DNA variants in \textit{CACNA1C} modify Parkinson disease risk only when vitamin D level is deficient

\textbf{ABSTRACT}

\textbf{Objective:} To evaluate the association between the genetic variants in \textit{CACNA1C}, which encodes the α1 subunit of the L-type voltage-sensitive calcium channel (LVSCC) and Parkinson disease (PD) while accounting for interactions with vitamin D concentration.

\textbf{Methods:} Two independent case-control data sets (478 cases and 431 controls; 482 cases and 412 controls) were used. Joint effects of single nucleotide polymorphisms (SNPs) and SNP-vitamin D interaction were analyzed by comparing models containing vitamin D deficiency, SNP genotypes, SNP-vitamin D interaction, and covariates to a restricted model with only vitamin D deficiency and covariates. Meta-analysis was used to combine the joint effects in the 2 data sets. Analysis was stratified by vitamin D deficiency to demonstrate the pattern of SNP-vitamin D interaction.

\textbf{Results:} Vitamin D deficiency was associated with PD in both data sets (odds ratio [OR] = 1.9-2.7, \(p \leq 0.009\)). SNP rs34621387 demonstrated a significant joint effect (meta-analysis, \(p = 7.5 \times 10^{-5}\); Bonferroni corrected, \(p = 0.02\)). The G allele at rs34621387 is associated with PD in vitamin D-deficient individuals in both data sets (OR = 2.0-2.1, confidence interval = 1.3-3.5, \(p = 0.002\)) but is not associated with PD in vitamin D-nondeficient individuals (\(p > 0.8\) in both data sets).

\textbf{Conclusions:} Previous studies suggest that vitamin D deficiency is associated with PD and sustained opening of LVSCC contributes to the selective vulnerability of dopaminergic neurons in PD. Our data demonstrate that the association between genetic variations in \textit{CACNA1C} and PD depends on vitamin D deficiency, providing one potential mechanism underlying the association between vitamin D deficiency and PD. \textit{Neurol Genet} 2016;2:e72; doi: 10.1212/NXG.0000000000000072

\textbf{GLOSSARY}

\begin{itemize}
\item 25(OH)D = 25-hydroxyvitamin D
\item DHP = dihydropyridine
\item GWAS = genome-wide association studies
\item LD = linkage disequilibrium
\item LVSCC = L-type voltage-sensitive calcium channel
\item NGRC = NeuroGenetics Research Consortium
\item PD = Parkinson disease
\item SN = substantia nigra
\item UDALL = Morris K Udall Parkinson Disease Research Center of Excellence
\item VDR = vitamin D receptor
\end{itemize}

Parkinson disease (PD) is characterized by the loss of dopaminergic neurons in the substantia nigra (SN). Sustained opening of L-type voltage-sensitive calcium channels (LVSCC) in adult dopaminergic neurons in the SN has been suggested to cause excessive \(\text{Ca}^{2+}\) entry, leading to excessive mitochondrial stress and production of reactive oxygen species, rendering SN neurons vulnerable to cell death.\(^1\) In the brain, \(\text{Ca}_{1.2}\), encoded by \textit{CACNA1C}, accounts for approximately 90\% of LVSCC activity, with \(\text{Ca}_{1.3}\), encoded by \textit{CACNA1D}, accounting for the remaining activity.\(^2\) \(\text{Ca}_{1.2}\) and \(\text{Ca}_{1.3}\) are antagonized by dihydropyridine (DHP) derivatives.

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Both animal and retrospective human studies support a protective role of DHP derivatives in PD development.3–9 Nonetheless, genetic variations in CACNA1C or CACNA1D have not been studied with PD risk.

