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Vitamin D Insufficiency and Schizophrenia Risk: Evaluation of Hyperprolinemia as a Mediator of Association

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Abstract

25-hydroxyvitamin D (25(OH)D) deficits have been associated with schizophrenia susceptibility and supplementation has been recommended for those at-risk. Although the mechanism by which a deficit confers risk is unknown, vitamin D is a potent transcriptional modulator and can regulate proline dehydrogenase (*PRODH*) expression. *PRODH* maps to chromosome 22q11, a region conferring the highest known genetic risk of schizophrenia, and encodes proline oxidase, which catalyses proline catabolism. L-Proline is a neuromodulator at glutamatergic synapses, and peripheral hyperprolinemia has been associated with decreased IQ, cognitive impairment, schizoaffective disorder, and schizophrenia.

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Conflict of Interest. Dr C. Clelland and Dr J. Clelland are the inventors on a US patent application that is based in part upon this study data. If awarded, the patent will be owned by their respective institutions, and Drs' Clelland may benefit financially in the future if the patent is licensed. Dr C. Clelland and Dr J. Clelland declare no other conflict of interest. Dr Read, Dr Drouet, Ms. Kaon, Ms Kelly, Dr Duff, Dr Nadrich and Dr Rajparia declare no conflict of interest.

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We investigated the relationship between 25(OH)D and schizophrenia, comparing fasting plasma 25(OH)D in 64 patients and 90 matched controls. We then tested for a mediating effect of hyperprolinemia on the association between 25(OH)D and schizophrenia.

25(OH)D levels were significantly lower in patients, and 25(OH)D insufficiency associated with schizophrenia (OR 2.1, adjusted p=0.044, 95% CI: 1.02-4.46). Moreover, 25(OH)D insufficient subjects had three times greater odds of hyperprolinemia than those with optimal levels (p=0.035, 95% CI: 1.08-8.91), and formal testing established that hyperprolinemia is a significantly mediating phenotype that may explain over a third of the effect of 25(OH)D insufficiency on schizophrenia risk. This study presents a mechanism by which 25(OH)D insufficiency confers risk of schizophrenia; via proline elevation due to reduced *PRODH* expression, and a concomitant dysregulation of neurotransmission. Although definitive causality can not be confirmed, these findings strongly support vitamin D supplementation in patients, particularly for those with elevated proline, who may represent a large subgroup of the schizophrenia population.

Keywords

Vitamin D insufficiency; hyperprolinemia; proline; schizophrenia; mediator; treatment

Introduction

$1\alpha,25(OH)_2D_3$, the active form of 25-hydroxyvitamin D (25(OH)D), is a pleotropic steroid hormone, for which synthesis is initiated in the skin via enzymatic conversion of 7-dehydrocholesterol in the presence of ultraviolet B (UVB) light (Rosen, 2011) and can also be derived from some food sources. In addition to its well-established and vital role in the maintenance of calcium homeostasis and bone mineral density (Rosen, 2011; Heaney, 2008), insufficiency or deficiency of 25(OH)D (the sum of $25(OH)D_2$ and $25(OH)D_3$), has been associated with cognitive impairment (Llewellyn et al., 2011), metabolic, immune, and malignant disease (reviewed in (Rosen, 2011) and more recently depression (Milaneschi et al., 2013). Epidemiological studies have also implicated 25(OH)D deficits in the risk for other psychiatric illness (Eyles et al., 2013), in particular susceptibility to schizophrenia (McGrath et al., 2010a).

Direct and compelling evidence for the association of low vitamin D with increased schizophrenia risk comes from two infant cohort studies. From analysis of a large birth cohort, McGrath et al. reported a significantly reduced risk of schizophrenia in male infants receiving vitamin D supplements (≥2000 IU/day) during their first year of life (McGrath et al., 2004). Retrospective measurement of $25(OH)D_3$ in neonatal blood spots from over 800 schizophrenia patients and matched controls, then demonstrated that infants with low $25(OH)D_3$ had a significantly increased risk of schizophrenia (McGrath et al., 2010b). Consistent with these findings, higher $25(OH)D_3$ has been associated with a lower risk of psychotic experiences in children (Tolppanen et al., 2012). This body of work has led to the recommendation of maternal, neonatal, infant, or early childhood vitamin D supplementation for those at-risk (McGrath, 2010). A recent finding of 25(OH)D deficiency in patients with first-episode psychosis (Crews et al., 2013), further supports a role for low vitamin D as a risk factor for psychotic illness.

A number of small studies have also suggested that 25(OH)D deficits also extend into adulthood schizophrenia: Significantly lower circulating 25(OH)D levels have been reported in adult patients with chronic schizophrenia compared to other psychiatric patients (Humble et al., 2010; Menkes et al, 2012; Itzhaky et al., 2012) as well as healthy controls (Rey-Sánchez et al., 2009; Partti et al., 2010; Doknic et al., 2011; Itzhaky et al., 2012). The importance of 25(OH)D level maintenance in the adult population has also been highlighted by a large cohort study, from which it was reported that women with high dietary vitamin D consumption had a 37% lower risk of psychosis-like symptoms as compared to women with low consumption (Hedelin et al.. 2010). To date no mechanism has been found to underpin the increased schizophrenia risk associated with vitamin D insufficiency.

The highest known genetic risk of schizophrenia, aside from that shared by monozygotic twins, is conferred by hemizygous microdeletion of chromosome 22q11 (Karayiorgou and Gogos, 2004). The proline dehydrogenase gene (*PRODH*), located within the common deleted region, encodes the PRODH/proline oxidase (POX) enzyme that catalyzes the first step in proline catabolism (see Supplementary Data, Figure S1). Proline is a neuromodulator at glutamatergic synapses (reviewed in Phang et al., 2001) and in humans the peripheral hyperprolinemia that can arise from mutations in *PRODH*, and which likely reflects CNS hyperprolinemia (Gogos et al., 1999; Paterlini et al., 2005; Dingman and Sporn, 1959; Efrom, 1965; Baxter et al., 1985; Jacquet et al., 2003; Shanti et al., 2004), has been significantly associated with cognitive impairment and decreased IQ (Raux et al., 2007), schizoaffective disorder (Jacquet et al., 2005) and schizophrenia (Clelland et al., 2011; Orešič et al., 2011).

25(OH)D is a potent modulator of gene expression, and has been shown to significantly upregulate *PRODH* gene expression in the presence of a p53 activator (Thompson et al., 2010). Screening the Gene Expression Omnibus (GEO) database to identify reproducible, biological regulators of *PRODH*, we also identified two GEO studies showing direct upregulation of *PRODH* in response to 25(OH)D_3 treatment (GEO accession numbers **GDS2628** and **GDS1847**). We hypothesized that schizophrenia risk may be mediated by proline elevation arising due to 25(OH)D deficits and the resultant decrease of *PRODH* expression. We therefore measured levels of 25(OH)D in the same plasma samples from 64 schizophrenia patients and 90 matched controls that were also assayed for fasting proline (Clelland et al., 2011), to investigate a causal relationship between 25(OH)D, hyperprolinemia, and schizophrenia.

Materials and Methods

Database Screening

Using a nonbiased approach, the GEO database (accessible at http://www.ncbi.nlm.nih.gov/geo/) was screened for molecules that regulate *PRODH*, using the Gene Profiles query term "*PRODH*". *PRODH* expression data was extracted from two retrieved studies, GEO accession numbers **GDS2628** and **GDS1847**. The materials and methods employed to generate this microarray expression data were published in (Bossé et al., 2007) and (Wood et al., 2004) respectively.

