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Investigating the Effects of H1N1 Influenza Infection on Vitamin D Metabolism and Response Gene Expression

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ABSTRACT

It has long been speculated that vitamin D plays an immunological role in the response to influenza infection. Previous studies by the Cantorna lab have shown that vitamin D deficiency or inability to produce $1,25(OH)_2D$, the active metabolite of vitamin D, throughout H1N1 influenza infection in mice resulted in increased lung inflammation and respiratory distress, as well as worse survival outcomes. To further understand the role of vitamin D in the H1N1 immune response, we investigated the expression levels of vitamin D metabolism and response genes, *Cyp24a1*, *Cyp27b1*, and the vitamin D receptor (*Vdr*) in mice that were vitamin D sufficient (D+) or deficient (D-), and in wildtype (WT) or knockout (KO) for *Cyp27b1* mice. *Cyp24a1* expression in the kidneys revealed that D+ WT mice exhibited the greatest vitamin D metabolism, especially post-infection. Lung experiments revealed that *Cyp24a1* expression peaked with immune response at day 6, although neither *Cyp27b1* nor *Vdr* expression followed a similar trend. Together, this data indicates that H1N1 infection may increase vitamin D metabolism, and future experiments will include investigating H1N1 M gene and proinflammatory cytokine expression to elicit a greater understanding of the role of vitamin D in the immune response and clearing the infection.

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Chapter 1

INTRODUCTION

Vitamin D Metabolism

Vitamin D is an important fat-soluble nutrient for the body (Figure 1), most known for its role in calcium absorption and bone maintenance but is also becoming implicated with the immune system. Vitamin D can be obtained through the diet, in the forms of mushrooms, fatty fish, and fortified dairy, or endogenously through a light reaction in the skin after UV radiation exposure[1]. Vitamin D comes in two main forms: vitamin D_3, which comes from animal sources and UV exposure, and vitamin D_2, which is obtained from plant sources such as mushrooms.

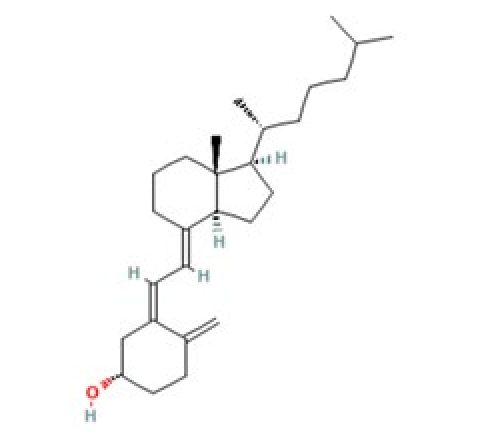

Figure 1. Structure of Vitamin D_3[2].

Vitamin D is a fat-soluble vitamin that is part of the secosteroid family. Secosteroids are steroids that contain a broken ring. (https://pubchem.ncbi.nlm.nih.gov/compound/Cholecalciferol)

When obtaining vitamin D from the sun, there are a series of reactions that must occur to generate vitamin D. The skin naturally produces a precursor molecule known as 7-dehydrocholesterol (7-DHC) as a side product from cholesterol biosynthesis (Figure 2)[3]. When

exposed to UV radiation, 7-DHC is photochemically converted to pre-D_3. Pre-D_3 is then thermally converted to vitamin D_3 (Figure 2). Both forms of vitamin D, D_3 and D_2, cannot be readily used by the body, so they must undergo a series of reactions to become biologically active.

Before the following series of reactions begins, vitamin D must first travel through the bloodstream. Because vitamin D is fat-soluble, vitamin D and its metabolites must bind the vitamin D binding protein (DBP) to facilitate travel in the bloodstream[4]. DBP transports vitamin D from the bloodstream to the first destination in the process of metabolism, the liver. In the liver, the cytochrome P450 family 2 subfamily R member 1 (CYP2R1) enzyme adds a hydroxyl group to vitamin D, creating calcidiol, or 25-hydroxyvitamin D (25OHD) (Figure 2)[5]. Calcidiol is the main circulating metabolite of vitamin D and has a stable half-life of two to three weeks in the body[6].

DBP binds calcidiol for travel to its next step in the pathway, which mainly occurs in the kidneys. The gene cytochrome p450 27B1 (CYP27B1), encodes the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), which is responsible for hydroxylating calcidiol to calcitriol ($1,25(OH)_2D$), the biologically active vitamin D metabolite in the body (Figure 2)[7]. Calcitriol, in comparison to calcidiol, has a much shorter half-life of four hours, making it much less stable in the body. Calcitriol has both a nuclear genomic receptor and a membrane-bound receptor[8]. The nuclear receptor allows calcitriol to control transcription of certain genes.