As a complex disease, PD has both genetic and environmental risk factors that act individually or through complex interactions to influence disease pathogenesis.10–18 Recently, several studies, including ours,19 have reported that lower circulating vitamin D concentration is associated with the risk of PD.20–22 Vitamin D is known to exert its biological actions by regulating the expression of many genes, providing a plausible molecular mechanism for gene-environment interactions. Previous studies have shown that vitamin D treatment decreases CACNA1C messenger RNA expression through the vitamin D receptor (VDR) in primary neuronal cultures.23,24 We hypothesize that CACNA1C and CACNA1D would be good candidate genes for PD and that the association of SNPs in these genes with PD might be modulated by circulating vitamin D concentrations.

**METHODS Subjects.** Subjects from 2 genome-wide association studies (GWAS) were included in the study. The first GWAS was conducted by the Morris K Udall Parkinson Disease Research Center of Excellence (UDALL) at the University of Miami,25 and the second GWAS was conducted as part of the NeuroGenetics Research Consortium (NGRC).26 Participant enrollment and clinical assessment were described previously.25–26 In brief, cases were ascertained by neurology clinics associated with UDALL (from 1997 to 2003) or NGRC (from 2003 to 2009). Controls were ascertained from community outreach efforts or were spouses of individuals with PD or Alzheimer disease (M.A.P.-V., Principal Investigator). All participants with stored plasma samples available for vitamin D assessment were included in the study. Participants in this study are non-Hispanic whites by self-report and confirmed by principal component analysis of population structure in the GWAS.

**Standard protocol approvals, registrations, and patient consents.** All participants gave informed consent prior to these studies. All ascertainment protocols were approved by each contributing center’s institutional review board.

**Genetic markers.** The UDALL data set was genotyped using Illumina 610-quad BeadChip (Illumina, San Diego, CA).25 The NGRC data set was genotyped using Illumina HumanOmni1-Quad_v1-0_B BeadChip (Illumina).26 Final marker sets after quality control (described in detail in these previous publications) were used for the imputation of untyped SNPs separately in each of the 2 data sets. Imputation allowed us to obtain genetic information on additional variants through the genome for a more thorough investigation. Both data sets were imputed up to 38 million SNPs using IMPUTE2 and the 1000 Genomes reference panel (phase 1, March 2012).27 During quality control, SNPs that had low imputation quality (info score <0.4) and low minor allele frequency (less than 1%) were removed from further analysis. SNPs within the 5-kb flanking regions of the start and end of CACNA1C and CACNA1D were examined for interaction with vitamin D status and association with PD.

**Plasma vitamin D measurement.** Stored plasma samples were analyzed using liquid chromatography tandem mass spectrometry at the Emory Clinical Translational Research Laboratory as described previously.28 In brief, the samples were analyzed using the methods outlined before.28 Three quality control samples (approximately 6, 21, and 62 ng/mL) were included in duplicate at the beginning and end of each run. The assay was linear up to 200 ng/mL and had a limit of detection of 1 ng/mL. Total imprecision ranged from 10.8% to 17.1%. The assay was validated using the NIST 972a standards. The laboratory participates in the Vitamin D External Quality Assessment Scheme proficiency scheme. For both the discovery and replication data sets, the samples were organized into multiple batches for vitamin D measurement: each batch included ~220 samples with a balanced number of cases and controls, analyzed in random order with respect to affection status. Laboratory technicians were blinded to affection status.

**Statistical analysis.** Logistic regression analysis was used to evaluate the association between vitamin D status and PD, adjusting for age at sampling, sex, and sampling season in each data set separately using the R software package. Vitamin D deficiency was defined as having total 25-hydroxyvitamin D [25(OH)D] <20 ng/mL, and vitamin D insufficiency was defined as having total 25(OH)D <30 ng/mL. A joint test of SNP main effect and SNP-vitamin D interaction effect was conducted using a 2 degrees-of-freedom likelihood-ratio test.29 This joint test compares a full model containing vitamin D deficiency, SNP dosage (additive genetic model), an SNP-vitamin D deficiency interaction term, and covariates (age at sampling, sex, and sampling season) to a restricted model with only vitamin D deficiency and covariates. Such an analysis will detect loci missed by main-effects analysis conducted in previous GWAS if the combined effect of the SNP effect and environmental interaction is stronger than the main effect of the SNP alone.