Study Participants

Male and female, African American, Caucasian and Hispanic patients, aged 18-65, were recruited from inpatient psychiatry wards at Bellevue Hospital Center (BHC). A schizophrenia diagnosis was confirmed using the Structured Clinical Interview for DSM IV Disorders (SCID). Patients received a standardized hospital diet based upon the American Diabetes Association guidelines of 20% protein, 25% fat, and 55% carbohydrate. Volunteer controls were recruited from the BHC community, with recruitment targeted to reflect patients on age, ethnicity, and gender. A SCID-NP interview was conducted for all controls, who were excluded if they reported symptoms from modules A-D. Capacity to give informed consent was determined in accordance with the New York University Langone Medical Center (NYU) IRB regulations. After complete description of the study to all subjects, written informed consent was obtained from all subjects in accordance with all NYU IRB regulations.

Determination of Fasting Plasma Levels

A fasting morning blood draw was performed as reported (Clelland et al., 2011). Immediately following each blood draw, heparinized bloods were sent to the national reference laboratory ARUP Laboratories (500 Chipeta Way, SLC, UT 84108) for quantitative plasma amino acid analysis (reference number 0080710). Proline was measured in μmoles/liter. To adjust for previously reported proline gender differences (Jacquet et al., 2005; Clelland et al., 2011), hyperprolinemic status was assessed according to the definition of Jacquet et al., as a proline level two standard deviations (SDs) or more above the gender-specific mean of controls.

25(OH)D (the sum of $25(OH)D_2$ and $25(OH)D_3$), was measured in ng/ml by quantitative chemiluminescent immunoassay, also at ARUP Laboratories (reference number 0080379). The Institute of Medicine (IOM) has suggested that levels of circulating 25(OH)D of ≥20ng/ml are sufficient for bone health in the general population (IOM, 2011; Rosen et al., 2012). However, attempting to provide guidance to clinicians caring for patients, the Endocrine Society (ES) most recently set the optimum level to ≥30ng/ml (Holick et al., 2011; Heaney et al., 2011; Holick et al., 2012). For this study of schizophrenia patients, 25(OH)D insufficiency was defined according to the clinically relevant ES definition of <30ng/ml (and thus encompassed both insufficiency and deficiency). 25(OH)D was measured for all subjects from a stored frozen plasma sample stabilized with dithiothreitol 0.1%(w/v) final concentration. These fasting plasma samples were frozen at -80°C immediately upon collection, and were not thawed prior to processing for 25(OH)D measurement in a single batch (n=154).

Statistical Analysis

Demographic variables were tested for diagnostic group differences using the Satterthwaite t-test or ANOVA (with a correction for multiple testing when n>2 groups), and χ^2 or Fisher exact tests. To assess the relationship across 25(OH)D strata, a Mantel-Haenszel Extension test was employed. Associations between the primary outcome variables of 25(OH)D insufficiency and hyperprolinemia, with schizophrenia, were examined via a causal mediation analysis, which utilizes a series of logistic regression models to test the primary

hypothesis that hyperprolinemia mediates the association between 25(OH)D insufficiency and schizophrenia. The hypothesized mediation model is shown in Figure 1. Mediation analysis allows the direct estimate of the effect size and significance of the hypothesized causal mechanism (the relationship between the independent variable-IV (vitamin D insufficiency), and the mediator variable-MV (hyperprolinemia) on the outcome variable of interest (the dependent variable, DV- schizophrenia) (Baron and Kenny, 1986; Kenny et al., 1998) calculating the coefficients for path c (Figure 1A, the direct association between 25(OH)D insufficiency and schizophrenia with no mediation), paths a and b (Figure 1B, the hypothesized associations between 25(OH)D insufficiency and hyperprolinemia, and hyperprolinemia and schizophrenia respectively).

To assess the relationship between 25(OH)D insufficiency (<30ng/ml) and diagnostic group (path c), covariates were examined in bivariate analyses. Terms found to have a relationship with both vitamin D insufficiency and diagnostic group (p values of <0.10) were subsequently evaluated in the multivariate model. Model goodness-of-fit was determined using the Likelihood Ratio test (LRT: [-2ln(likelihood for null model/likelihood for alternative model)]), testing for the significant influence of covariates plus the main explanatory variables in each sequential multivariate model. LRT p-values ($p > \chi^2$) were reported as both unadjusted and Bonferroni adjusted (for n=4 model tests).

For the final model, the coefficients (standardized to allow the indirect effect to be computed as the product of coefficients) for paths c, a, b, and c′ (the direct IV to DV, mediated by the MV) were calculated, with bias-corrected confidence intervals (CIs) determined via the non-parametric bootstrapping procedure based upon 5000 permutations. This method computes an empirically generated sampling distribution from which a 95% CI can be determined. Mediation was considered significant if the 95% CI for the indirect pathway coefficient (a +b) did not contain the null value. A sensitivity analysis was also performed to assess robustness of the final model in the presence of model violations (Supplementary Results Three: Sensitivity Analysis). Statistical analyses were performed in SAS v9.1, and Stata IC v11.

For completeness of the analysis, proline and 25(OH)D levels were also assessed as continuous variables, with normality assessed via Skewness and Kurtosis tests, and subsequently analyses performed using the Mann-Whitney or Kruskal-Wallis non-parametric tests, and Spearman's rank correlation. As secondary analyses, we also assessed the relationship between 25(OH)D levels, and cognitive and psychiatric symptoms in patients. A score of ≥27 on the Mini-mental-State Exam (MMSE) was utilized to indicate normal cognitive function (Crum et al., 1993), seeking to avoid distribution violations due to ceiling effects.

Results

Vitamin D Positively Regulates *PRODH* Gene Expression *in vitro*

Searching for regulators of *PRODH* expression, we identified two microarray gene expression studies in which treatment with vitamin D upregulated *PRODH* expression *in vitro* (Supplementary Data, Results One).

25(OH)D Insufficiency is Significantly Associated with Schizophrenia

Patients and control subjects were well matched on demographic characteristics, including variables with a likely role in determining vitamin D levels, such as ethnicity (within each diagnostic group, 33-35% were African-American, 33-34% Caucasian, and 33% Hispanic), Body Mass Index (BMI), and season of recruitment (Table 1). In the entire sample (n=154), fasting plasma 25(OH)D levels were significantly lower in patients as compared to controls (Mann-Whitney z=2.023, p=0.043, Table 1).

There have been conflicting definitions of 25(OH)D insufficiency, with recommendations of sufficiency of ≥30ng/ml for clinical care (Holick et al., 2011; Heaney et al., 2011; Holick et al., 2012) as opposed to population-based bone health guidelines of ≥20ng/ml (IOM, 2011; Rosen et al., 2012). Categorizing vitamin D levels into three strata (≥30ng/ml = optimal levels; 29–20ng/ml = ES insufficient; and 19-10 ng/ml = IOM insufficient), there was a significant linear trend for schizophrenia with decreasing 25(OH)D (χ^2 1df = 5.18, p=0.023). However, the odds of schizophrenia were not different for ES insufficient versus sufficient subjects (n=43 v n=68, ORs=2.51, p=0.023) as compared to IOM insufficient versus sufficiency (n=36 v n=68, ORs=2.4, p=0.033). Based upon this finding and the ES clinical rather than population based recommendations for sufficiency, we employed the definition of <30ng/ml for further analyses.

