THE NEUROPROTECTIVE ROLE OF VITAMIN D ON NEURONS IN A CENTRAL NERVOUS SYSTEM AUTOIMMUNE DISEASE

Undergraduate Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Honors Research Distinction in Biomedical Science in the School of Health and Rehabilitation Sciences at The Ohio State University

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Defense Date: April 17th, 2015
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>Page 2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>Page 3</td>
</tr>
<tr>
<td>List of Figures</td>
<td>Page 4</td>
</tr>
<tr>
<td>Abstract</td>
<td>Page 5</td>
</tr>
<tr>
<td>Introduction</td>
<td>Page 6</td>
</tr>
<tr>
<td>Methods</td>
<td>Page 12</td>
</tr>
<tr>
<td>Results</td>
<td>Page 19</td>
</tr>
<tr>
<td>Discussion</td>
<td>Page 23</td>
</tr>
<tr>
<td>Conclusion</td>
<td>Page 31</td>
</tr>
<tr>
<td>References</td>
<td>Page 32</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I truly appreciate the time and energy that Dr. Amy Lovett-Racke has taken to mentor me throughout my undergraduate research career. Dr. Lovett-Racke is a role model to me both in my professional and personal life. She has always offered guidance and patience, which has helped me to grow as a scientist. Not only did Dr. Lovett-Racke play an important role in my research experience, but she also helped me decide my future career path. Without her, my undergraduate experience would not have been the same. I am thankful that she allowed me to join her lab two years ago.

In addition, I greatly appreciate all of the support from the lab members in the Dr. Racke and Dr. Lovett-Racke labs including Dr. Michael Racke, Dr. Yuhong Yang, Dr. Wei Pei, Yue Liu, Priscilla Lee, Mary Severin, Matt Gromley, Ellie Rieser, and Varun Shah. I would also like to thank the previous members of the lab including Dr. Mireia Guerau, Kyle Jablonski, Michelle Larson, and Patrick Kwadwo Nuro-Gyina. All the lab members are not only my colleagues, but also my friends. I am especially thankful for Priscilla Lee, the graduate student I worked with in lab each day. Priscilla was always patient with me as I slowly learned the many laboratory techniques. Her wisdom and time allowed me to learn more about research than I could have even imagined.

Finally, my sincere gratitude goes to all the other individuals who have helped in my learning process. This includes our collaborators Dr. Jonathan Godbout, Ashley Fenn, Diana Norden, and Dr. Geert Carmeliet as well as my third committee member Dr. Li Zuo. Finally, I would like to thank the Biomedical Science program under the leadership of Dr. John Gunn, Steven Mousetes, and Lori Martensen for the opportunity to participate in such an inspiring and individualized undergraduate major.
LIST OF FIGURES

Figure 1 (a and b): Hypothesized Mechanism of Immune Response in Developing Central Nervous System

Page 11

Figure 2 (a and b): Methods for BV-2 Microglia Treated with Neuronal Conditioned Medium From N2a Cells

Page 13

Figure 3: Mechanism for VDR Deletion in SLICK Mice

Page 17

Figure 4 (a and b): N2a Cells and Primary Neurons Treated with Calcitriol

Page 19

Figure 5: Primary Microglia Treated with IL-34

Page 20

Figure 6 (a, b, c, and d): BV-2 Microglia Treated with Neuronal Conditioned Medium From N2a Cells

Page 21

Figure 7 (a, b, and c): Cell Staining of Wild-type and SLICK.f/+ Mice

Page 22

Figure 8: EAE Disease Severity in Wild-type versus SLICK.f/+ Mice

Page 23
ABSTRACT

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS). Childhood vitamin D insufficiency is a known risk factor of MS. However, it remains unclear how vitamin D levels before adolescence affect the susceptibility to disease. We hypothesize that vitamin D increases IL-34 production, which directs microglia into an anti-inflammatory phenotype, preventing neurodegeneration in a developing CNS. We observed an increased expression of IL-34 mRNA in primary neurons and neurons derived from a mouse Neuroblastoma (N2a) cell line after treating with calcitriol, the biologically active form of vitamin D. Additionally, immortalized murine microglia (BV-2) and primary microglia treated with IL-34 showed a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory molecules compared to untreated controls upon lipopolysaccharide (LPS) stimulation. These findings indicate that microglia can be directed to an anti-inflammatory phenotype by IL-34, which is produced by vitamin D-treated neurons. To study how vitamin D signaling in childhood influences the risk of developing disease in vivo, we generated a transgenic mouse model using single-neuron labeling with inducible Cre-mediated knockout (SLICK) mice. This allows manipulation of VDR levels on neurons during different ages of life. We found that SLICK f/+ mice have decreased VDR expression when evaluated by immunohistochemistry. These mice showed that low levels of vitamin D during development caused increased disease severity through inducing experimental autoimmune encephalomyelitis (EAE). By understanding the mechanism by which vitamin D levels influence multiple sclerosis susceptibility, vitamin supplements could be administered to prevent high risk populations from developing this incurable disease.
INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) caused by auto-reactive T lymphocytes (T-cells) which recognize self myelin as foreign. This autoimmune disease causes neurodegeneration by damaging the myelin sheath and axons, interrupting the signals through these myelinated nerves. Symptoms of MS include vision loss, incontinence, pain, depression, and eventually a decrease in motor function. Disease onset is typically 20-40 years of age. In addition, there is no cure for MS and the treatments are very expensive. Our goal is to find a pathway that causes an increase in MS disease susceptibility that can be manipulated in order to prevent disease onset.