Besides a different genotyping platform, the 2 GWAS data sets are also different in distribution of vitamin D concentration, age, sex, and sampling season covariates (table 1). Therefore, joint tests were conducted in each data set separately. To achieve better statistical power while accommodating the heterogeneity between the 2 data sets, the joint test results in the 2 data sets were then combined using a meta-analysis approach. This meta-analysis used the joint meta-analysis extension of METAL.30 To adjust for multiple testing, simpleM was used to estimate the effective number of independent tests taking into account linkage disequilibrium (LD) between SNPs;31 this estimate of independent tests was used to determine a significant threshold for each gene, adjusting for the number of independent tests. To unravel the nature of the interactions between SNP dosage and vitamin D deficiency, stratification analysis was performed to evaluate the associations between SNPs and PD risk in vitamin D-deficient and vitamin D-nondeficient strata separately. The use of prevalent cases could lead to potential survival bias. Sensitivity analysis was carried out by duration of disease to test the effect of considering disease duration in the case definition on the identified association, i.e., cases with shorter duration (“near-incident” cases with less than 3 years of duration) or longer duration (3 years or longer).
RESULTS Characteristics of subjects in 2 data sets.

Table 1 displays the characteristics of participants from the 2 data sets. In both data sets, the percentage of women is lower in the PD cases than controls, consistent with prior observations of increased prevalence of PD in men. In the UDALL data set, the controls have older age at sampling than cases because of the intentional recruitment of controls at older ages to reduce the possibility of including controls who would develop PD after ascertainment. In the NGRC data set, the controls have younger age at sampling than cases. There is no difference in sampling season between cases and controls in UDALL; however, in NGRC, more PD cases were sampled between July and December (when higher vitamin D levels are usually observed for the same individual) than in controls. Owing to these differences in clinical characteristics and genotyping platforms between the 2 data sets, we elected to perform individual analyses in each data set separately.

Lower vitamin D concentrations are associated with PD in both data sets. Total plasma 25(OH)D concentrations are significantly lower in PD cases than in controls in both data sets (table 1). Total 25(OH)D was also analyzed categorically, using the established clinical criteria for vitamin D deficiency (25(OH)D < 20 ng/mL) and vitamin D insufficiency (25(OH)D < 30 ng/mL). Cases with PD are significantly more likely than controls having vitamin D deficiency (25(OH)D < 20 ng/mL) and vitamin D insufficiency (25(OH)D < 30 ng/mL) in both data sets (table 2). In addition, a dosage effect was consistently observed in both data sets, with vitamin D deficiency more strongly associated with PD than was vitamin D insufficiency (odds ratio [OR] = 2.63 vs 2.13 in UDALL, OR = 1.56 vs 1.34 in NGRC).

Analysis of interaction between vitamin D deficiency and SNPs in CACNA1C and CACNA1D. Since the strongest association was found between vitamin D deficiency and PD, the initial gene-environment interaction analysis was focused on analyzing the association of PD with the joint effects of SNP dosage, and its interaction with vitamin D deficiency. In total, 1,244 SNPs in CACNA1C and 504 SNPs in CACNA1D were available for analysis. The effective number of independent tests after adjusting for LD is 274 and 175 in CACNA1C and CACNA1D, respectively. To maximize statistical power, we performed a meta-analysis across both data sets. The strongest evidence for a joint effect was found at SNP rs34621387 (meta-joint test \( p = 7.5 \times 10^{-5} \), Bonferroni-adjusted \( p = 0.02 \)) in the third intron of CACNA1C (figure, A). Within CACNA1D, the lowest meta-joint test \( p \) value is 0.02, which would not remain significant after adjusting for multiple testing (figure, B). To further evaluate the nature of the interaction, associations between rs34621387 and PD were evaluated in individuals who were vitamin D deficient. Additional sensitivity analyses were performed by age at onset and sex to test whether the identified association is restricted to early or later age at onset of PD and whether it is sex specific.

