (Kuriyama *et al.*, 1990) which may impair the ability of the basal ganglia to control motor function and the pharmacological modulation of GABA (ex. by modafinil) has been shown to confer some DAergic neuroprotection in MPTP models (Fuxe et al., 1992, Jenner et al., 2000). Thus, it is possible that VPA-mediated neuroprotection may to some extent involve

increases in GABA in the basal ganglia. Another possible mechanism of VPA-mediated neuroprotection may relate to its ability to inhibit GSK3-β (Chen et al., 2007, Leng et al., 2008). Both *in vitro* and *in vivo* studies using Parkinson-producing toxins (i.e. rotenone, MPP^+ and 6-OHDA) have implicated GSK3-β in DAergic cell death and have shown that inhibition of GSK3-β to be partially protective in these models (Chen et al., 2004, Wang et al., 2007, Chen et al., 2008). However, studies by Melamed et al (Melamed *et al.*, 1986) and Lagrue et al (Lagrue *et al.*, 2007) in which low levels of VPA were administered (below that needed to induce histone hyperacetylation) showed no protective effects against an acute MPTP-induced toxicity. This may suggest that a critical concentration of VPA (possibly that which is needed for HDAC inhibition) may be needed for VPA to protect against MPTP-induced parkinsonism.

In summary, VPA administration, at the dosing regimen used in the current study, partially protected the nigrostriatal dopamine system from injury in a mouse model of sub-acute MPTP toxicity. Additional studies using other PD model systems and other VPA dosing regimens are warranted in order to more fully define the potential neuroprotective activity of this drug. However, considering the extensive clinical experience with VPA (in patients with epilepsy and bipolar disorder) and its good clinical safety profile, clinical studies examining VPA-mediated HDAC inhibition as a potential disease modifying therapy in PD may be warranted.

Highlights

- Systemic administration of valproate promotes hyperacetylation of histone 3 lysine 9 in the mouse substantia nigra of FVBn mice
- Valproate increases striatal dopamine levels in saline and MPTP treated mice
- Valproate partially protects against MPTP-mediated dopamine cell loss in the substantia nigra of FVBn mice while maintaining dopamine phenotype

Abbreviations

AcH3 Lys 9	acetylated histone 3 lysine 9
DA	dopamine
DAergic	dopaminergic
GABA	γ-amino butyric acid
GSK3-β	glycogen synthase kinase-3β
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HPLC	high pressure liquid chromatography
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine
PD	Parkinson’s disease
SN	substantia nigra
SNc	substantia nigra pars compacta
TH	tyrosine hydroxylase

VPA	valproic acid

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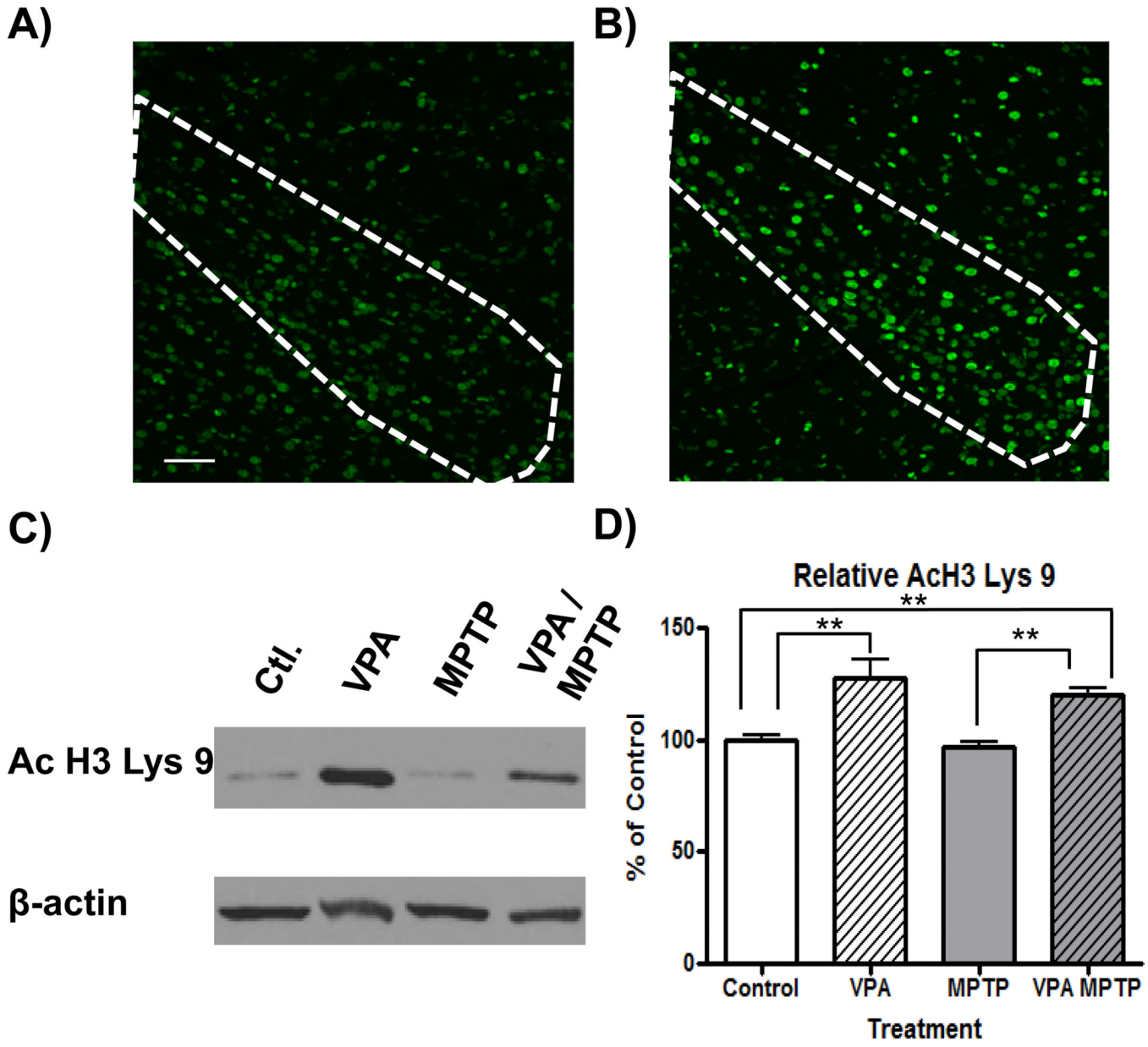