25(OH)D insufficiency was significantly associated with schizophrenia (p=0.007, 95% CI: 1.3-4.9, Figure 1A, path c, and Table 2: Model 1). As expected, covariate analysis showed the explanatory variables of ethnicity (African American versus Caucasian, β=-1.20, p=0.004; African American versus Hispanic, β=-0.49 p=0.24), and season of recruitment (winter/spring versus summer/autumn, β=0.57, p=0.083), were predictors of 25(OH)D insufficiency at p<0.1, but neither were related to diagnostic group (p>0.1, Supplementary Data Table S1). A subject's level of education predicted both 25(OH)D insufficiency (β=-0.53, p=0.033) and schizophrenia (β=-1.49, p<0.001, Table S1) and therefore this variable was taken forward to the multivariate model to investigate its potential to confound the relationship between insufficiency and schizophrenia. The variables of age (p=0.99), gender (p=0.49), alcohol use (p=0.13), vitamin D supplementation (≤400IU/day, p=0.73), body mass index (BMI, p=0.27), and for the patients, duration of their hospital stay prior to the fasting blood draw (p=0.38) and antipsychotic medication (as measured by chlorpromazine (CPZ) equivalents, p=0.434), were determined to have no relationship with 25(OH)D insufficiency. While smoking status was a significant predicator of diagnostic group, the odds of insufficiency among current or former smokers were not significantly different to those subjects who had never smoked (p=0.25). We also investigated variables related to the storage of the plasma samples (Table S1), because although the stability of vitamin D after decades in storage has been reported (McGrath et al., 2010b), in our study fasting plasma 25(OH)D levels were measured at the study end. However, the number of days in storage from blood draw to measurement did not predict vitamin D insufficiency (Table S1: β=0.0008, p=0.317).

As secondary analyses we investigated the relationship between 25(OH)D levels and cognitive and psychiatric symptoms. In the entire sample 25(OH)D levels were not related to MMSE score (n=153, Mann-Whitney p=0.245), with similar findings in a stratified

analysis on patients (n=63, Mann-Whitney p=0.696) and controls (n=90, Mann-Whitney p=0.992). In patients, 25(OH)D levels were also not related to psychiatric symptom severity: Brief Psychiatric Rating Scale (n=64, Spearman's rho=0.01, p=0.93), Scale for Assessment of Positive Symptoms (n=64, rho=0.2, p=0.11), and Scale for Assessment of Negative Symptoms (n=64, rho=-0.07, p=0.61). There was no significant relationship between 25(OH)D levels and age at first hospitalization (n=47, rho=0.07, p=0.66), or duration of illness (n=47, rho=0.10, p=0.49), and levels did not differ across the schizophrenia subtypes of the patient population (disorganized, paranoid, residual, and undifferentiated, Kruskal-Wallis χ^2=0.31, 3df, p=0.95). Similarly, there was no significant relationship between categorical vitamin D insufficiency and psychiatric symptom measures or clinical characteristics (Mann-Whitney p>0.05 for each outcome).

Hyperprolinemia Mediates the Relationship between 25(OH)D Insufficiency and Schizophrenia

Testing our mediation hypothesis, we examined the relationship between the clinically relevant outcomes of 25(OH)D insufficiency and hyperprolinemia: Insufficient subjects had three times greater odds of hyperprolinemia than those with optimal levels (n=154, OR=3.1, p=0.035, 95% CI: 1.08-8.91, Figure 1B, path a)). The levels of 25(OH)D in patients and controls, by both insufficiency and hyperprolinemic status are shown in Figure 2. As a continuous variable, 25(OH)D levels were also negatively correlated with fasting plasma proline levels (n=154, rho= -0.21, p=0.01, Supplementary Results Two Figure S3).

Table 2 shows the multivariate models employed to determine the effect of 25(OH)D insufficiency on diagnostic group, with adjustment for education level, followed by addition to the model of the hypothesized MV, hyperprolinemic status. Sequential model goodness-of-fit was determined, with the final model retaining the variables of insufficiency, education, and hyperprolinemic status (model 3). Notably, upon the addition of hyperprolinemia to the model, the adjusted β coefficient of the significant relationship between 25(OH)D insufficiency and schizophrenia decreased (from an OR of 2.13 to 1.86) with a widening of the 95% CI and inclusion of the null value, suggesting that hyperprolinemic status may mediate this relationship, but does not moderate the association (no significant interaction between insufficiency and hyperprolinemia in model 5). Calculating the standardized coefficients for paths a, b, c, and c′, from the adjusted and unadjusted models with bias-corrected confidence intervals (Table 3), we demonstrated significant mediation in both models (the 95% CI of the indirect pathway did not contain the null value), concluding that over one third, or 37.7% of the total association between vitamin D insufficiency and schizophrenia is mediated by the presence of hyperprolinemia. A formal sensitivity analysis, which provides a measure of the model robustness to the possible existence of an unmeasured confound, supports our causal interpretation (Supplementary Results Three).

Discussion

A series of large cohort studies has implicated insufficiency of vitamin D in the susceptibility to both psychosis and schizophrenia (McGrath et al., 2004; McGrath et al.,

2010b; Tolppanen et al., 2012; Hedelin et al., 2010), However, the mechanism by which this deficit confers risk remains unknown. Based upon data showing regulation of *PRODH* by vitamin D, this current study in which we performed a formal test of causal mediation, suggests that over one third of the association between 25(OH)D insufficiency and schizophrenia may be explained by the presence of hyperprolinemia.

A mediation model seeks to identify and explain the mechanism that underlies an observed relationship: In this case, does proline elevation mediate the relationship between 25(OH)D insufficiency and schizophrenia. According to the Baron and Kenny test of mediation, three criteria must be met to support and formally test a mediation hypothesis: *Criterion 1)The independent variable (25(OH)D insufficiency), is associated with the outcome (schizophrenia)*. We observed a significant association, which supports the findings from recent studies (Rey-Sánchez et al., 2009; Partti et al., 2010; Doknic et al., 2011; Itzhaky et al., 2012). We also considered covariates with a known role in regulating vitamin D, and their potential to explain our study findings. As expected, we found a significant relationship between 25(OH)D deficiency and ethnicity with darker skin pigmentation, as well as season of recruitment. However, patient and control groups were well matched on these two variables, and neither could account for the significant finding between insufficiency and diagnostic group. In our study, we found no relationship between BMI and insufficiency, which is consistent with four out of five studies that investigated 25(OH)D insufficiency in psychotic patients as compared to controls (reviewed in Belvederi Murri et al., 2013). We also found no relationship between vitamin D supplementation and insufficiency, possibly because the vitamin D supplement dose required to merely maintain blood levels has been estimated as 500 IU/day (Heaney et al., 2003), with a rise in vitamin D of 8-11 ng/ml per 1,000 IU of daily supplementation for people with levels in the insufficiency range (Garland et al., 2011). In our study no subject received supplements greater than 400IU/day.

