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25-Hydroxyvitamin D Depletion Does Not Exacerbate MPTP-Induced Dopamine Neuron Damage in Mice

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Abstract

Recent clinical evidence supports a link between 25-hydroxyvitamin D insufficiency (serum 25-hydroxyvitamin D [25(OH)D] levels < 30 ng/mL) and Parkinson’s disease. To investigate the effect of 25(OH)D depletion on neuronal susceptibility to toxic insult, we induced a state of 25(OH)D deficiency in mice and then challenged them with the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We found there was no significant difference between control and 25(OH)D-deficient animals in striatal dopamine levels or dopamine transporter and tyrosine hydroxylase expression after lesioning with MPTP. Additionally, we found no difference in tyrosine hydroxylase expression in the substantia nigra pars compacta. Our data suggest that reducing 25(OH)D serum levels in mice has no effect on the vulnerability of nigral dopaminergic neurons in vivo in this model system of parkinsonism.


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Introduction

Multiple factors, including increased oxidative stresses, varying genetic susceptibility, mitochondrial dysfunction, proteasomal dysfunction, inflammation and premature apoptosis contribute to the pathophysiology of Parkinson’s disease (PD) [1]. Furthermore, such environmental factors as vitamin D status may modify PD risk and/or progression [2]. Deficient and insufficient vitamin D status has been associated with PD in one case report and several epidemiologic studies [3,4]. Whether 25(OH)D deficiency states are causal or merely correlating in PD remains poorly understood. Despite this knowledge limitation, many clinicians recommend vitamin D supplementation to patients as a means to prevent or delay progression of PD. Therefore, it is important to clarify the role, if any, that hypovitaminosis plays in PD-related pathology. The goal of this study is to address this gap in our knowledge.

The term “vitamin D” frequently refers to the family of compounds that include the nutritional supplements (cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2)), 25-hydroxyvitamin D (25(OH)D), and the biologically active vitamin D hormone, 1,25-dihydroxyvitamin D (1,25(OH)2D). Both vitamin D2 and vitamin D3 are rapidly hydroxylated to 25(OH)D, the body’s primary storage form for “vitamin D” and the best indicator of vitamin D status. This 25(OHD) metabolite has much higher circulating concentrations in serum and has a much longer half-life than the serum 1,25(OH)2D, which is considered the hormonal form of vitamin D. The hormonal form of vitamin D, 1,25(OH)2D is dependent on the substrate 25-hydroxyvitamin D. Therefore, circulating 1,25(OH)2D concentrations generally correlate with 25(OH)D concentrations. However, concentrations of the biologically active vitamin D hormone (1,25(OH)2D) are tightly regulated and in our neurological clinical settings it is rare to find patients who have normal renal function and abnormally low 1,25(OH)2D concentrations, even when 25(OH)D concentrations suggest severe vitamin D deficiency.

Several lines of clinical evidence suggest the vitamin D hormone may play a mechanistic role in the pathogenesis of PD. First, genetic factors, such as finding the vitamin D hormone receptor b allele BsmI polymorphism is more frequent in patients with PD than healthy controls and 5’ end SNPs on the VDR gene may modulate risk and/or survival in patients with PD [3,4]. Second, higher 25(OH)D serum levels are associated with reduced risk of developing PD [5]. Third, we and others report that patients with PD are more likely to have 25(OH)D insufficiency (defined as a
Laboratory evidence also suggests plausibility that the vitamin D physiology may play a role in the PD. The hormone (1,25(OH)2D) has been shown in multiple injury models relevant to stroke, multiple sclerosis, and Alzheimer’s disease to differentially protect both neuronal populations and glia via upregulation of L-type calcium channels, immune modulation, increased antioxidant function, improved neurotrophin expression [11,12,13,14,15,16,17]. Similarly, 1,25(OH)2D appears to support homestasis of the dopaminergic neuronal populations affected in PD. Both vitamin D hormone receptors and 1-alpha hydroxylase enzyme (the activating enzyme for vitamin D hormone) are expressed throughout the human brain, but are particularly enriched in the large (likely dopaminergic) neurons of the substantia nigra [18]. These findings have led to speculation that vitamin D deficiency may leave nigrostriatal neurons more vulnerable to insult [10,19]. Indeed, animal and cell culture studies demonstrate that pre-treatment with vitamin D hormone (1,25(OH)2D) protects dopaminergic neurons from 6-hydroxydopamine and N-methyl-4-phenylpyridinium (MPP+) toxicity [16,17]. Up to a point, increasing vitamin D hormone appears to be neuroprotective. Alternatively, as Newmark and Newmark suggest and as seen in traumatic brain injury models, functional vitamin D deficiency (from reduced 25(OH)D, 1,25(OH)2D or vitamin D receptor dysfunction) may leave nigrostriatal neurons more vulnerable to insult [10,19,20]. However, the effect of reducing 25(OH)D concentrations (as seen in patients with PD) or vitamin D hormone levels on the vulnerability of the nigrostriatal system to insult has not been tested in vivo. To explore this possibility, we determined if 25(OH)D deficiency in mice renders the nigrostriatal system of those mice more vulnerable to insult by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