Multiple sclerosis affects 2.5 million people worldwide¹ and an estimated 400,000 people in the United States². Additionally, the prevalence of MS is increasing worldwide. Each year MS costs the United States more than $10 billion², and the disease costs each patient an average of $47,000 a year³. The high cost of MS makes prevention essential. Considering the risk factors of MS, vitamin D could be easily and cost effectively implemented into diets of at risk patients. This could then help to decrease the risk of disease onset later in life. Currently, there is no way to prevent MS, so finding a way to decrease disease onset is a critical step in addressing this costly and devastating disease. Additional research would lead to the implication of a new public health policy to increase vitamin D levels in people with high MS susceptibility.

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis: The disease course of MS is clinically observed as both a relapsing/remitting and progressive disease. In the relapsing/remitting form of the disease, the patients have relapses of neurological dysfunction followed by a period of full or partial remission of these defects. This later develops into a secondary progressive phase where there is a continuous decrease in neurological function.
Some patients begin with this progressive form of MS which is identified as primary progressive multiple sclerosis. During disease, auto-reactive immune cells and microglia cause inflammation, myelin damage, and eventually axonal death. MS is commonly studied using experimental autoimmune encephalomyelitis (EAE). This animal model of MS resembles the disease and is caused by an immune response targeting the neurons. B6, B10.PL, and SJL mice are common breeds of mice, which can be easily induced to develop EAE. However EAE susceptibility and disease course does vary based on the mouse strain. EAE is induced by immunization with myelin proteins emulsified in complete Freund’s adjuvant (CFA) or adoptive transfer of myelin specific CD4+ cells\(^4\). The steps for these procedures have been clearly identified in the literature\(^4\). Using this model, \textit{in vivo} studies are conducted to further the advancement of MS research.

\textbf{Risk Factors:} There are currently three known risk factors of multiple sclerosis, which including genetics, environment, and an immunological chance component. Twin studies show that 25-40\% of monozygotic twins both develop MS while there is only a 5\% concordance rate in dizygotic twins\(^5\). This demonstrates that genetic factors play a part in disease susceptibility, but it is not the only factor. Additionally, the human leukocyte antigen (HLA) Class II gene has been found to have the highest association in MS patients\(^6\), also indicating a genetic component. Environmental factors, such as Epstein Barr virus (EBV) and smoking, play another role in susceptibility. Almost all (99.5\%) of MS patients have antibodies against EBV\(^7\). However, 94\% of the population has EBV antibodies, and they do not all develop MS\(^7\). Therefore, this is another contributing risk factor. It is also proven that there is a positive correlation between smoking before disease onset and developing MS\(^8\). Finally, an immunological chance factor can be seen in the twin studies. Only 25-40\% of monozygotic twins both develop MS, though these
children have identical genetics and very similar environmental factors. Therefore, there is a “chance factor” because many immunological defects or challenges that shape the immune system need to occur at the same time to trigger disease onset. These factors all play a role in disease susceptibility, but vitamin D is an additional factor that has promising implications for MS prevention.

**Vitamin D:** The association between vitamin D and MS has been evaluated since the 1960’s when the first epidemiological study showed that there is an increase in MS prevalence farther from the Equator than closer to the Equator. This correlation has been shown in United Kingdom migrants. Those who moved to Tasmania, a higher latitude, developed greater MS frequency than those who migrated to Queensland, a lower latitude, even though the migrants were from similar genetic backgrounds. The worldwide MS distribution correlates with UV exposure and vitamin D levels in the body. Additionally, migration studies have shown that children over the age of 15 who move to higher latitudes develop the same MS risks as their counterparts in their original home. In contrast, children under the age of 15 will adapt risks from their new environment. This suggests that the role of vitamin D in MS risk is critical before puberty. The data implies that there is a vitamin D mediated mechanism which causes an increased risk for MS early in life, even though disease onset occurs at 20-40 years of age.

Vitamin D is commonly known to regulate calcium levels in the body, but it is also important in the immune system. The pathway for vitamin D3 involves two biochemical reactions. Epidermal skin layers have 7-dehydrocholesterol which is converted to cholecalciferol (vitamin D3) by ultraviolet light from the sun. The liver then hydroxylates cholecalciferol to calcifediol (25-hydroxyvitamin D3). Calcifediol is then hydroxlated to calcitriol (1,25-dihydroxycholecalciferol) by 25-hydroxyvitamin D 1-alpha-hydroxylase.
hydroxylase) in the kidneys\(^\text{13}\). Calcitriol is then the active form of vitamin D in the body. In innate immunity, macrophages recognize infection or injury. Toll-like receptor (TLR) binding increases expression of vitamin D receptor (VDR) and the hydroxylase enzyme to increase the production of calcitriol\(^\text{13}\). This active form of vitamin D can then interact with VDR on the cells which induces anti-microbial activity by releasing cathelicidins and defensins. These factors fight the insult and recruit additional immune cells\(^\text{14}\). Along with this pathway, vitamin D influences the adaptive immune system in a variety of ways. High levels of vitamin D are associated with decreased in T-cell proliferation\(^\text{15}\) and increased development of T-regulatory cells\(^\text{16}\). There is also a shift in T-cell populations from Th1 to Th2 with a decrease in Th17 cells\(^\text{17}\). Along with the effect on T-cells, vitamin D decreases B-cell differentiation\(^\text{18}\), production of inflammatory cytokines from monocytes\(^\text{19}\), and dendritic cell proliferation\(^\text{20}\).