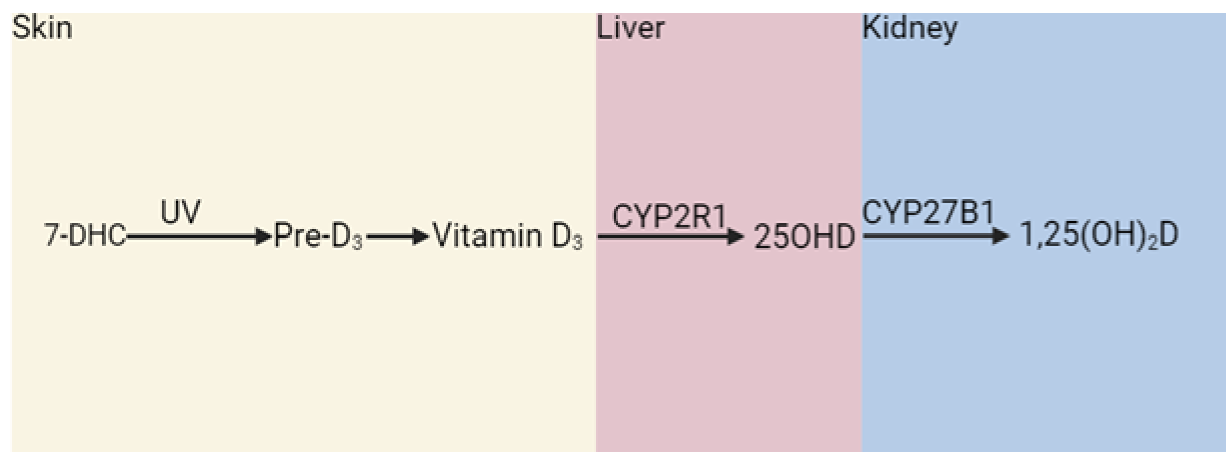

Figure 2. The Metabolism of Vitamin D.

The basic steps of vitamin D metabolism, starting with 7-DHC and ending with active $1,25(OH)_2D$, are illustrated. These processes take place throughout the body, beginning in the skin, then traveling through the bloodstream to the liver and kidneys.

To prevent vitamin D toxicity, there is a negative feedback mechanism to regulate the levels of calcitriol preferentially, but calcidiol may also be affected in this process. The regulatory gene CYP24A1, found in all target tissues of calcitriol, encodes 24-hydroxylase, which hydroxylates $1,25(OH)_2D$ and 25OHD[1,6,9]. These metabolites will then undergo catabolism into calcitroic acid, and this product can then be excreted through the feces from the bile[10]. This feedback loop regulates the levels of vitamin D in the body, protecting the body from hypercalcemia, the build-up of calcium in the body, and renal issues such as kidney stones.

Vitamin D Response in the Cell

In order for transcriptional control by calcitriol to occur, calcitriol is transported to a target cell through a chaperone protein, heat shock protein 70 (HSP70)[7]. Inside the target cell, calcitriol binds to the nuclear receptor and transcription factor, the vitamin D receptor (VDR)[11]. When bound to calcitriol, VDR has a high affinity for the VDR/retinoid X receptor (RXR) heterodimer, a member of the steroid receptor family. Together, 1,25D-bound VDR and RXR heterodimerize,

forming a complex capable of binding to vitamin D response elements (VDREs) in the DNA. Found in genes usually associated with calcium and phosphorous regulation and homeostasis, VDREs contain high-affinity binding sites for the 1,25D-VDR-RXR. This complex binds to the promoter region of the response genes, with the VDR binding upstream and RXR binding downstream in the promoter[12]. This allows for the formation of a transcriptional complex, as general transcription factors and coactivators are then recruited. Through this mechanism, target genes of vitamin D are then activated and actively transcribed.

CYP27B1

CYP27B1 is the gene that encodes enzyme 25-hydroxyvitamin D-1α-hydroxylase. This enzyme, expressed in the inner mitochondrial membrane, then converts 25OHD to active $1,25(OH)_2D$, mainly in the kidneys. Due to its prevalence in the renal system, CYP27B1 is predominantly regulated by parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), along with $1,25(OH)_2D$[1]. PTH and FGF23 are directly and indirectly associated with the body's calcium and phosphate levels, respectively, and elevated concentrations of these ions lead to CYP27B1 suppression. $1,25(OH)_2D$ also regulates CYP27B1 through these processes, as well as inducing CYP24A1 expression to reduce $1,25(OH)_2D$ levels in a negative feedback loop.