Materials and Methods

Ethics Statement, Animals and Behavioral Testing

All animal procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by Emory University Institutional Animal Care and Use Committee for these specific experiments. Retired breeder (8–10 months of age) male C57Bl/6j mice were purchased from Jackson Laboratory. Vesicular monoamine transporter 2 hypomorph (VMAT2 LO) and wild type mice were collected for pretreatment baseline measures. Mice then were allowed to walk to their home cage. Stride length was only measured for the nigrostriatal system of those mice more vulnerable to insult by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).
DOPAC and HVA were detected using electrodes set at potentials 58x174 were then spun at 10,000 x 10 proteins. 20 Scientific). 20 concentrations were determined by BCA protein assay (Thermo 305). Densitometric analyses were performed using secondary antibody (Sigma) was incubated with the blots for 1 359. Primary antibodies (rabbit polyclonal anti-TH and rat polyclonal anti-DAT, Chemicon, Temecula, CA and mouse anti-tubulin, Sigma-Aldrich, St. Louis, MO) were incubated with blots at 4 423. An Agilent 1100 Series Capillary HPLC system coupled with an Agilent Model 1200 HPLC equipped diode array detector operated at a wavelength of 290 nm. Separation was achieved on a reverse phase ALCa C18 column (5 μm, L = 150 mm, ID = 4.6 mm, Catalog No. 88052; Alltech Associates Inc., Deerfield, IL) with a mobile phase consisting of 89% 50 mM KH2PO4 and 11% acetonitrile. MPP+ levels were identified by comparison of retention time with known standards and concentrations were calculated from a 6-point standard curve of known concentrations of MPP+. Protein concentrations were determined by Bradford Assay.

Immunohistochemistry of Striatal and Nigral Dopaminergic Markers

Perfused brains were kept at 4°C for 12 hours in 4% PFA. Brains were then transferred to 30% sucrose and stored at 4°C. Forty μm sections were prepared (Microm HM450) and transferred to Tris-buffered saline and stored at 4°C until use. For immunohistochemical staining, thin sections were washed six times in 1x TBS to remove all traces of PFA. Thin sections were incubated with a rat monoclonal anti-dopamine transporter (DAT) (1:750; Chemicon), or a rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:2000; Chemicon) overnight at 4°C and then incubated in a biotinylated-goat anti-rat or goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. Visualization was performed using 0.03% 3,3’-diaminobenzidine (Sigma Aldrich, St Louis, MO) at room temperature.

Statistical Analyses

All statistical analyses were performed using Graph Pad Prism 5.0. Student t-tests were performed for initial two group comparisons with significant values reported as p<0.05. Two-way ANOVA analyses were performed on all multi-group comparisons with Bonferroni post-hoc tests to determine significant interactions. Significant values are reported as p<0.05.
Results

Short Term 25(OH)D Depletion had no Effect the Overall Health or Behavior of the Mice

In order to test the effect of 25(OH)D deficiency on the vulnerability of nigrostriatal neurons, we first depleted mice of serum levels of 25(OH)D. Since a laboratory animal’s sole source of vitamin D is dietary, mice were transferred from standard rodent chow (Purina) to a vitamin D deficient diet to specifically deplete serum 25(OH)D levels. Serum 25(OH)D levels are widely regarded as the most reliable predictor of vitamin D status as it is done in most animal and human studies. After 6 weeks on depleted chow, serum 25(OH)D levels were depleted by nearly 80% (Figure 2A). Vitamin D control chow-fed and depletion chow-fed mice had a mean ± SEM 25(OH)D serum level of 43.9±0.7 ng/ml and 7.17±0.4 ng/ml, respectively (n = 6; p<0.0001).