Vitamin D also aids in the development of the CNS. This is evident since both neurons and microglia express VDR, and 25(OH)D3-1α-hydroxylase is found in the CNS\(^\text{21}\). This makes the production of calcitriol possible. Vitamin D aids in the release of nerve growth factor (NGF) and glial cell line-derived neurotropic factor (GDNF) to allow for differentiation of the cells in the CNS\(^\text{21}\). Finally, vitamin D controls the calcium balance in the brain especially through calcium ion channels which aid in normal function of the CNS\(^\text{21}\). Recent studies have shown that calcitriol can regulate neurotrophic levels in glial cells\(^\text{22}\). These studies have led to the overall hypothesis that vitamin D has a neuroprotective function by decreasing inflammation in the CNS. Vitamin D insufficiency is also seen in other neurological disorders including Parkinson’s disease, Schizophrenia, depression and cognitive decline,\(^\text{23}\) suggesting vitamin D is essential for normal CNS function.
However, even with this knowledge, *it is still unknown how low vitamin D levels can increase MS susceptibility at a young age, even though the disease onset occurs 2-4 decades later in life.*

**Microglia and IL-34:** Microglia are macrophages in the CNS, which are constantly surveying their immediate environment. During development, microglia aid in CNS development. Later in life, microglia respond to damage or infection in order to protect the CNS. It is known that early-active MS lesions contain an activated form of microglia which express pro-inflammatory properties\(^{24}\). It is now agreed that microglia activation is one of the key steps in MS disease pathogenesis. Interleukin 34 (IL-34) is a newly discovered cytokine that is produced by neurons. IL-34 has also been proven to be essential for microglia development and homeostasis\(^{25}\). This implies that IL-34 aids in the development of an anti-inflammatory phenotype of microglia in the CNS. Despite this research, *it still unknown if an absence of IL-34 would cause an increase in microglia activation.*

**Hypothesis:** Although vitamin D has been extensively studied, it is still unknown how vitamin D affects neurons before disease onset in MS. Since vitamin D would be the easiest manipulated risk factor in MS, we believe that investigating this topic is a critical step in prevention. The correlation between MS onset and vitamin D deficiency before puberty must be fully investigated to understand MS pathogenesis and prevent disease onset. It is known that increased vitamin D levels cause an increase in IL-34 production in endothelial cells\(^{26}\) and that vitamin D can prevent microglia activation by keeping them in an anti-inflammatory phenotype\(^{27}\). Additionally, IL-34 production peaks during childhood and declines during adulthood\(^{28}\) making IL-34 a logical component in our hypothesized vitamin D mediated neuroprotective pathway for MS. Our hypothesized model (*Figure 1*) shows the mechanism a
child’s CNS goes through during an insult to the CNS. Although serious viral CNS infections such as meningitis and encephalitis can occur, mild CNS insults are common in children. Many viruses do have some neurotropic capabilities and can cause minor inflammation in the CNS without any initial symptoms. These types of insults are modeled in our hypothesized mechanism.

We hypothesize that when there are high vitamin D levels during childhood (Figure 1a), neuron and myelin damage is prevented by vitamin D mediating high levels of IL-34 production from the neurons. The IL-34 then binds to the microglia, causing them to become minimally

Figure 1: Our hypothesized mechanism showing the effects of vitamin D in a developing CNS. Figure 1a shows a CNS with high vitamin D levels, and Figure 1b shows a CNS with low vitamin D levels.
activated. The low levels of pro-inflammatory cytokines produced help to fight off the infection without causing damage to the CNS. This could then decrease the chances of T-cells becoming primed later in life by maintaining a healthy CNS. However, when a virus penetrates the CNS and there are low levels of vitamin D (Figure 1b), the vitamin D receptors are not saturated which causes low levels of IL-34 to be released. This prevents IL-34 receptors on microglia from achieving saturation. We believe that this can lead to overly activated microglia which release high levels of pro-inflammatory cytokines and nitric oxide. This inflammation causes myelin and neuronal damage, but there is not a detectable effect of this damage during childhood. Later in life, this neurodegeneration could initiate auto-reactive T-cell formation because of the debris in the CNS and the decrease in CNS protection. Therefore, this demyelination could increase the chance of T-cells becoming primed, increasing the risk of MS onset. Therefore, our hypothesis states: **Vitamin D increases IL-34 production, which directs microglia into an anti-inflammatory phenotype preventing neurodegeneration in a developing central nervous system.**

**METHODS**

**Cell Line Cultures:** The mouse neuroblastoma (N2a) cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were thawed and cultured for 7 days in Dubecco’s Modified Eagle’s Medium (DMEM) with 10% Fetal Bovine Serum (FBS). The cells were then treated with 10nM retinoic acid for an additional 7 days. Differentiated cells were then washed and treated with calcitriol at 0nM, 10nM, 100nM, or 1000nM for 24 hours. After treatment, the cells were stored in 1mL of TRIzol at -80°C to be used for Quantitative Real-Time PCR.
The murine microglial (BV-2) cell line was obtained from Dr. Godbout’s Lab (The Ohio State University, Columbus, OH). These cells were thawed and cultured in microglia medium containing DMEM with 10% FBS. The cells were then treated with the neuronal condition medium from the N2a cell line and cultured with LPS stimulation for either 4 or 18 hours in fresh medium (Figure 2a). LPS stimulation lasted 4 hours for Quantitative Real-Time PCR and 18 hours for ELISA. The supernatant was collected and stored for ELISA analysis at -20°C and the cells were stored in 1mL of Trizol at -80°C to be analyzed with Quantitative Real-Time PCR. In the experiment where the cells were treated with anti IL-34, the anti IL-34 (2µg/ml) was added in with the neuronal condition medium (Figure 2b).

**Figure 2**: Pictured above is the experimental design for the BV-2 Microglia Cell Line treated with neuronal conditioned medium. The N2a cells were treated with 0, 10, 100, or 1000 nM of calcitriol for 24 hours. The medium was then used to treat the BV-2 Microglia Cell Line. After incubation with LPS stimulation for 4 or 18 hours, the supernatant and cells were examined for inflammatory markers using Real-Time PCR or ELISA. Figure 2a is testing the effect of calcitriol while Figure 2b is also examining the effect of anti IL-34 on the cell culture.