Figure 1.

Systemic valproate administration promotes histone hyperacetylation in the brain. Immunoflourescent images show the presence of acetylated histone 3 Lys9 (AcH3 Lys 9) in the substantia nigra (outline) of saline (A) or VPA treated (B) mice (scale bar = 100μm). Representative western blots show increased acetylation of AcH3 Lys 9 in frontal cortex in animals receiving VPA as well as VPA and MPTP, compared to control animals (C). Densitometric analysis showed significant increases in AcH3 Lys 9 (relative to the level of β-actin in each sample) in VPA and VPA/MPTP-treated animals, compared to their respective controls (D). **: $p<0.01$.

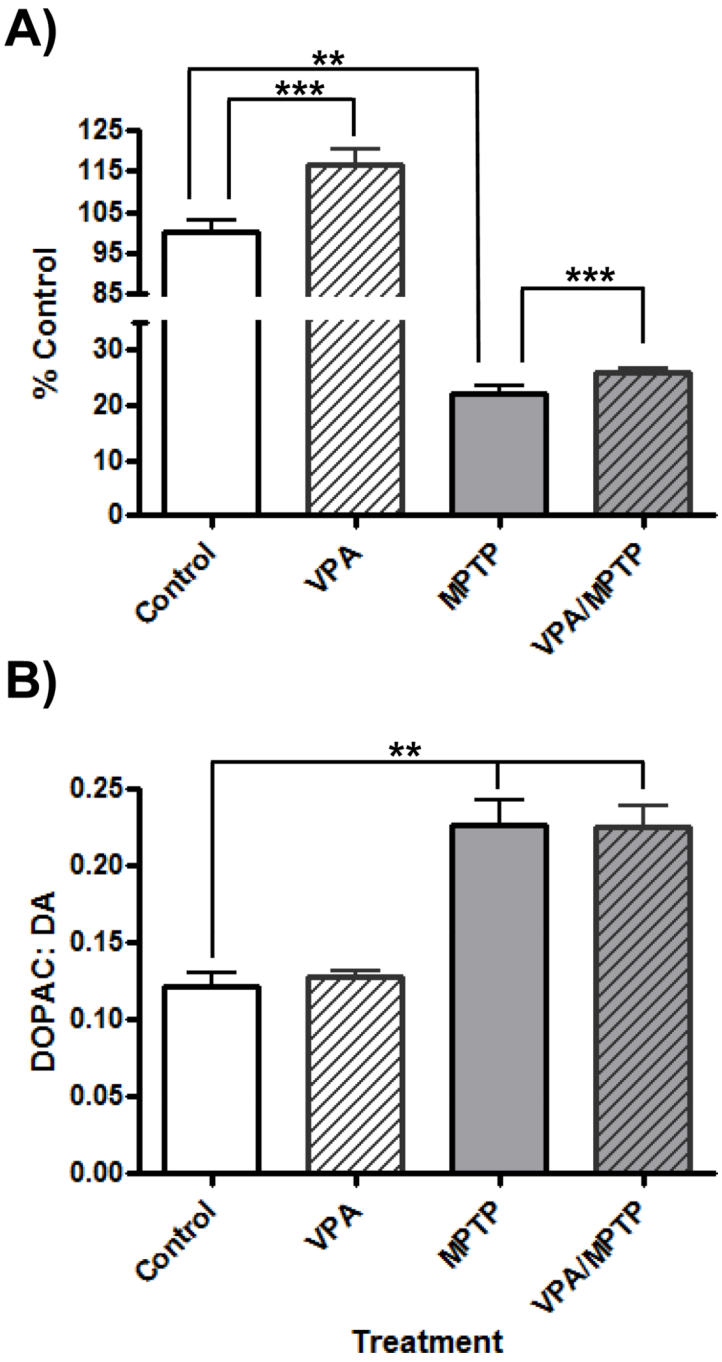

Figure 2.

Effects of Valproate (VPA) on striatal dopamine (DA) and DA turnover following administration of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP). Treatment with VPA increased striatal DA levels (A), as measured by high pressure