Next, we confirmed that 25(OH)D deficiency had no effect on the mice’s ability to learn or perform a behavioral index of nigrostriatal damage. Previously, gestational 25(OH)D deficiency in mice’s ability to learn or perform a behavioral index of nigrostriatal damage. Previously, gestational 25(OH)D deficiency has been shown to affect dopamine-dependent locomotor behavior. 

Figure 2. Short term 25(OH)D depletion has no observable effect on mouse locomotor behavior. A) Serum 25(OH)D levels after 6 weeks on either control or vitamin D depletion rodent chow. Results are presented as absolute mean values (ng/ml) ± SEM for eight animals per group (***p<0.0001) B) Pre-MPTP lesion stride length measures. doi:10.1371/journal.pone.0039227.g002

25(OH)D Depletion did not Further Exacerbate the Effects of MPTP in Mice

We hypothesized that 25(OH)D depletion would not affect the dopamine system alone, rather render the system more vulnerable to future insult. To test this, we challenged 25(OH)D-depleted mice with the dopaminergic neurotoxin MPTP (4 x 15 mg/kg) to produce a mild lesion resulting in approximately 50–60% reduction of striatal dopamine. This dose was chosen so that excarnation or protection could be detected in the vitamin D-depleted animals. MPTP lesion was confirmed by measuring changes in dopamine and markers of dopamine neuron. Striatal dopamine (Figure 3A) was significantly reduced by 60% in MPTP-lesioned animals as compared to saline-treated animals (Control/Saline: 25.546±3.6 pmol/mg of protein, Control/MPTP-10.523±1.2 pmol/mg of protein; n = 4). Next, we investigated the effect of 25(OH)D status on the magnitude of dopamine injury. 25(OH)D depletion had no effect on striatal dopamine levels after MPTP lesion. A similar 60% reduction in dopamine levels after MPTP treatment in 25(OH)D depleted animals was observed (Vitamin D Depletion/Saline: 32.735±1.4 pmol/mg of protein, Vitamin D Depletion/MPTP: 13.369±5.0 pmol/mg of protein; n = 4). While 25(OH)D depleted animals trended towards having higher dopamine, there was no significant difference in striatal dopamine levels observed between vitamin D control chow-fed and depleted chow fed animals with or without MPTP lesion.

In order to determine whether 25(OH)D deficiency exacerbates MPTP-induced behavioral changes, forepaw stride length measures were performed. MPTP treatment did not significantly affect forepaw stride length measured at the end of the study (Control/Saline Group- 6.46±0.26 cm; Control/MPTP Group- 6.47±0.26 cm; Vitamin D Depletion Saline Group- 7.02±0.29 cm; Vitamin D Depletion MPTP Group- 5.97±0.25 cm; n = 7–9) (Figure 3B). Interestingly, 25(OH)D deficient-MPTP lesioned mice had significantly shorter forepaw stride lengths than mice that were 25(OH)D deficient-saline treated (*, p<0.05).

Since changes in markers of dopamine neuron integrity can be seen in the absence of any behavioral deficit and are often changed when dopamine levels are altered, we next investigated the effect of 25(OH)D depletion of expression of markers of dopamine neurons in the nigrostriatal system. A significant 46% and 49% reduction in striatal tyrosine hydroxylase (the rate limiting step in dopamine synthesis) and dopamine transporter protein levels were observed with MPTP lesion, respectively (Figures 4); however, 25(OH)D depletion did not exacerbate the total loss of terminal markers in this paradigm. Additionally, immunohistochemical analyses revealed a dramatic reduction in both of the striatal TH (Figure 5A) and DAT levels (data not shown) and the nigral TH levels (Figure 5B) of MPTP lesioned animals that were not further exacerbated by 25(OH)D depletion. Thus, 25(OH)D deficiency has no effect on expression of markers of dopamine neuron health in mice.