**Primary Cell Cultures**: Primary neurons were isolated from wild-type B10.PL (Jackson Labs, Bar Harbor, Maine) neonatal mice at one day old. The skin and skulls of the mice were cut in half to allow for removal of the entire brain. The cortex was separated from the hippocampus,
and the meninges were removed. The tissues were suspended in Phosphate Buffered Saline (PBS) during the rest of the process. The tissue was minced with a razor blade to approximately 0.5mm³. After washing three times with PBS, the cells were suspended in PBS (10mL). Digestion occurred in trypsin (0.025%) for 15 minutes at 37°C. The digestion was stopped by an addition of 20mL of PBS. The cells were repeatedly pipetted to breakdown the tissue further and passed through a cell dissociation sieve. After, the suspended cells were centrifuged at 170 x g for 5 minutes at 37°C, PBS was removed and the cells were reconstituted in neuronal medium containing Neuronal Basal Medium with B-27 Serum-Free Supplement (2%), GlutaMAX™-1 (0.5mM), and gentamicin (20µg/mL). The cells were plated at 200,000 cells per well in a 24 well plate coated with Poly-L-lysine. The cells were cultured for approximately 14 days with replacement of half of the medium every 3 days. Calcitriol was used to treat the neurons at a concentration of 0nM, 10nM, 100nM, or 1000nM for 24 hours. The supernatant was removed and the cells were stored in TRIzol at -80°C.

Primary microglia were isolated from wild-type B10.PL neonatal mice at 0-4 days old. An incision was made through the skin and skull, and the brain was removed. The brain samples were minced into 0.5mm³ size pieces using a scapula while the tissue was suspended in 10mL PBS. Digestion occurred in trypsin (0.025%) for 15 minutes at 37°C with Deoxyribonuclease I (DNAse1, 1mL). The solution had 15mL of PBS added to the culture to dilute the trypsin and centrifuged 170 x g for 5 minutes. Once again, the cells were washed with PBS and spun down at 170 x g for 5 minutes. The cells were resuspended in 15mL of microglial medium containing DMEM with FBS (20%), gentamicin (20µg/mL), L-glutamine (1%), and penicillin streptomycin (1%). The particles were removed from the culture by a cell dissociation sieve. Then, the cells were resuspended in 5mL of microglial medium per brain sample. Cultures were plated in a
150cm² flask with 5mL of resuspended cells and 20mL of microglial medium. The medium was replaced every 3 days. After approximately 10 days of culturing at 37°C, the cells were shaken at 80 x g for 3 hours at 37°C. The supernatant was then plated on 24 well Poly-L-lysine coated plates at 1x10⁵ cells per well. The cells were incubated at 37°C for 48 hours and treated with serum-free microglial medium, lipopolysaccharide (LPS, 0ng/mL or 10ng/mL), and IL-34 (0ng/mL, 2ng/mL, or 10ng/mL) for 8 hours at 37°C. A half hour before collection, adenosine triphosphate (ATP) was added to the culture to release produced Interleukin-1 beta (IL-1β)²⁹. The supernatant was collected for ELISA analysis and stored at -20°C.

**Enzyme-Linked Immunosorbent Assay (ELISA):** Supernatants were collected from cell cultures as previously described. The supernatants were analyzed for Interleukin 6 (IL-6), Interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), and Interleukin 10 (IL-10). A sandwich ELISA protocol was used. In general, a purified monoclonal antibody specific for the protein was plated in the wells overnight. After washing, samples or standards were added to the well to allow for antigen binding. The standards were the recombinant protein of interest in a serial dilution. After overnight incubation, the biotinylated detection antibody was added to bind to the antigen. For the IL-6 ELISA, IL-6 purified anti-mouse capture antibodies and biotinylated goat anti-mouse detection antibodies from BD Biosciences were used. The IL-6 standard was setup using recombinant proteins. IL-1β TNFα, and IL-10 levels were examined using purified anti-mouse capture antibodies and biotinylated rat anti-mouse detection antibodies as well as standards from BioLegend. After the detection antibodies were added, a substrate was used to cause an enzymatic reaction, resulting in color change if the antigen was present. The substrates used were avidin-peroxidase for IL-6 and 3,3',5,5'-Tetramethylbenzidine (TMB) for IL-1β, TNFα, and IL-10. An Emax Precision Microplate Reader was used to measure the enzymatic
reaction in the ELISA. Between each antibody or antigen addition, the plate was washed using Wellwash 4 MK 2 with 1x PBS. The cytokine concentrations were calculated using SoftMax Pro Software in respect to the linear curve created by the standards.