In our sample lower education levels were associated with both 25(OH)D insufficiency and schizophrenia. While the relationship between education level and vitamin D has been largely unreported in studies of psychiatric populations (Belvederi Murri et al., 2013), Partti et al., also found significantly lower mean serum 25(OH)D levels in forty-eight schizophrenia patients whose levels of 25(OH)D were compared to a large sample of over 6000 healthy survey participants (Partti et al., 2010), who appeared to be appropriately matched to patients' on education level (Fisher's exact p=1.04). Moreover in our study, adjusting for this factor did not notably attenuate the association between insufficiency and schizophrenia.

A continuum for Vitamin D levels across the spectrum of psychotic illness has been suggested (Belvederi Murri et al., 2013), with schizophrenia patients manifesting the lowest levels. Correlations between 25(OH)D levels and some negative/depressive symptoms have also been reported among patients with psychotic symptoms (Berg et al., 2010), and depressive disorders (Milaneschi et al., 2013). However, in our schizophrenia sample we found no relationship between 25(OH)D levels and total symptom scale scores for positive and negative symptoms, which is consistent with another study of schizophrenia (Itzhaky et al., 2012). This may suggest that assessment of total scores does not allow for evaluation of the discreet symptoms that associate 25(OH)D with schizophrenia or alternatively, that

investigation of additional assessments, for example assessments targeting cognitive dysfunction, is warranted.

Criterion 2) The independent variable (25(OH)D insufficiency), significantly predicts the mediator, (hyperprolinemia). This in turn significantly predicts the dependent variable, (schizophrenia). We observed a significant association between insufficiency and hyperprolinemia: Those subjects with 25(OH)D insufficiency were three times more likely to be hyperprolinemic than subjects with sufficient levels of 25(OH)D. Furthermore, in this gender and ethnically mixed US sample we have also demonstrated a significant association of hyperprolinemia with schizophrenia (Clelland et al., 2011), a finding consistent with recent studies of both Japanese female schizophrenia patients (Tomiya et al., 2007) and European (Orešič et al, 2011) schizophrenia patients. After taking into account discrepancies in hyperprolinemia study findings that may have arisen due to the use of a non-fasting protocol (Davis, 1972), or differential patient diagnoses based upon lifetime DSM III (Jacquet et al., 2005) versus more recent DSM IV diagnostic criteria (Clelland et al., 2011; Orešič et al., 2011), only one study did not find a significant relationship between elevated proline and schizophrenia or schizoaffective disorder (Rao et al., 1990). There is thus a body of work from more recent studies that demonstrates a positive association between hyperprolinemia and schizophrenia-spectrum disorders (Jacquet et al., 2005; Tomiya et al., 2007; Orešič et al., 2011; Clelland et al., 2011).

Criterion 3) When the mediator and the independent variable are simultaneously employed in the model, the path between the independent and dependent variable is reduced. In our final unadjusted and adjusted models, controlling for hyperprolinemia decreased the strength of the relationship, with over one third of the association between 25(OH)D insufficiency and schizophrenia explained by the presence of hyperprolinemia.

Proline has multiple properties similar to classical excitatory amino acid neurotransmitters, including its release at the synapse after K^+-induced depolarization, its synthesis within synaptosomes, and its uptake into synaptosomes by a high-affinity Na-dependent transport system (Nadler, 1987; Nadler et al., 1992) and reviewed in (Phang et al., 2001). Proline can also modulate glutamatergic neurotransmission, as supported by the specificity of proline active uptake to a subset of glutamatergic terminals, its ability to inhibit glutamate release at high concentrations, and the finding of reduced glutamate uptake in the hyperprolinemic rat brain (Phang et al., 2001; Cohen and Nadler, 1997; Ferreira et al., 2012). While studies of elevated proline in humans (22q11DS patients (Karayiorgou et al., 2004), hyperprolinemia types I and II (Phang et al., 2001)) and a chronic proline administration model (Shanti et al., 2004), have documented the pathogenic properties of hyperprolinemia, the consequences of elevated proline for CNS neurotransmission have been best demonstrated by work on the hyperprolinemic Prodh null mouse (Gogos et al., 1999; Paterlini et al., 2005), which in the presence of POX deficiency and elevated proline (peripheral and CNS), exhibits a deficit in sensorimotor gating, increased sensitivity to amphetamine, and impairments in declarative memory, coupled with locally decreased CNS glutamate and GABA, increased neurotransmitter release at glutamatergic synapses, and possible activation of dopaminergic signaling. The results of our current study therefore present a mechanism by which vitamin D insufficiency confers risk of schizophrenia; via reduced PRODH expression, proline

elevation, and the concomitant dysregulation of neurotransmission. That elevated proline may also impact recovery from a recent psychotic episode, has also been suggested from our previous finding that hyperprolinemic patients had inpatient hospital stays nearly 50% longer than their non-hyperprolinemic counterparts (Clelland et al., 2011).

A potential caveat to our mediation finding is that patients with schizophrenia may present with 25(OH)D insufficiency simply due to their lifestyle circumstances, such as the use of antipsychotic medication or lack of UVB exposure due to, for example, their period of inpatient hospitalization. Regarding medication, in our patient sample we found no relationship between exposure to antipsychotic medication and 25(OH)D insufficiency, which is consistent with reports of patents with schizophrenia (Itzhaky et al., 2012), and of first-episode psychosis patients (Crews et al., 2013). Regarding sunlight, although direct exposure was not assessed in our study, patients were relatively short-stay inpatients at a primary care public hospital and we found no relationship between the duration of hospitalization and 25(OH)D levels, which again supports previous reports assessing the duration of hospitalization (Crews et al., 2013; Partti et al., 2012; Menkes et al., 2012), as well as direct sunlight exposure (Itzhaky et al., 2012). Similarly, in a previous study the variables of diet and exercise habits have also not been able to explain the differences between 25(OH)D levels in patients and control subjects (Berg et al., 2010). It is also important to note that we assessed statistically the potential for untested lifestyle variables to attenuate the study findings, and demonstrated the robustness of the associations even in the presence of additional, untested confounds that may causally affect each outcome. While the cross-sectional study design reported here precludes definitive conclusions regarding the direction of the relationship between insufficiency and schizophrenia, data from cohort studies attributing vitamin D insufficiency to the risk of schizophrenia (McGrath et al., 2004; McGrath et al., 2010b) or psychosis (Hedelin et al., 2010), strongly support our causal hypothesis.

Two additional considerations warrant further discussion in light of our findings. Firstly, although we report that one third of the association between low vitamin D and schizophrenia is mediated by elevated proline, our data conversely suggests that nearly two-thirds of the measured association does not depend on hyperprolinemic status. It is possible that loss of functional PRODH may also mediate the risk of schizophrenia incurred by vitamin D insufficiency: PRODH is expressed widely in the human brain (Gogos et al., 1999), and there are multiple molecular signaling and apoptotic pathways that would likely be impacted by PRODH downregulation (see Supplementary Data, Supplementary Background and Figure S1). In that case, plasma proline level may represent a surrogate of PRODH downregulation. Alternatively, the additional risk for schizophrenia conferred by insufficiency may be independent of PRODH and/or proline: Experimental studies have shown that vitamin D strongly promotes CNS cell differentiation, positively regulates axonal growth, can regulate calcium transients via its ability to down-regulate voltage sensitive L-type calcium channels, and may protect against reactive oxygen species (Eyles et al., 2013), and as documented in a recent review, the vitamin D receptor (*VDR*), which complexes with the active form of 25(OH)D, is also widely expressed in the human brain (Eyles et al., 2013. Moreover, maternal vitamin D deficiency induces neonatal CNS cellular

proliferation, reduces apoptosis and alters neurogenesis ((Eyles et al., 2013 and references therein) suggesting additional mechanisms by which insufficiency may modify schizophrenia risk.