It is possible that 25(OH)D deficiency could affect the metabolism of the MPTP neurotoxin obscuring any effect that 25(OH)D depletion has on the vulnerability of the nigrostriatal system. Therefore, we tested the ability of 25(OH)D deficiency to interfere with MPTP metabolism. In a separate cohort of mice, we

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found that MPP+ levels were similar in mice challenged with a single dose of MPTP after 25(OH)D depletion (Figure 6). Therefore, MPTP metabolism as measured by MPP+ levels is not altered by 25(OH)D depletion.

25(OH)D Levels are not different in Mice with Acute or Chronic Dopamine Deficiency

Because 25(OH)D levels are reportedly lower in patients with more advanced PD, we hypothesized that perhaps acute or chronic dopamine loss drives the lower levels of serum 25(OH)D observed in patients with PD. To test this possibility, we also determined whether dopaminergic neuron damage acutely affected 25(OH)D serum levels. In mice on the vitamin D complete chow, there were no differences in 25(OH)D serum levels between mice treated with MPTP and those treated with saline after ten days of lesion (Control/Saline Group- 40.40±1.1 ng/ml; Control/MPTP Group- 40.60±1.1 ng/ml; Vitamin D Depletion/ Saline Group- 6.60±0.7 ng/ml; Vitamin D Depletion/MPTP Group- 5.91±1.4 ng/ml; n = 4; ***, p<0.0001); Figure 7). However, the half-life of 25(OH)D serum levels are sufficiently long that 25(OH)D concentration changes could have been obscured over the course of only 10 days.

Therefore to test the effect of chronic dopamine deficiency as seen in PD on 25(OH)D serum levels, we used a mouse model that more closely matches the chronic state of dopamine deficiency, the vesicular monoamine transporter 2 hypomorph (VMAT2 LO) mouse model. These mice have severe impairment of their ability to package dopamine into synaptic vesicles for later release resulting in enhanced turnover of dopamine with a chronic state of hypodopinerigia similar to that seen in patients with PD [21,22]. We measured serum 25(OH)D levels in both young mice and old mice maintained on standard vitamin D complete rodent chow to detect any changes in 25(OH)D status in these mice. Interestingly, lifelong deficiency in dopamine levels has no effect on 25(OH)D levels (Figure 8). There were no differences in serum 25(OH)D levels in VMAT2 WT and LO mice in young (<3 months) or old (>12 months) mice. Also, older mice had significantly higher serum 25(OH)D levels than younger mice (***, p<0.0001). Therefore, in this model chronic dopamine deficiency does not promote 25(OH)D deficiency when fed a standard rodent chow diet.
Discussion

With exception of bone and tooth health, the role that vitamin D supplements, 25OHD and vitamin D hormone play in the pathogenesis of disease has been understudied and underappreciated. Because epidemiological data have suggested a link between low 25(OH)D status and PD, we sought to investigate the effects of 25(OH)D depletion in the MPTP mouse model-
levels under our experimental conditions (Figures 4 and 6). The any effect of 25(OH)D depletion on MPTP metabolism or DAT surface of target cells, and the inability to reproduce the non-

dopamine neuronal markers, striatal dopamine, and gait analysis. Also, we found no difference in the status of dopaminergic nigrostriatal neurons as measured above with serum 25(OH)D deficiency alone. This suggests that in animals, low serum 25(OH)D levels may not predict dopamine neuronal health. It is unlikely that 25(OH)D deficiency in PD patients is a surrogate marker for some other metabolic changes such as in calcium or parathyroid hormone levels as these levels are rarely altered in our clinical population with 25(OH)D insufficiency (data not shown). It is also unlikely based on previous studies that calcium levels are affected in our model system [31]. While similarly designed studies largely reduce serum vitamin D hormone levels (~60% reduction), it is possible that vitamin D hormone levels remained high enough throughout our study in 25(OH)D depleted mice to prevent any exacerbation of MPTP toxicity [32]. Additionally, while 25(OH)D concentrations are low early in the course of the disease [8], and in one epidemiologic study lower concentrations of 25OHD were associated with increased risk of PD [5], the duration of 25(OH)D insufficiency in patients with PD prior to diagnosis is unknown. Therefore, to better model chronic 25(OH)D insufficiency (and obtain chronically low-normal or even deficient vitamin D hormone levels), future studies should include longer duration of 25(OH)D depletion in animals.