**Quantitative Real-Time Polymerase Chain Reaction (PCR):** RNA was isolated using the TRIzol Reagent Isolation Protocol. The cells were stored in TRIzol as previously described with 1ml of TRIzol per reaction. Chloroform (200µL) was added to each reaction, shaken for 15 seconds, incubated for 3 minutes at 37°C, and then centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was collected. Isopropanol (500µL) was added to the aqueous phase of each reaction along with GlycoBlue (1µL). The samples were incubated for 5 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. Finally, the RNA pellet was washed with 75% ethanol, centrifuged at 7,500 x g for 5 minutes at 4°C, and dried at 37°C. The pellets were resuspended in RNase-free water. NanoDrop was used to find the concentration of the RNA. The concentrations were then used for the calculations reverse transcription. The general procedure was conducted by reverse transcribing RNA (2.5 μg) into cDNA using the SuperScript™ II Reverse Transcriptase manufacturer’s protocol. Briefly, each reaction contained 100ng/uL random primer (1µL), 10mM dNTP (1µL), 5X First-Strand Buffer (4µL), 0.1 M DDT (1µL), RNaseOUT™ (1µL), and SuperScript™ II RT (1µL). The reactions were temperature controlled by the Mastercycler. The mRNA levels were determined by Real-Time PCR with SYBR Green RT-PCR Kit using the manufacturer’s protocol. Each reaction contained the forward and reverse primer (0.5µL, 10nM) and 2x Master Mix (5µL). The results were normalized to 1 using the hprt1 no treatment group. The reactions were initiated and read with the Applied Biosystems ViiA 7 Real-Time PCR System. The results were calculated using $2^{-\Delta\Delta Ct}$ method and expressed as relative values.
**Mouse Model:** We generated a transgenic mouse model with inducible VDR deletion specifically on neurons. We developed this transgenic mouse by breeding $VDR^{f/f}$ mice (Dr. Geert Carmeliet, University of Leuven, Belgium) and single-neuron labeling with inducible Cre-mediated knockout (SLICK) mice from Jackson Laboratories. Cre recombinase-estrogen receptor T2 ($\text{Cre}^{\text{ERT2}}$) is a fusion protein of estrogen receptor and cre-recombinase. Cre-recombinase recognizes flox/flox sites. After one cross of these mice, we obtained SLICK $f/+\text{ mice}$, which contained only one $\text{loxP}$-flanked VDR allele. The $\text{Cre}^{\text{ERT2}}$ protein is normally located in the cytoplasm. However when tamoxifen is administered, this causes translocation of the protein to the nucleus so it can bind to the $\text{loxP}$ sites and cause deletion. The $\text{Cre}^{\text{ERT2}}$ proteins as well as an YFP tag are under the promoter of mouse $\text{Thy1}$ gene, which is only found on neurons. Therefore, this will cause a deletion of one VDR allele specifically in the neurons of the mice. Additionally, the YFP tag can be used to identify the neurons in the SLICK mice. The model used can be seen in Figure 3.

**Immunohistochemistry:** The mice were perfused using 4% paraformaldehyde (PFA) with pH 9.4. The brain and spinal cord were removed. The tissue samples were incubated in 10%, 20%, and then 30% sucrose each for 24 hours. The tissues were frozen into Optimal Cutting Temperature (O.C.T) Compound and sectioned into 10 µm slices. The slides were stored at -
20°C. The tissues were incubated in 0.4% Triton-X with PBS for 30 minutes to permeabilize the sample. The tissues were then incubated in 1% BSA, 0.4% Triton-X, 5% goat serum, and 0.001% sodium azide in PBS for 1 hour as a blocking solution. The primary antibody, vitamin D-receptor (VDR) rabbit polyclonal IgG, was then added at a concentration of 1:500 in blocking solution and was incubated overnight. The slides were then washed three times for 10 minutes each in PBS. The secondary antibody, Alexa Flour chrome 546 goat anti-rabbit IgG, was added at a concentration of 1:4000 in blocking solution for 1 hour. The slides were again washed three times for 10 minutes each. The slides were fixed with VECTASHIELD Hard Set Mounting Medium with DAPI. Pictures were taken at 40x with Olympus 8X41 microscope.

**EAE Induction:** Myelin oligodendrocyte glycoprotein (MOG) peptide fragment 35-55 (50µg) and Proteolipid Proteins 139-151 (50µg) with adjuvant complete freund (CFA, 50µl) and *Mycobacterium tuberculosis* (final concentration of 1mg/ml) was mixed for 20 minutes to form an emulsion. The mice were immunized with the emulsion (25µl) by subcutaneous injection into four sites, medial to each leg. Pertussis toxin (100ng, 200µl/injection) was then administered to each mouse. Clinical EAE development occurred approximately 10 days after the initial immunization. The mice were scored on a scale from 0-6. The scoring system states: 0 = no clinical disease, 1 = limp tail, 2 = moderate hind limb weakness, 3 = severe hind limb weakness, 4 = complete hind limb paralysis, 5 = quadriplegia or premorbid state, and 6 = death from disease. The Institutional Animal Care and Use Committee at The Ohio State University Wexner Medical Center approved all animal protocols.

**Statistical Analysis:** GraphPad Prism software was used to perform the statistical analysis. The unpaired T-test was used for all statistical analysis except EAE disease course.
EAE significance was evaluated using the Mann-Whitney U test. One asterisk indicates a \( p \)-value <0.05, two asterisks is a \( p \)-value <0.01, and three asterisks is a \( p \)-value <0.001.

**RESULTS**

**Vitamin D Increases IL-34 Levels:** To investigate the initial step of the hypothesized mechanism, neurons were treated with calcitriol and analyzed for IL-34 production. N2a cells, a Neuroblastoma cell line, were differentiated into “neuron-like” cells with retinoic acid for 7 days. Calcitriol was then used to treat the cells for 24 hours. The cells had a dose dependent increase in IL-34 production as the concentration of calcitriol was increased using Quantitative Real-Time PCR (Figure 4a). To verify that calcitriol induces IL-34 in neurons, the same procedure was used to measure the IL-34 production of primary neurons from wild-type neonatal mice. The primary cells were cultured for 14 days and treated with calcitriol for 24 hours. There was an increase in IL-34 production which peaked at 100nM of calcitriol in primary neuron cultures (Figure 4b) confirming that vitamin D can induce IL-34 production in neurons.

**IL-34 Inhibits Inflammatory Response:** Our next question addressed IL-34 modulating microglia phenotype. Primary microglia were isolated from neonatal mice and cultured for 48 hours. The cells were then placed in serum free medium and treated with LPS (0ng/mL or...
10ng/mL) and IL-34 (0ng/mL, 2ng/mL, or 10ng/mL) for 8 hours. LPS is a Toll-like receptor 4 (TLR4) agonist that activates microglia and induces pro-inflammatory cytokine production. The supernatant was collected and used to analyze cytokine production via ELISA. Levels of IL-6, TNFα, IL-1β, and IL-10 production were analyzed.