Despite its overwhelming prevalence in the kidneys, CYP27B1 has been found in a variety of other cell types: epithelial cells, cells in the endocrine glands, and even immune cells. Within the immune system, CYP27B1 was found to be most highly expressed in macrophages, monocytes, dendritic cells, T cells, and B cells[13]. The innate immune response occurs when immune cells such as macrophages and dendritic cells are activated, and this increases these cells' CYP27B1 expression. In turn, expression of molecules that help recognize and attack pathogens, like TLRs (toll-like receptors) and cathelicidins, are also increased. This increase is

not seen in mice lacking CYP27B1[14]. The upregulation of CYP27B1 in macrophages and dendritic cells may result in greater production of 1,25$(OH)_2$D, which has been known to suppress the adaptive immune response of T cells and B cells[13]. This suppression may help to alleviate certain conditions, such as in the cases of inflammation and autoimmunity[14].

VDR

The VDR gene encodes the VDR, which is responsible for binding 1,25$(OH)_2$D, a result of CYP27B1 activity. VDR gene regulation is complex, potentially involving multiple signaling pathways and not as well-studied as the genes the VDR itself regulates[15]. The most significant regulator of the VDR is 1,25$(OH)_2$D. As 1,25$(OH)_2$D levels increase, so does the expression of the VDR. This allows for a more significant response and increased expression of VDRE's[16]. The VDR may also be regulated by other vitamin D metabolites, the state of cell proliferation, steroid and peptide hormones, and growth factors among others. The VDR is found in many different tissues of the body, such as the bone and kidneys, which are significant to the well-known mechanisms of vitamin D[17]. However, the VDR is also found to highly expressed in many immune cells.

The VDR is highly expressed in macrophages, monocytes, dendritic cells, T-cells, and B-lymphocytes[18]. VDR expression in the adaptive immune system has been found to be proportional to the proliferation of T and B cells[19]. The vitamin D response, mediated by the VDR, results in greater antimicrobial molecule production, as discussed with CYP27B1. VDR also contributes to adaptive immune suppression through stimulation of regulatory T-lymphocytes. However, the increased expression of VDR has an indiscernible effect on cytotoxic T-lymphocytes.

CYP24A1

CYP24A1 encodes the enzyme 24-hydroxylase. CYP24A1 is involved in the negative feedback loop where high levels of 1,25$(OH)_2$D induce CYP24A1 activity to reduce the circulating amount of 1,25$(OH)_2$D. This is possible through CYP24A1's two VDREs within its promoter, making it inducible by 1,25$(OH)_2$D and VDR activity[1]. Given that CYP24A1's main role in the body is to regulate the levels of vitamin D and its activity is strongly induced by 1,25$(OH)_2$D, CYP24A1 activity has been understood as a strong marker for the presence of a 1,25$(OH)_2$D response within a cell.

CYP24A1 activity has been found in a variety of tissues, such as kidney, bone, intestine, and skin tissues[10]. These are usual targets of vitamin D. However, much like CYP27B1 and VDR, CYP24A1 expression has been found in the immune system. Much like the CYP27B1 and VDR genes, CYP24A1 can be found in monocytes, dendritic cells, helper and killer T-cells, and B-cells[20]. While CYP24A1 does not have a direct role in the immune response, its expression in immune cells indicates the presence of a vitamin D response in both innate and adaptive immune responses.

The Role of Vitamin D in Influenza

For centuries, it has been suspected that the seasons play an integral role in immunity. One of the most significant indicators of this phenomenon is influenza[21]. The influenza epidemic results in about one billion cases and 290,000 to 650,000 deaths annually, according to WHO[22]. The influenza epidemic peaks in the cold, dark winter months, and then becomes scarce during the summer months at temperate latitudes. The more prominent the change in seasons in a certain region, the more likely this effect would be seen. Given the change in daylight over the course of

the changing seasons, R. Edgar Hope-Simpson speculated that the greater solar radiation during summertime played some type of role against influenza[21].

With this knowledge and vitamin D's background previously explored, vitamin D is a prominent candidate for the cause of this seasonal protection against the influenza epidemic. Vitamin D is produced in the skin through a photochemical reaction, and vitamin D is known to have effects on the immune system. This, along with other data indicating vitamin D deficiency in vulnerable elderly populations, low serum levels of calcidiol in winter months, and general vitamin D deficiency that coincides with seasonal variations, point to vitamin D as a significant contributor to this effect.

The immune system has a complex response to influenza that has been shown to be affected by vitamin D. For example, macrophages release cytokines into infected tissues, and while cytokines signal cells to respond to the infection, too many cytokines may lead to adverse outcomes. Vitamin D has been shown to impair macrophage development and function, which may alleviate these adverse effects[23,24]. Studies have also shown that vitamin D stimulates expression of antimicrobial peptides in a variety of immune cells[21].