Secondly, a full interpretation of our findings with respect to their relevance to schizophrenia may be limited by study of this adult population. Much of the epidemiological and experimental basis underlying vitamin D risk comes from study of the developing embryo or early infant. Although a recent cross-sectional study reported the significant association of insufficiency with psychotic features in adolescents (ages 12-18) (Gracious et al., 2012), to our knowledge only one study has explored dietary provided vitamin D and risk of psychotic symptoms in adults (Hedelin et al., 2010). Therefore, further study of hyperprolinemia and vitamin D in neonates and children, as well as those at-risk prior to symptom onset, is warranted to fully explore the timing of vitamin D insufficiency exposure, proline elevation, psychotic symptom onset, and the relationship with schizophrenia.

Nevertheless, there is a clear need for new schizophrenia therapies: First and second generation antipsychotics have significant side-effects, negatively impacting tolerance and long-term adherence, a third of patients do not respond to currently available treatments, and residual symptoms persist in over 60% (Girgis et al., 2008; Barbui et al., 2008). Furthermore, there are currently no medications approved for the treatment of negative or cognitive symptoms. On the other hand, Vitamin D supplementation is well tolerated with minimal side-effects, and the findings reported here support the possibility that supplementation might reduce symptoms or augment recovery of patients with schizophrenia, particularly targeting those with elevated proline, who may represent over one quarter of the schizophrenia population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Barbui C, Signoretti A, Mule S, Boso M, Cipriani A. Does the Addition of a Second Antipsychotic Drug Improve Clozapine Treatment? Schizophr Bull. 2008; 35(2):458–68. [PubMed: 18436527]

Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. J Pers Soc Psychol. 1986; 51(6):1173–82. [PubMed: 3806354]

Baxter CF, Baldwin RA, Davis JL, Flood JF. High proline levels in the brains of mice as related to specific learning deficits. Pharmacol Biochem Behav. 1985; 22(6):1053–9. [PubMed: 4023022]

Belvederi Murri M, Respino M, Masotti M, Innamorati M, Mondelli V, Pariante C, Amore M. Vitamin D and psychosis: mini meta-analysis. Schizophr, Res. 2013; 150(1):235–9. [PubMed: 23906618]

Berg AO, Melle I, Torjesen PA, Lien L, Hauff E, Andreassen OA. A cross-sectional study of vitamin D deficiency among immigrants and Norwegians with psychosis compared to the general population. J Clin Psychiatry. 2010; 71(12):1598–604. [PubMed: 20441728]

Bossé Y, Maghni K, Hudson TJ. 1alpha,25-dihydroxy-vitamin D3 stimulation of bronchial smooth muscle cells induces autocrine, contractility, and remodeling processes. Physiol Genomics. 2007; 29(2):161–8. [PubMed: 17213369]

Clelland CL, Read LL, Baraldi AN, Bart CP, Pappas CA, Panek LJ, Nadrich RH, Clelland JD. Evidence for association of hyperprolinemia with schizophrenia and a measure of clinical outcome. Schizophr Res. 2011; 131(1-3):139–45. [PubMed: 21645996]

Cohen SM, Nadler JV. Proline-induced inhibition of glutamate release in hippocampal area CA1. Brain Res. 1997; 769:333–9. [PubMed: 9374203]

Crews M, Lally J, Gardner-Sood P, Howes O, Bonaccorso S, Smith S, Murray RM, Di Forti M, Gaughran F. Vitamin D deficiency in first episode psychosis: A case-control study. Schizophr Res. 2013; 150(2-3):533–7. [PubMed: 24060571]

Crum RM, Anthony JC, Bassett SS, Folstein MF. Population-based norms for the Mini-Mental State Examination by age and educational level. JAMA. 1993; 269(18):2386–91. [PubMed: 8479064]

Davis JM, Spaide JK, Himwich HE. Effects of tranylcypromine and L-cysteine on plasma amino acids in controls and schizophrenic patients. Am J Clin Nutr. 1972; 25(3):302–10. [PubMed: 5011913]

Dingman W, Sporn MB. The penetration of proline and proline derivatives into brain. J Neurochem. 1959; 4(2):148–53. [PubMed: 13665376]

Doknic M, Maric NP, Britvic D, Pekic S, Damjanovic A, Miljic D, Stojanovic M, Radojicic Z, Jasovic Gasic M, Popovic V. Bone remodeling, bone mass and weight gain in patients with stabilized schizophrenia in real-life conditions treated with long-acting injectable risperidone. Neuroendocrinol. 2011; 94(3):246–54.

Efrom ML. Familial hyperprolinemia. Report of a second case, associated with congenital renal malformations, hereditary hematuria and mild mental retardation, with demonstration of an enzyme defect. N Engl J Med. 1965; 272:1243–54. [PubMed: 14290545]

Eyles DW, Burne TH, McGrath JJ. Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. Front Neuroendocrinol. 2013; 34(1):47–6. [PubMed: 22796576]

Ferreira AG, da Cunha AA, Scherer EB, Machado FR, da Cunha MJ, Braga A, Mussulini BH, Moreira JD, Wofchuk S, Souza DO, Wyse AT. Evidence that hyperprolinemia alters glutamatergic homeostasis in rat brain: neuroprotector effect of guanosine. Neurochem Res. 2012; 37(1):205–13. [PubMed: 21935728]

Garland CF, French CB, Baggerly LL, Heaney RP. Vitamin D supplement doses and serum 25-hydroxyvitamin D in the range associated with cancer prevention. Anticancer Res. 2011; 31(2): 607–11. [PubMed: 21378345]

Girgis RR, Javitch JA, Lieberman JA. Antipsychotic drug mechanisms: links between therapeutic effects, metabolic side effects and the insulin signaling pathway. Mol Psychiatry. 2008; 13:918–29. [PubMed: 18414407]

Gogos JA, Santha M, Takacs Z, Beck KD, Luine V, Lucas LR, Nadler JV, Karayiorgou M. The gene encoding proline dehydrogenase modulates sensorimotor gating in mice. Nat Genet. 1999; 21(4): 434–9. [PubMed: 10192398]

Gracious BL, Finucane TL, Friedman-Campbell M, Messing S, Parkhurst MN. Vitamin D deficiency and psychotic features in mentally ill adolescents: A cross-sectional study. BMC Psychiatry. 2012; 12:38. [PubMed: 22571731]

Heaney RP. Vitamin D in health and disease. Clin J Am Soc Nephrol. 2008; 3(5):1535–41. [PubMed: 18525006]

Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr. 2003; 77(1):204–10. [PubMed: 12499343]

Heaney RP, Holick MF. Why the IOM recommendations for vitamin D are deficient. J Bone Miner Res. 2011; 26(3):455–7. [PubMed: 21337617]

Hedelin M, Löf M, Olsson M, Lewander T, Nilsson B, Hultman CM, Weiderpass E. Dietary intake of fish, omega-3, omega-6 polyunsaturated fatty acids and vitamin D and the prevalence of psychotic-like symptoms in a cohort of 33,000 women from the general population. BMC Psychiatry. 2010; 10:38. [PubMed: 20504323]

Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM. Endocrine Society. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96(7):1911–30. [PubMed: 21646368]

Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM. Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. J Clin Endocrinol Metab. 2012; 97(4):1153–8. [PubMed: 22442274]

Humble MB, Gustafsson S, Bejerot S. Low serum levels of 25-hydroxyvitamin D (25-OHD) among psychiatric out-patients in Sweden: relations with season, age, ethnic origin and psychiatric diagnosis. J Steroid Biochem Mol Biol. 2010; 121(1-2):467–70. [PubMed: 20214992]

Institute of Medicine (IOM). Dietary Reference intakes for calcium and vitamin D. The National Academies Press; Washington DC: 2011. released November 2010

Itzhaky D, Amital D, Gorden K, Bogomolni A, Arnson Y, Amital H. Low serum vitamin D concentrations in patients with schizophrenia. Isr Med Assoc J. 2012; 14(2):88–92. [PubMed: 22693787]

Jacquet H, Berthelot J, Bonnemains C, Simard G, Saugier-Veber P, Raux G, Campion D, Bonneau D, Frebourg T. The severe form of type I hyperprolinaemia results from homozygous inactivation of the PRODH gene. J Med Genet. 2003; 40(1):e7. [PubMed: 12525555]

Jacquet H, Demily C, Houy E, Hecketsweiler B, Bou J, Raux G, Lerond J, Allio G, Haouzir S, Tillaux A, Bellegou C, Fouldrin G, Delamillieure P, Ménard JF, Dollfus S, D'Amato T, Petit M, Thibaut F, Frébourg T, Campion D. Hyperprolinemia is a risk factor for schizoaffective disorder. Mol Psychiatry. 2005; 10(5):479–85. [PubMed: 15494707]

Karayiorgou M, Gogos JA. The molecular genetics of the 22q11-associated schizophrenia. Brain Res Mol Brain Res. 2004; 132(2):95–104. [PubMed: 15582150]

Kenny, DA.; Kashy, D.; Bolger, N. Data analysis in social psychology. In: Gilbert, D.; Fiske, S.; Lindzey, G., editors. Handbook of social psychology. fourth. McGraw-Hill Press; New York: 1998. p. 233-265.

Llewellyn DJ, Lang IA, Langa KM, Muniz-Terrera G, Phillips CL, Cherubini A, Ferrucci L, Melzer D. Vitamin D and risk of cognitive decline in elderly persons. Arch Intern Med. 2011; 170(13):1135–41. [PubMed: 20625021]

McGrath J. Is it time to trial vitamin D supplements for the prevention of schizophrenia? Acta Psychiatr Scand. 2010; 121(5):321–4. [PubMed: 20525021]

McGrath J, Saari K, Hakko H, Jokelainen J, Jones P, Järvelin MR, Chant D, Isohanni M. Vitamin D supplementation during the first year of life and risk of schizophrenia: a Finnish birth cohort study. Schizophr Res. 2004; 67(2-3):237–45. [PubMed: 14984883]

McGrath JJ, Burne TH, Féron F, Mackay-Sim A, Eyles DW. Developmental vitamin D deficiency and risk of schizophrenia: a 10-year update. Schizophr Bull. 2010a; 36(6):1073–8. [PubMed: 20833696]

McGrath JJ, Eyles DW, Pedersen CB, Anderson C, Ko P, Burne TH, Norgaard-Pedersen B, Hougaard DM, Mortensen PB. Neonatal vitamin D status and risk of schizophrenia: a population-based case-control study. Arch Gen Psychiatry. 2010b; 67(9):889–94. [PubMed: 20819982]

Menkes DB, Lancaster K, Grant M, Marsh RW, Dean P, du Toit SA. Vitamin D status of psychiatric inpatients in New Zealand's Waikato region. BMC Psychiatry. 2012; 12:68. [PubMed: 22734499]

Milaneschi Y, Hoogendijk W, Lips P, Heijboer AC, Schoevers R, van Hemert AM, Beekman AT, Smit JH, Penninx BW. The association between low vitamin D and depressive disorders. Mol Psychiatry. 2013 Apr 9. Epub ahead of print. 10.1038/mp.2013.36

Nadler J. Sodium-dependent proline uptake in the rat hippocampal formation: association with ipsilateral-commissural projections of CA3 pyramidal cells. J Neurochem. 1987; 49:1155–60. [PubMed: 2887633]

Nadler JV, Bray SD, Evenson DA. Autoradiographic localization of proline uptake in excitatory hippocampal pathways. Hippocampus. 1992; 2:269–78. [PubMed: 1308188]

Orešič M, Tang J, Seppänen-Laakso T, Mattila I, Saarni SE, Saarni SI, Lönnqvist J, Sysi-Aho M, Hyötyläinen T, Perälä J, Suvisaari J. Metabolome in schizophrenia and other psychotic disorders: a general population-based study. Genome Med. 2011; 3(3):19. [PubMed: 21429189]

Partti K, Heliövaara M, Impivaara O, Perälä J, Saarni SI, Lönnqvist J, Suvisaari JM. Skeletal status in psychotic disorders: a population-based study. Psychosom Med. 2010; 2(9):933–40. [PubMed: 20841556]

Paterlini M, Zakharenko SS, Lai WS, Qin J, Zhang H, Mukai J, Westphal KG, Olivier B, Sulzer D, Pavlidis P, Siegelbaum SA, Karayiorgou M, Gogos JA. Transcriptional and behavioral interaction between 22q11.2 orthologs modulates schizophrenia-related phenotypes in mice. Nat Neurosci. 2005; 8(11):1586–94. [PubMed: 16234811]

Phang, JM.; Hu, CA.; Valle, D. Disorders of proline and hydroxyproline metabolism. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. Metabolic and molecular basis of inherited disease. McGraw-Hill Press; New York: 2001. p. 1821-1838.

Rao ML, Gross G, Strebel B, Bräunig P, Huber G, Klosterkötter J. Serum amino acids, central monoamines, and hormones in drug-naive, drug-free, and neuroleptic-treated schizophrenic patients and healthy subjects. Psychiatry Res. 1990; 34(3):243–57. [PubMed: 1981623]

Raux G, Bumsel E, Hecketsweiler B, van Amelsvoort T, Zinkstok J, Manouvrier-Hanu S, Fantini C, Brévière GM, Di Rosa G, Pustorino G, Vogels A, Swillen A, Legallic S, Bou J, Opolczynski G, Drouin-Garraud V, Lemarchand M, Philip N, Gérard-Desplanches A, Carlier M, Philippe A, Nolen MC, Heron D, Sarda P, Lacombe D, Coizet C, Alembik Y, Layet V, Afenjar A, Hannequin D, Demily C, Petit M, Thibaut F, Frebourg T, Campion D. Involvement of hyperprolinemia in cognitive and psychiatric features of the 22q11 deletion syndrome. Hum Mol Genet. 2007; 16(1): 83–91. [PubMed: 17135275]

Rey-Sánchez P, Lavado-García JM, Canal-Macías ML, Gómez-Zubeldia MA, Roncero-Martín R, Pedrera-Zamorano JD. Ultrasound bone mass in schizophrenic patients on antipsychotic therapy. Hum Psychopharmacol. 2009; 24(1):49–54. [PubMed: 19016257]

Rosen CJ. Clinical practice. Vitamin D insufficiency. N Engl J Med. 2011; 364(3):248–54. [PubMed: 21247315]