There was no change in the groups without LPS stimulation. However, when stimulated with LPS, there was a dose-dependent decrease in IL-6, TNFα, IL-1β, and IL-10 (Figure 5). IL-34 reduced all cytokine expression, both pro-inflammatory (IL-6, TNFα, and IL-1β) and anti-inflammatory (IL-10), indicating that IL-34 suppresses microglia activation and cytokine production. This suggests that IL-34 promotes a resting phenotype in microglia.

**Vitamin D Inhibits Inflammatory Response:** We next analyzed whether calcitriol induction of IL-34 by neurons induces microglia activation. N2a cells were treated with calcitriol for 24 hours. The neuronal conditioned medium was collected and used to treat BV-2 microglia. These microglia were also activated with LPS. The supernatant was collected for ELISA and the RNA was extracted for Quantitative Real-Time PCR. After analysis, there was
no difference in any of the groups without LPS stimulation. There was a decrease in the production of IL-6 as the concentration of calcitriol to treat the N2a cells was increased from 0nM to 1000nM (Figure 6a) in the LPS stimulated group. In addition, this experiment was repeated with 1000ng/ml of LPS and there was significance at 100nM and 1000nM of calcitriol. There was also a decrease in major histocompatibility complex (MHC) class II and inducible nitric oxide synthase (iNOS). The mRNA levels of anti-inflammatory markers arginase 1 (ARG1) and heme oxygenase 1 (HO-1) were increased as the concentration of calcitriol used to treat the neurons was increased. These results are shown in Figure 6b and Figure 6c. To determine if IL-34 was responsible for the decreased cytokine production, anti IL-34 was added.
to the neuronal conditioned medium before it was used to treat the microglia. There was not the same decrease in IL-6 as previously seen; illustrating that IL-34 was the cytokine that mediated the change in IL-6 production (Figure 6d).

**SLICK f/+ Mice Have Decreased Vitamin D Receptor:** The SLICK mice were crossed with VDR f/f mice to obtain SLICK f/+ mice which had inducible vitamin D receptor deletions specifically on neurons. The cells were then stained for VDR (red) and DAPI (blue). There is also an autofluorescent YFP tag (green) on the SLICK mice. The YFP tag was in the SLICK mice under the *Thy1* promoter which is located only in neurons. The cells with vitamin D receptor were counted in both wild-type and SLICK f/+ mice, and there was a significant decrease in the number of cells with vitamin D receptor in the SLICK mice compared to the wild-type mice. An example of the samples that were used in quantification can be seen in **Figure 7a and Figure 7b.** In the wild-type mice 77.6% of the cells were VDR+ while only 44.8% were positive in the SLICK f/+ mice. Therefore, there was a significant decrease in cells that were VDR positive in the SLICK mice compared to the wild-type mice. The percentage of VDR positive cells can be seen in **Figure 7c.**

![Figure 7: Wild-type and SLICK f/+ mice were perfused and the tissue was stained for VDR (red) and DAPI (blue). YFP (green) signaling can also be in the SLICK mice, tagging each of the neurons. Figure 7a shows a WT f/f mouse, and figure B is a SLICK f/+ mouse. After quantification of VDR positive cells, we found a significant decrease of VDR in the SLICK f/+ mice. Figure 7c shows the average percent of VDR positive cells using 5 images for each group.](image-url)
Decreased Vitamin D Signaling in Neurons Increases Disease Severity: To determine if decreased VDR signaling in neurons may increase susceptibility to CNS autoimmunity, the SLICK f/+ and wild-type mice were evaluated for disease severity using the EAE model. The SLICK f/+ mice were fed a tamoxifen chow diet from 3-5 weeks of age to cause VDR deletion during early life, and then EAE was induced at 7 weeks of age.

All of the mice in the experiment did develop disease. Although there was no significant change in disease onset, there was a significant increase in disease severity of the SLICK f/+ mice when compared to the wild-type mice (Figure 8). This suggested that decrease VDR signaling in neurons enhances CNS autoimmunity.

**DISCUSSION**

Though the first epidemiological study conducted by Acheson in 1960 proved there was a correlation between MS and childhood vitamin D levels⁹, the mechanism by which vitamin D prevents MS later in life is still unknown. We propose a mechanism where vitamin D stimulates IL-34 production in neurons, which mediates an anti-inflammatory phenotype in a developing CNS. If the CNS becomes damaged during childhood, this could aid in the process of priming T-cells that could cause MS onset later in life. In addition, the damage could make the CNS...
more susceptible to an MS attack. Therefore, by more fully understanding this mechanism, we could work to prevent MS onset in susceptible patients. The data shown here does support our hypothesis; however, additional experiments are needed in order to completely prove this hypothesis.

Initially, we showed that by treating a neuronal cell line with calcitriol there was a dose dependent increase in mRNA levels of IL-34. Calcitriol is the form of vitamin D that is circulating throughout the body. Therefore, the response of these cells can occur without the need of synthesizing or activating the vitamin D. In the primary neuronal cultures, there was an increase in IL-34 as the concentration of calcitriol increased. However, it was not dose-dependent. This result can be attributed to the fact that normal levels of calcitriol in a mouse are between 10nM and 100nM. Therefore, at 1000nM, the vitamin D is above physiological levels, and the vitamin D receptors are most likely completely saturated. We hypothesize that this is the reason we did not see the increase in the levels of IL-34 mRNA at 1000nM of calcitriol. In the future, we would like to repeat these experiments and investigate IL-34 production through ELISA.