The mechanisms of action of vitamin D are still being explored, but the research field of vitamin D and immunology has expanded rapidly due to its importance with the 2009-2010 H1N1 influenza pandemic and the COVID-19 pandemic. Many studies have yielded inconclusive results, but a meta-analysis from 2021 determined that vitamin D does indeed have preventative effects against influenza, and vitamin D supplementation during the winter months has a more significant effect than during other seasons[25]. The Cantorna lab has done extensive research further exploring the effects of vitamin D during H1N1 influenza infection. A study published in 2022 by the lab showed that vitamin D deficiency increased lung inflammation

independent of infection, and demonstrated more severe disease outcomes, such as increased lung inflammation and respiratory distress throughout influenza infection[26]. D- (vitamin D deficient) and *Cyp27b1* KO (*Cyp27b1* knockout, unable to convert 25OHD to active $1,25(OH)_2D$) mice had worse survival outcomes than D+ (vitamin D sufficient) WT (wildtype) mice. Overall, this data indicated that vitamin D deficiency and inability to produce $1,25(OH)_2D$ led to greater vulnerability of these mice to H1N1 infection and severity of disease.

These previous studies have illustrated the potential immunological effects of vitamin D on H1N1 influenza infection. However, a molecular perspective on vitamin D's actions within the infected organism might provide a fuller picture of how vitamin D contributes to these prognoses. Therefore, this project seeks to understand the molecular impacts of vitamin D throughout H1N1 influenza infection through investigating the expression of three significant enzymes or receptors employed throughout the vitamin D response in the immune system: CYP24A1, CYP27B1, and VDR.

Chapter 2

MATERIALS AND METHODS

Mice

C57BL/6 WT mice were used throughout all experiments and were originally purchased from Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME, USA). *Cyp27b1* knockout (*Cyp27b1* KO) mice on the same background were utilized during kidney experiments and were originally gifted from Dr. Hector DeLuca (University of Wisconsin, Madison, WI, USA). The mice were bred and housed at the Pennsylvania State University, University Park, under approved IACUC protocols. The mice were age, infection status, and sex-matched throughout the experiments.

Vitamin D Status

Mice were raised on identical diets that differed only in the vitamin D content: vitamin D sufficient (D+, Envigo, TD.89124, Madison, WI, USA) or deficient (D-, Envigo, TD.89123) diets. Serum collected from the experimental mice was used to monitor vitamin D sufficient or deficient status. The serum was analyzed via an ELISA kit and against standards set by the manufacturer's instructions (25-OH D, Eagle Biosciences, Amherst, NH, USA). The limits of detection were 1.6 ng/mL 25D.

H1N1 Infection and Sample Collection

At 8 to 10 weeks of age, D+ mice were intranasally dosed with 30 TCID50 mouse-adapted A/H1N1/California/04/2009 influenza under isofluorane-induced anesthesia (Figure 3). This dosing resulted in an H1N1 influenza infection mouse model for the study[26].

For the kidney experiments, the D+ WT and *Cyp27b1* KO mice were infected for a period of 14 days. The mice were then sacrificed at the end of the 14-day period (D14) (Figure 3). Immediately following euthanasia, kidneys and lungs were harvested from all experimental mice, suspended in TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), and frozen at -80$^{\circ C}$ until further experiments were performed.

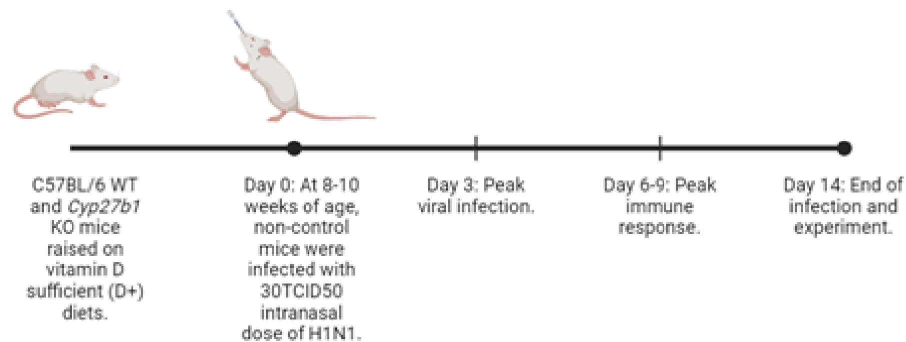

Figure 3. Experimental Design of H1N1 Infection for Kidney Studies.