MPTP is a reliable toxicant used to selectively lesion dopamine neurons and is the only toxicant model available for animal studies that also undoubtedly causes a parkinsonian syndrome in humans [33]. While the MPTP mouse is the most common animal model of PD, it is limited by several factors including peripheral and central metabolism of MPTP to MPP+, expression of DAT on the surface of target cells, and the inability to reproduce the non-motor and progressive symptoms of the disease. We did not detect any effect of 25(OH)D depletion on MPTP metabolism or DAT levels under our experimental conditions (Figures 4 and 6). The MPTP dosing paradigm described in the present study is designed to cause a moderate loss of dopamine and terminal markers, which allows for detection of exacerbation or attenuation of toxicity. Therefore, any effect of 25(OH)D alone on MPTP susceptibility should be revealed, and we feel that we selected the appropriate dosage of MPTP for this study. Additionally experiments using a milder 4 X 10 mg/kg MPTP dosing paradigm also revealed no effects of 25(OH)D depletion on MPTP toxicity (data not shown).
animals did have a significantly smaller forepaw stride length whereas control chow fed animals had no change (Figure 3B). It is unclear whether the observed differences in dopamine levels and forepaw stride length are a result of 25(OH)D status; however, changes in dopamine homeostasis are strongly correlated to changes in performance in several behavioral tasks, including gait analyses by forepaw stride length [23].

In humans, middle 25(OH)D insufficiency is associated with higher PD risk while higher serum levels are associated with reduced risk of PD [5]. Whether 25(OH)D insufficiency is causal or correlational remains unknown, but it has been proposed that the vitamin D hormone (1,25(OH)2D) plays a protective role in the health of the neurons affected in PD and that lower serum levels of vitamin D predispose these neurons to damage. Indeed, pretreatment of rat mesencephalic cultures with 1,25(OH)2D (the biologically active form of vitamin D) confers resistance of dopaminergic neurons to MPP+ (the active form of the toxin) [17]. More recently, it was shown that administration of 1,25(OH)2D partially restored TH expression in the nigra of 6-hydroxydopamine models of PD (such as the transgenic A53T).

However, the VMAT2 LO mouse [21,34] would yield different results. It is hypothesized that vitamin D status and other factors associated with chronic deficiency that may be necessary to affect neuronal vulnerability. Alternatively, some interaction between vitamin D status and other
genetic factors may affect risk of PD. It is also possible that vitamin D supplementation may protect neuronal populations against insult, but reduced 25(OH)D levels have little or no effect on the viability and vulnerability of dopamine neurons. Thus, additional studies are warranted to explore the role of 25(OH)D deficiency on the long term health of dopaminergic neurons.

Conclusions
Our data suggest that short term 25(OH)D depletion over the course of a few weeks does not render dopamine neurons more susceptible to the neurotoxin, MPTP as we initially hypothesized. However, additional studies are warranted to explore whether more long-standing deficiency of 25(OH)D or gestational depletion have a role in the laboratory in vivo and in vitro models of dopaminergic neuronal vulnerability to toxins. These large future studies are necessary to establish the role, if any, that vitamin D supplements, vitamin D status or the active 1,25(OH)2D (hormone) play PD pathophysiology.

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Author Contributions
Conceived and designed the experiments: MLE EDD GWM. Performed the experiments: EDD LMM NLC JEM KDP JAD VT. Analyzed the data: EDD. Contributed reagents/materials/analysis tools: LMM NLC JEM KDP JAD VT. Performed the behavioral analyses: EDD LMM NLC JEM KDP JAD VT. Analyzed the gene expression data: EDD. Contributed reagents/materials/analysis tools: LMM NLC JEM KDP JAD VT. Performed the statistical analysis: EDD. Contributed reagents/materials/analysis tools: LMM NLC JEM KDP JAD VT. Contributed reagents/materials/analysis tools: LMM NLC JEM KDP JAD VT. Performed the analyses by forepaw stride length [23].

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