Our next experiment further investigated the second part of our hypothesized mechanism. Primary microglia were directly treated with IL-34 in order to investigate their change in cytokine production. The ELISA data did indicate that IL-34 directs the microglia into an anti-inflammatory phenotype, since there was a significant decrease in pro-inflammatory cytokines IL-6, IL-1β, and TNFα. All of these cytokines are mediators in the immune response. The decrease in these pro-inflammatory cytokines in the CNS indicates the environment is not stressed, and it is not producing harmful products such as nitric oxide. There was also a decrease in IL-10, which is normally considered an anti-inflammatory cytokine. The decrease in this
cytokine indicates that there could be an overall decrease in cytokine production in the immediate environment. IL-34 is presumed to be in a surveying state which would not induce cytokine production. In multiple sclerosis, it is known that low IL-10 levels cause an increase in TNFa, which leads to inflammation\textsuperscript{31}. However, since we saw a decrease in TNFa, we do not believe this IL-10 to be causing this inflammatory response. Even with this knowledge, the role of IL-10 needs to be more fully evaluated in order to understand this mechanism. Additional experiments need to be conducted to see if there is an overall decrease in cytokine production or an increase in other anti-inflammatory cytokines.

The final \textit{in vitro} experiment investigated the overall mechanism by combining the methods of the two previous experiments. The neuronal cell line was treated with calcitriol and cultured to allow for the hypothesized IL-34 to be produced. The neuronal conditioned medium was then used to treat a microglial cell line. After treatment, ELISA was used to investigate the cytokine production. As previously seen in the IL-34 treated primary microglia, there was a decrease in IL-6 production as the concentration of calcitriol used to treat the neurons was increased. We believe that this change was due to the increase in IL-34 production that occurred when a higher concentration of calcitriol was used to treat the neuronal cultures. There was also a decrease in MHC class II and iNOS. The expression of MHC class II is normally on immune antigen presenting cells\textsuperscript{32} and is up regulated in response to foreign antigens such as LSP. A decrease in this marker indicates that there was reduced activation of the microglia.

Additionally, iNOS is known to be involved in immune disease pathogenesis and is defined as an inflammatory mediator\textsuperscript{33}. High levels of nitric oxide, from iNOS, are known to play a role in MS progression. Nitric oxide causes damage to the blood-brain barrier, axonal degeneration, and demyelination from oligodendrocyte damage\textsuperscript{34}. Therefore, iNOS could be a key factor in myelin
damage during childhood. A decrease in this mRNA exemplifies that there is an anti-inflammatory response being activated. Although IL-10 in the previous experiment indicated that there could be an overall decrease in cytokine production, we did find an increase in anti-inflammatory markers arginase-1 and HO-1. Arginase is an important anti-inflammatory marker in the CNS because of its neuroprotective function. In addition, HO-1 is normally induced during oxidative stress and can decrease free oxygen species to aid in the inflammatory response. Therefore, we saw a decrease in pro-inflammatory cytokines as well as an increase in anti-inflammatory cytokines. Overall, this indicates that the system has an anti-inflammatory phenotype when treated with calcitriol to increase IL-34 levels in the cells. These experiments need to be repeated in primary neurons and primary microglia in order to completely prove the hypothesis. In addition, we confirmed that the production of IL-6 in these cultures was due to IL-34 production, because anti IL-34 neutralized the IL-6 response. Adding anti IL-34 to the culture would decrease IL-34 production even in the high calcitriol samples. This assures that there is not another cytokine in the medium that is inducing changes in the inflammatory response. Therefore, anti IL-34 needs to be added to the primary cultures as well. The anti IL-34 antibody should stop the changes in cytokine production and prove that IL-34 is mediating this response.

The in vitro experiments help to show the steps of the hypothesized mechanism in a controlled setting. However, the cell cultures do not necessarily reflect the processes that occur in vivo. This is why it was important for us to continue these experiments in an animal model. It is not easy to achieve a mouse model with vitamin D deficiency for a variety of reasons. If vitamin D is completely removed from the mouse since birth by systemically deleting vitamin D receptor, the mouse does not grow normally. After birth the mice appear similar to wild-type
mice. However, after weaning the mice have hypocalcemia, infertility, impaired bone formation, and do not survive past 15 weeks of age\textsuperscript{37}. Mice that develop in a low vitamin D environment also have distortion in brain shape, reduced differentiation, and lower neurotropic factors\textsuperscript{38}. Adult mice even have decreased attentiveness and behavioral sensitivity\textsuperscript{38}. Therefore, we would not be able to induce EAE in these mice to study the effects of decreased vitamin D levels. Also, feeding mice a vitamin D deficient diet is not enough to decrease vitamin D levels in the body. Mice can obtain vitamin D from the light in the housing facility despite the fact that most of their bodies are covered in fur. Furthermore, vitamin D deficiency also has a significant effect on the immune response in these mice. This is why we found it important to create a mouse model which had inducible vitamin D receptor deletion specifically on neurons.

Breeding VDRf/+ and SLICK CreER\textsuperscript{T2} mice created our transgenic mouse model. After tamoxifen administration, the SLICK f/+ mice were found to have significantly decreased VDR levels while still appearing to be healthy mice. The vitamin D receptors positive cells were counted using a stain that marked the nucleus of all cells in the tissue. This could potentially include astrocytes, microglia, oligodendrocytes, ependymal cells, as well as neurons. Therefore, this count needs to be repeated by staining only neurons in order to more accurately identify the decrease of VDR specifically on the neurons.