Rosen CJ, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Manson JE, Mayne ST, Ross AC, Shapses SA, Taylor CL. IOM committee members respond to Endocrine Society vitamin D guideline. J Clin Endocrinol Metab. 2012; 97(4):1146–52. [PubMed: 22442278]

Shanti ND, Shashikumar KC, Desai PV. Influence of proline on rat brain activities of alanine aminotransferase, aspartate aminotransferase and acid phosphatase. Neurochem Res. 2004; 29(12): 2197–206. [PubMed: 15672540]

Thompson T, Andreeff M, Studzinski GP, Vassilev LT. 1,25-dihydroxyvitamin D3 enhances the apoptotic activity of MDM2 antagonist nutlin-3a in acute myeloid leukemia cells expressing wild-type p53. Mol Cancer Ther. 2010; 9(5):1158–68. [PubMed: 20406950]

Tolppanen AM, Sayers A, Fraser WD, Lewis G, Zammit S, McGrath J, Lawlor DA. Serum 25-hydroxyvitamin d(3) and d(2) and non-clinical psychotic experiences in childhood. PLoS One. 2012; 7(7):e41575. [PubMed: 22848531]

Tomiya M, Fukushima T, Watanabe H, Fukami G, Fujisaki M, Iyo M, Hashimoto K, Mitsuhashi S, Toyo'oka T. Alterations in serum amino acid concentrations in male and female schizophrenic patients. Clin Chim Acta. 2007; 380(1-2):186–90. [PubMed: 17367771]

Wood RJ, Tchack L, Angelo G, Pratt RE, Sonna LA. DNA microarray analysis of vitamin D-induced gene expression in a human colon carcinoma cell line. Physiol Genomics. 2004; 17(2):122–9. [PubMed: 14996990]

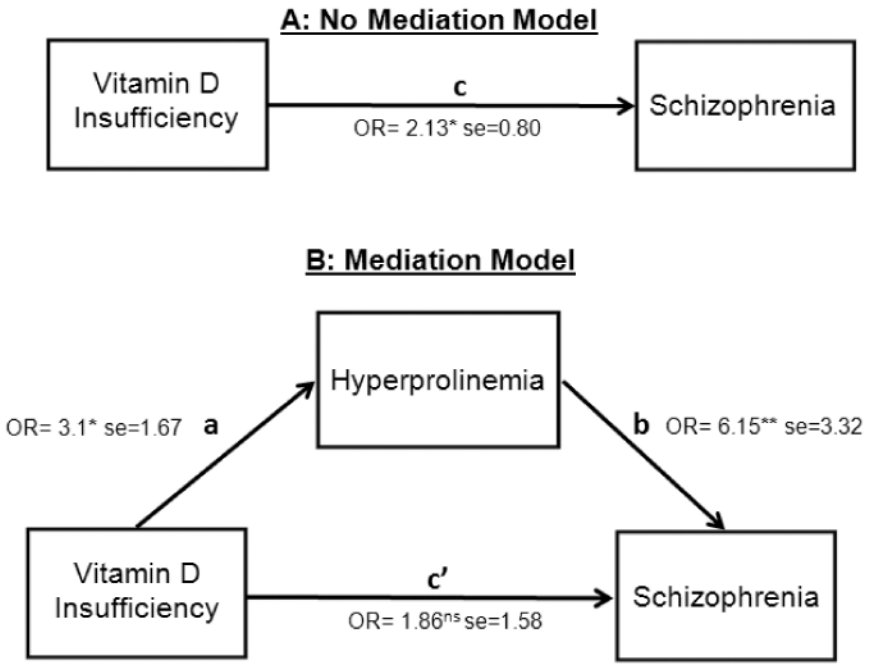

Figure 1. Hypothesized Mediation Model for the Association Between 25-hydroxyvitamin D insufficiency and Schizophrenia

Panel A depicts the direct association between insufficiency and schizophrenia, adjusted for education level, with no mediator (path c). Panel B depicts the association mediated (in part) by the presence of hyperprolinemia (indirect paths a + b (for path b the coefficient was previously reported (Clelland et al., 2011)). The total effect of 25(OH)D insufficiency on schizophrenia risk, adjusted for education, is defined by the sum of the indirect plus direct paths (a+b+c′). A causal mediation analysis tested the effect size (Odds Ratio (OR) and standard error (se)) and significance of each path in a series of logistic regression models. *P<0.05, **P<0.005, ns = not significant.

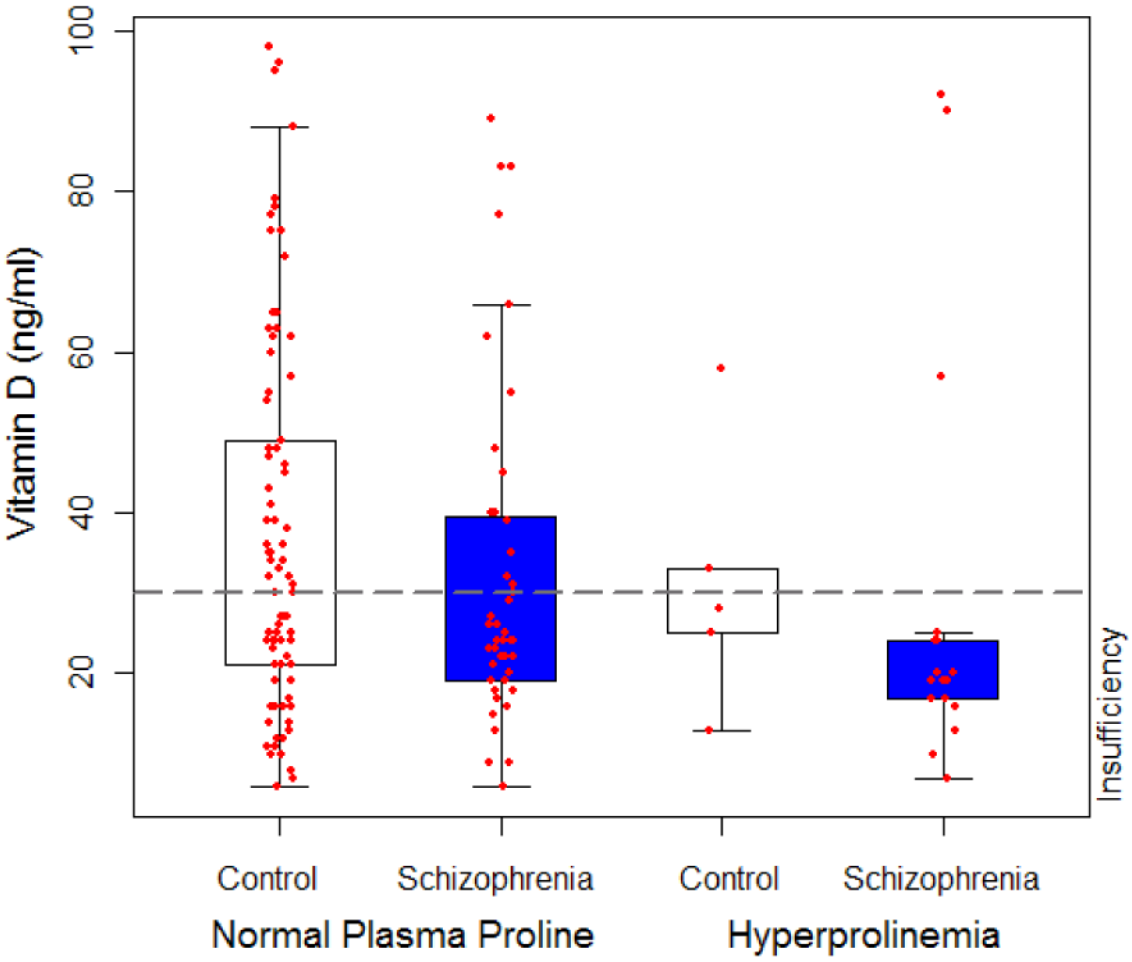

Figure 2. Relationship between 25-hydroxyvitamin D Insufficiency and Hyperprolinemia