To investigate the potential biological basis for the gene-vitamin D interaction at the most significant SNP, 1000 Genomes phase 3 data were used to identify all SNPs that are in LD within a 1 million base pair region surrounding the significant SNP. In addition, publicly available data from CHIP-Seq experiments with VDR were also used to evaluate the functional relevance of SNPs.

Table 2 Association between vitamin D status and PD risk

|                      | Prevalence, % | OR       | 95% CI     | \( p \) Value |
|----------------------|--------------|----------|------------|--------------|
|                      | Case | Control |       |           |
| Vitamin D deficiency | 36   | 17      | 2.64     | 1.88-3.71   | <0.0001      |
| Vitamin D insufficiency | 71  | 54      | 2.13     | 1.58-2.89   | <0.0001      |
| Vitamin D deficiency | 26   | 19      | 1.56     | 1.12-2.18   | 0.009        |
| Vitamin D insufficiency | 61  | 55      | 1.34     | 1.01-1.77   | 0.042        |

Abbreviations: CI = confidence interval; NGRC, NeuroGenetics Research Consortium; OR = odds ratio; PD = Parkinson disease; UDALL = Morris K Udall Parkinson Disease Research Center of Excellence.
deficient or vitamin D nondeficient separately in each data set. This analysis revealed that the association of rs34621387 and PD depends on vitamin D concentration: allele G is associated with PD in the vitamin D-deficient stratum in both data sets (OR = 2.0, \(p = 0.002\) and OR = 2.1, \(p = 0.002\)) but is not associated with PD in vitamin D-nondeficient stratum in both data sets (\(p > 0.8\)) (table 3).

Sensitivity analysis suggested that this pattern of association (restricted to vitamin D-deficient individuals) is not affected by age at onset, disease duration, or sex (table e-1 at Neurology.org/ng).

**Functional relevance of the intronic SNP rs34621387 in CACNA1C.** No SNPs in substantial LD (\(r^2 > 0.2\)) with rs34621387 were found to be located in a
known VDR binding site, as delimited by reported Chip-Seq experiments.\textsuperscript{32,33} We used RegulomeDB\textsuperscript{34} and HaploReg\textsuperscript{35} to determine whether rs34621387 or its proxies are located within any other regulatory elements. This analysis found that rs34992881 ($\chi^2 = 0.76$ with rs34621387) is located within a region displaying epigenomic signatures for chromatin enhancer, which include histone H3 lysine 4 trimethylation (H3K4me3), H3K27 acetylation (H3K27ac), and H3K9ac. In addition, the enhancer signatures are only detected in some of the tissues or cell types surveyed by the Roadmap Epigenome project, suggesting the possibility of the tissues or cell types surveyed by the Roadmap enhancer signatures are only detected in some of

DISCUSSION

It is well established that vitamin D exerts its main biological actions by regulating the expression of a large array of genes and one of them is \textit{CACNA1C}.\textsuperscript{23,24,32,33} Using 2 case-control data sets where vitamin D deficiency is significantly associated with PD, we found that vitamin D status significantly modified the association between SNPs in \textit{CACNA1C} and PD: genetic effects of \textit{CACNA1C} only manifest in individuals with vitamin D deficiency, but not in individuals who are not vitamin D deficient. As such, the effect of \textit{CACNA1C} in PD would have become difficult to detect in a sample where a substantial portion of individuals have sufficient vitamin D levels, if only SNP main effects were examined. Our study demonstrates the importance of incorporating environmental exposure to understand the genetic underpinning for interindividual phenotypic variance in PD.