Finally, EAE was induced in the SLICK f/+ mice at 7 weeks of age. When compared to the control mice, the SLICK f/+ had a significant increase in disease severity. EAE is a highly studied mouse model of MS and induction is well defined in literature\textsuperscript{4}. However, we induced sub-optimal EAE by only having one injection of the pertussis toxin. This allowed us to see the effects of low vitamin D without having a large number of mice die from the disease. EAE is a T-cell mediated autoimmune disorder. This shows that vitamin D signaling in neurons is playing
a role in the CNS inflammation, which is consistent with our proposed hypothesis. Our hypothesis does state that vitamin D affects susceptibility in a developing CNS. We deleted VDR from 3-5 weeks old, when the mice are still consider juvenile. Typically, 8 to 9 weeks old is considered an adult mouse. In the future, we want to identify the exact point in development when vitamin D affects EAE disease severity. Therefore, we plan to repeat this experiment multiple times and change the age at which the mice have VDR deleted. We anticipate the most severe disease to be seen before the mice undergo puberty, because the epidemiological migration data states that this is when vitamin D is most important in humans to prevent MS

Other EAE studies have also seen changes in disease severity when vitamin D was altered in adolescent rats. In addition, we preformed our experiments in SLICK f/+ in order to represent a vitamin D deficient central nervous system. This model most accurately represents a MS susceptible person, since most individuals do have some levels of vitamin D. SLICK f/f mice could also be tested in order to study a model with an even greater decrease of VDR. We do need to be careful with this model because vitamin D is important for normal CNS development. Therefore, these mice could have additional neurological problems which we do not initially anticipate. Although the data seems to correlate with the in vitro model, we cannot conclude that this change is mediated by the same mechanism without examining IL-34 levels. It would be difficult to identify IL-34 levels in these mice by Quantitative Real-Time PCR because IL-34 is produced by a variety of cells in the CNS. Therefore, we would most likely not see a change in IL-34 levels by only altering the production in neurons. Instead, we plan to perform intravenous injections to introduce IL-34 back into these mice after the VDR deletion, and then induce EAE. With the supplementation of IL-34, we hypothesize that these mice will have a similar disease course to the wild-type mice. The IL-34 that is administered should replace the IL-34 that would
be reduced from the VDR deletion. This experiment would be the final experiment needed to prove the proposed hypothesis.

Studies on vitamin D as a mediator in immunological diseases have been increasing during the last few years. The focus on vitamin D is due to the increase in number of children and adults with vitamin D deficiency. The increase in vitamin D deficiencies has been attributed to the increased concerns about UV light as a risk factor for skin cancer. The concern for dieting and eating less calories has also lead to an increase in individuals who do not consume milk products which are a major source of vitamin D. In addition, fewer individuals have jobs which required them to be outside for long periods unlike a few decades ago. This change in human vitamin D levels has led to vitamin D studies on a wide variety of diseases such as Parkinson’s disease, Alzheimer’s disease, heart disease, and a variety of cancers.

The hypothesis that vitamin D could be associated with multiple sclerosis is not a new idea to the field. Currently, vitamin D is being investigated as a therapeutic supplement for MS in a clinical trial. Despite this ongoing research, it is important to realize that our hypothesis stated here addresses vitamin D as a risk factor for MS, not a treatment method. Other risk factor studies have also been conducted to evaluate vitamin D changes before EAE onset. Adzemovic conducted a similar study in rats where the diets were either supplemented or deprived of vitamin D. The diet changes occurred in pre-and early post-natal mice by altering the mothers’ diets as well as in juvenile/adolescent and adult rats. The mice that were supplemented with vitamin D during adolescence did have decreased EAE disease severity supporting our overall hypothesis. Our project builds on these ideas. Although vitamin D supplementation is the end goal of the experiment, vitamin D deficiency does need to be studied in order to fully understand our proposed mechanism. This study did not see a change in rats with vitamin D deficient
diets\textsuperscript{39}, but we believe this is due to the vitamin D the mice were synthesizing naturally during development. We hope our project can add to these ideas by providing a convincing model which has decreased VDR. In addition, we ultimately hope to find the exact time point in development which causes changes to EAE disease severity as well as prove that IL-34 is mediating this change.

As previously stated, vitamin D is only one of the many risk factors that are currently known for MS. However, if vitamin D is proven to be a major contributing risk factor, it would be the easiest to manipulate in children of at risk populations. Children who live at latitudes above 40\textdegree, premature infants, and individuals with darker skin complexion have increased risk of vitamin D deficiency\textsuperscript{42}. One study found that greater than 50\% of children hospitalized in the pediatric intensive care units during the fall and winter were found to be vitamin D deficient or insufficient\textsuperscript{42}. If these risk factors are combined with the risk factors for MS such as a genetic history of multiple sclerosis, history of EBV, and smoking, a specific population could be targeted to help prevent multiple sclerosis. In this population, vitamin D supplements could be administered at a young age. This supplementation would be easy to begin, because vitamin D already exists as a vitamin supplement in pill form. In addition, injections of vitamin D are available which are more effective and longer lasting. The cost of these supplements would be much lower than any other multiple sclerosis drug on the market, and they are already known to have minimal side effects. Very high levels of vitamin D do have negative side effects, especially in children and young adults. This would make it important to monitor these individuals while they receive the vitamin supplementation, especially during childhood. Overall, vitamin D could be easily and cost effectively administered to prevent multiple sclerosis in susceptible populations.
CONCLUSION

This study attempts to prove the mechanism that can explain the correlation between MS susceptibility and vitamin D insufficiency in early life. Our hypothesis is that vitamin D in the CNS signals to produce IL-34 which keeps microglia in their surveying state without causing excessive damage in a developing CNS insult. Our data supports our hypothesis. However, more data collection is needed to fully prove this hypothesized mechanism. This data sets the foundation for additional experiments to understand the relationship between vitamin D insufficiency and the risk of CNS autoimmunity. By proving this mechanism, we will one day be able to supplement at risk patients with vitamin D in order to help prevent the lifelong and devastating effects of multiple sclerosis.
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