liquid chromatography (HPLC) 2 weeks after the final MPTP injection, but had no effect on DA turnover (B) compared to control mice. . **p< 0.01, ***: p< 0.001.

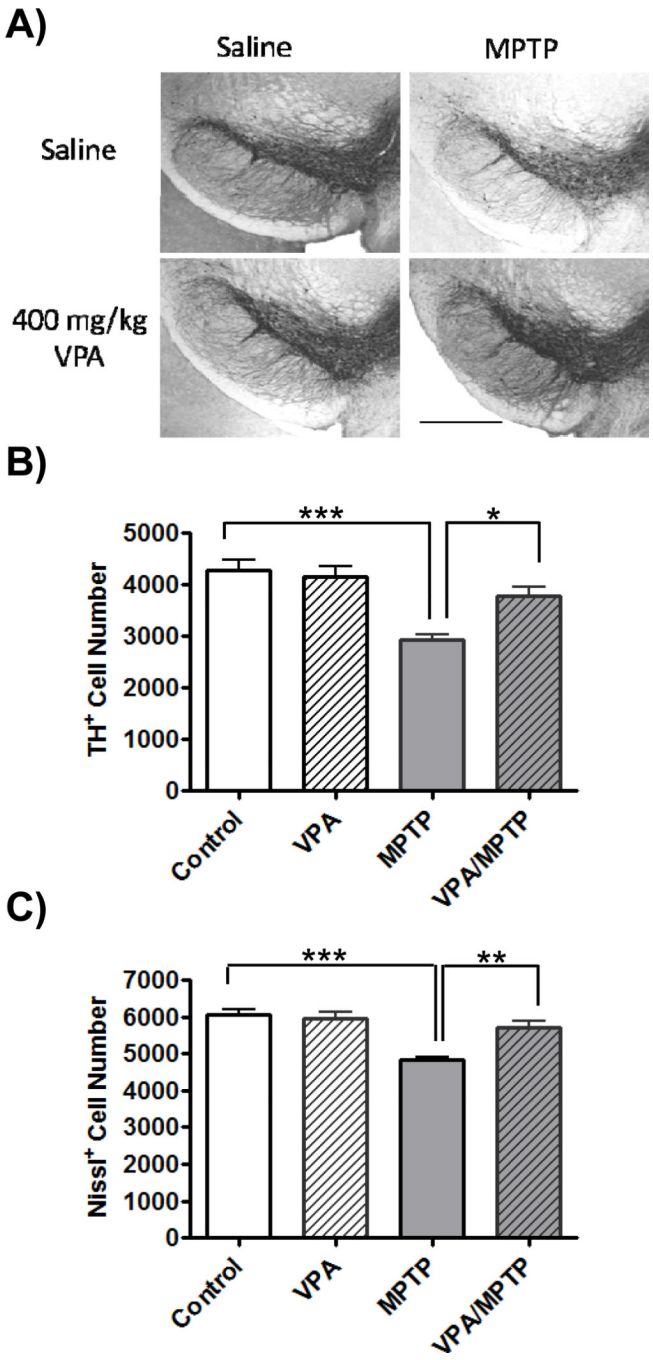

Figure 3.

Effects of valproate (VPA) treatment on substantia nigra pars compacta (SNc) neuronal survival following 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) treatment. A) Photomicrographs of tyrosine hydroxlase (TH) staining in the SNc (scale bar = 2mm) B) Stereological estimates confirmed a loss of TH immunopositive (TH^+) cells in the SNc following MPTP administration. The MPTP-induced decrease in TH^+ cells was prevented by VPA administration. C) Stereological estimates confirmed a loss of neurons (Nissl+ cells) in the SNc as a result of the MPTP exposure. Mice receiving VPA and MPTP show a smaller amount of cell loss than animals that received MPTP without VPA. *: $p< 0.05$, **$p< 0.01$, ***: $p< 0.001$.