Vitamin D levels are plotted for controls (white) and patients (blue), by hyperprolinemic status. The dashed line represents the threshold level for insufficiency. The odds of vitamin D insufficiency were significantly different across groups (LR χ^2, 3df = 10.04, p=0.018): Compared to non-hyperprolinemic controls (n=85), hyperprolinemic patients (n=17) were significantly more likely to be insufficient (OR = 5.5, 95% CI 1.47-20.56, p=0.01), as were non-hyperprolinemic patients (n=47, OR = 2.1, 95% CI 1.001-4.32, p =0.05), but not hyperprolinemic controls (n=5, OR = 1.7, 95% CI 0.28-11.13, p=0.54). Key: Red jittered points represent individual subject data. The box indicates the interquartile range (IQR), The whiskers extend to the most extreme data point <1.5 times the IQR. Mean levels of vitamin D ± SDs for each group are as follows: non-hyperprolinemic controls 37.39±23.04, non-hyperprolinemic patients 32.66±21.64, hyperprolinemic controls 31.4±16.59, hyperprolinemic patients 28.76±25.69.

Table 1
Characteristics of Schizophrenia Patients and Control Subjects, n=154

Characteristic	Patients *n=64*	Controls *n=90*	Prob[a]
Demographic Characteristics and Tests for Group Differences.			
Females, n (%)	33 (51.6)	46 (51.1)	0.96
Ethnicity, n (%)			0.98
African American	21 (32.8)	31 (34.5)	
Caucasian	22 (34.4)	30 (33.3)	
Hispanic	21 (32.8)	29 (32.2)	
Age (years), mean ± SD	38.5 ± 11.3	37.9 ± 12.0	0.72
Body Mass Index (BMI), mean ± SD	26.9 ± 1.51	26.9 ± 5.07	0.97
Season of Recruitment, n (%)			0.87
Winter/Spring	34 (53)	49 (54)	
Summer/Fall	30 (47)	41 (46)	
Vitamin D Supplementation[b], n (%)	12 (19)	20 (22)	0.60
Alcohol Abuse or dependence	10 (15.6)	10 (11.1)	0.41
Current or previous Smoker[c], n (%)	39 (60.93)	22 (24.4)	<0.0001
Education, n (%)			<0.0001
Did not complete high school	18 (28.1)	4 (4.5)	
Graduated high school	36 (56.3)	40 (44.4)	
Graduated 4 year college degree or higher	10 (15.6)	46 (51.1)	
Hospital Duration (days)[d], median (IQR)	12.5 (15)	n/a	
Primary Outcome Measures			
Fasting plasma proline (uM), mean ± SD	215.84 ± 63.0	174.28 ± 55.97	<0.0001
Fasting Hyperprolinemia[e]	17 (26.6)	5 (5.6)	< 0.001
Fasting vitamin D (ng/ml), mean ± SD	31.63 ± 22.64	37.06 ± 22.7	0.043
Fasting vitamin D Insufficiency[f]	44 (68.8)	42 (46.7)	0.007

[a] p-values calculated by Satterthwaite t-test, Mann-Whitney, Kruskal-Wallis, Fisher exact test, or Chi-Square, based upon the distribution of continuous variables and expected cell counts for categorical variables.

[b] Vitamin D supplementation ≤400IU/day. No subject received supplementation >400IU/day.

[c] 4 subjects not reported.

[d] Days in hospital prior to fasting blood draw.

[e] Hyperprolinemia defined as a fasting plasma proline level ± 2 SDs from the gender-specific mean of the control group (Clelland et al., 2011): ≥203.3uM for females and ≥327.6uM for males.

[f] 25-hydroxyvitamin D insufficiency defined as <30ng/ml (Rosen, 2011).

Table 2

Modeling Multivariate Predictors of Schizophrenia (n=154)

Covariate	OR	se	Z	p[a]	LR χ^2	p[b]
Model 1 (χ^2=7.52, df=1, p=0.0061)						
Vitamin D Insufficiency	2.51	0.86	2.69	0.007		
Model 2 (χ^2=34.58, df=2, p<0.0001)					27.06	<0.0001[c]
Vitamin D Insufficiency	2.13	0.80	1.84	0.044		(<0.0004)
Education	0.24	0.07	-4.47	0.000		
Model 3 (χ^2=46.04, df=3, p<0.0001)					11.46	0.0007 [d]
Vitamin D Insufficiency	1.86	0.73	1.58	0.115		(0.0028)
Education	0.23	0.07	-4.68	0.000		
Hyperprolinemia	6.46	3.86	3.13	0.002		
Model 4 (χ^2=18.44, df=2, p=0.0001)					27.06	<0.0001 [e]
Vitamin D Insufficiency	2.17	0.77	2.18	0.030		(<0.0004)
Hyperprolinemia	5.33	2.92	3.05	0.002		
Model 5 (χ^2=49.88, df=6, p<0.0001)					3.84	0.28 [f]
Vitamin D Insufficiency	0.12	0.20	-1.31	0.189		(1.12)
Hyperprolinemia	2.76	6.61	0.42	0.672		
Education	0.10	0.06	-3.77	0.000		
Interaction (insufficiency*hyperprolinemia)	1.57	2.00	0.35	0.725		
Interaction (insufficiency*education)	3.54	2.63	1.71	0.088		
Interaction (hyperprolinemia*education)	1.22	1.22	0.20	0.841		

[a] probability >|z|

[b] probability of likelihood ratio (LR) statistic (p> χ^2) unadjusted, (adjusted)

[c] Model 2 versus 1

[d] Model 3 versus 2

[e] Model 3 versus 4

$f_{\text{Model 5 versus 3}}$

Table 3
Testing for Mediation by Hyperprolinemia, n=154

Pathway	Standardized coefficient [a] (95% CI[b])	
	Unadjusted Model 4	Adjusted Model 3
Indirect (a+b)	0.089 (0.012, 0.222)	0.095 (0.005, 0.247)
Direct (c′)	0.196 (0.017, 0.366)	0.157 (-0.04, 0.349)
Total (a+b+c′)	0.285 (0.086, 0.460)	0.252 (0.04, 0.449)
No mediation (c)	0.246	0.203
Proportion of the effect Mediated[c]	0.089/0.285*100 = 31.2%	0.095/0.252*100 = 37.7%

[a] Rescaled based upon the variable SDs, to allow indirect effects to be computed as the product of coefficients.

[b] Bias-corrected CI based upon 4970 replications, as one or more parameters could not be estimated in 30 bootstrap replications. Significant if 95% CI does not contain zero.

[c] The proportion of the total effect between vitamin D insufficiency and schizophrenia (a+b+c′) mediated by hyperprolinemia (a+b) = standardized a+b / a+b+c′