Using existing databases, no definitive evidence for regulatory potential at rs34621387 or its proxies was observed. Therefore, the biological mechanism underlying the association of rs34621387 and PD in the presence of vitamin D deficiency is currently not understood. Interestingly, SNP rs1006737, in the same intron (intron 3) as rs34621387 in \textit{CACNA1C}, has been widely replicated in association with schizophrenia and other neuropsychiatric conditions, making it one of the most robust findings in psychiatric genetics.\textsuperscript{36-38} Similar to rs34621387, no compelling evidence for regulatory function was initially found for rs1006737 or proxies, using existing databases. Subsequent studies revealed that SNPs in LD with rs1006737 interact with the proximal promoter region and direct allelic-specific expression of \textit{CACNA1C} in human induced pluripotent stem cell-derived neurons, providing a molecular basis for the SNP-phenotype association.\textsuperscript{39,40} SNP rs1006737 and rs34621387 are not in strong LD with each other ($r^2 < 0.1$). Whether rs34621387 confers genetic risk to PD by directing allelic-specific expression of \textit{CACNA1C}, in a similar fashion as rs1006737 in schizophrenia, will require further, more in-depth functional studies.

Previous studies have shown that vitamin D inhibits the expression of \textit{CACNA1C}.\textsuperscript{23,24} One possible mechanism underlying the observed interaction is that high vitamin D levels effectively suppress LVSCC expression and thus reduce the activity of LVSCC in the dopaminergic neurons in SN, masking the effect of rs34621387.

It has been proposed that Ca\textsubscript{1,3}, encoded by \textit{CACNA1D}, might be particularly responsible for the selective death of dopaminergic neurons in the SN based on the observation that Ca\textsubscript{1,3} channels open at relatively hyperpolarized membrane potentials, which makes them more suitable for pacemaking.\textsuperscript{1} We did not find any evidence for association between genetic variants in \textit{CACNA1D} and PD, even after taking into account vitamin D status, which is consistent with the lack of evidence suggesting that \textit{CACNA1D} is regulated by vitamin D.\textsuperscript{32,24} Our study,
however, does not exclude the possibility that genetic variations in CACNAID are involved in PD pathogenesis as our study is limited by a modest sample size, a focus on common variants, and interactions with vitamin D concentration. In addition, we used an ethnically homogeneous population (i.e., non-Hispanic whites) to minimize confounding by population substructure. Additional studies are needed to assess generalizability to other populations.

As in other case-control association studies of vitamin D concentration and PD, our results could reflect “reverse causation,” i.e., lower vitamin D concentrations in patients with PD are a consequence of the disease (lower levels of sun exposure due to reduced mobility and less time spent outdoors) rather than a cause. However, a previous longitudinal study demonstrated that lower vitamin D levels at baseline are associated with increased risk of PD during a 29-year follow-up; a second study showed that patients with early, nondisabling PD and relatively normal mobility have lower vitamin D levels; and our own work demonstrated that lower vitamin D2, a form of vitamin D that is obtained independently from sunlight exposure, is associated with PD, as is vitamin D3 (the form of vitamin D that is mainly obtained from sunlight exposure).19

Our data demonstrate that genetic variations in CACNA1C and vitamin D deficiency act together to increase PD risk. This study also highlights the importance of incorporating environmental exposure to identify genetic factors influencing interindividual phenotypic variance in PD.

AUTHOR CONTRIBUTIONS
L.W. and W.K.S. conceived and designed the experiments. J.L.H., C.P.Z., H.P., M.A.P.-V., and J.M.V. were responsible for collecting biosamples and obtaining genotype data. M.L.E. and J.C.R. were responsible for measuring vitamin D concentrations. L.M., G.W.B., and E.R.M. imputed genotype data and performed statistical analyses. L.W., W.K.S., and J.M.V. interpreted the data. L.W. wrote the manuscript. W.K.S. and J.M.V. edited the manuscript. All authors read and approved the final manuscript.

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