D+ WT and *Cyp27b1* KO mice followed an H1N1 infection protocol beginning at 8 to 10 weeks of age and ending 14 days post-infection.

For all lung studies, only D+ WT mice were utilized (Figure 4). The mice were sacrificed periodically throughout the infection period: at day (D)3, D6, and D9 (Figure 4). Immediately following euthanasia, the lungs and kidneys were harvested from experimental mice, suspended in TRIzol reagent, and frozen at -80$^{\circ C}$ until further experiments were performed.

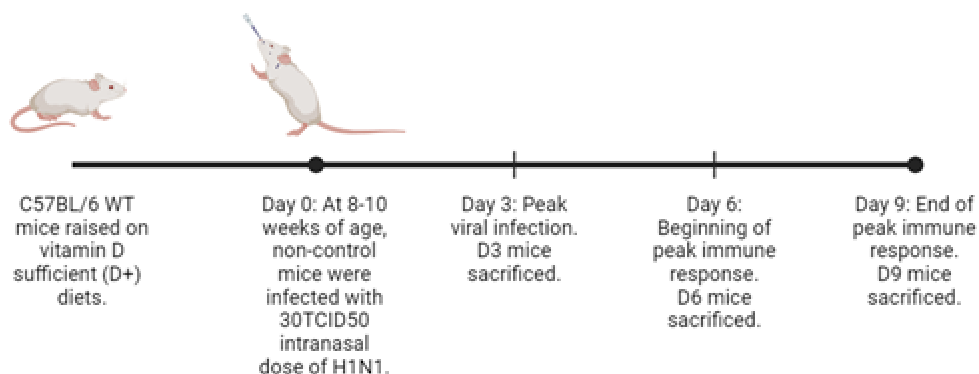

Figure 4. Experimental Design of H1N1 Infection for Lung Studies.

D+ WT mice followed an H1N1 infection protocol beginning at 8 to 10 weeks of age.

RNA Isolation

Both kidney and lung tissues were thawed and homogenized in TRIzol reagent. These tissues then followed a chloroform-isopropanol precipitation protocol to isolate RNA. The RNA was resuspended in 200-300 µL (kidneys) or 100-200 µL (lungs) diethyl pyrocarbonate (DEPC) water. The sample concentrations were then quantified using NanoDrop (ThermoFisher, Waltham, MA, USA). Sample RNA concentrations were diluted to 200 ng/µL.

RT-qPCR

1-2 µg of isolated RNA from the kidney or lung samples were reverse transcribed using AMV Reverse Transcriptase (Promega, Madison, WI, USA) to create 100 µL cDNA samples. 3 µL cDNA were amplified during qPCR using a mastermix created with SYBR green mix (Azura Genomics, Raynham, MA, USA) and primers. Primers for *Cyp24a1*, *Cyp27b1*, and *Vdr* were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). The primer sequences used were as indicated in Table 1. The qPCR was run on the StepOne Plus system (Applied Biosystems, Carlsbad, CA, USA). Gene expression was measured relative to the GAPDH

housekeeping gene expression and calculated with the $\Delta\Delta C_T$ method. The experimental data was normalized to a control ΔC_T. The means of the data were graphed with the standard error of the mean (SEM). Results were statistically analyzed using a one-way ANOVA test with the Bonferroni method and comparisons between each group, two-way ANOVA test with the Tukey method, or unpaired *t*-test on GraphPad Prism software ver. 9.3.1. Significance was determined as follows: **** p-value <0.0001, *** p-value <0.001, ** p-value <0.01, * p-value <0.05.

Table 1. Primer Sequences Used for RT-qPCR.

Target Gene	Forward Primer 5'—3'	Reverse Primer 5'—3'
Cyp24a1	ACC CCC AAG GTC CGT GAC ATC	CCA GTT GGG TCC AGG TAA GG
Cyp27b1	CCG CGG GCT ATG CTG GAA C	CTC TGG GCA AAG GCA AAC ATC TGA
Vdr	CTG CAC CTC CTC ATC TGT GA	CCC CTT CAA TGG AGA TTG C

Chapter 3

RESULTS

Investigating *Cyp24a1* Expression in Kidneys

The effects of vitamin D status and H1N1 influenza infection on enzymes important for the metabolism of vitamin D and the effects of the vitamin D receptor were determined. Experiments used WT or *Cyp27b1* knockout (KO) mice that were raised on D+ or D- diets. Both the D- and *Cyp27b1* KO groups were expected to be unable to produce 1,25$(OH)_2$D, therefore exhibiting lower expression of downstream *Cyp24a1*. *Cyp24a1* expression was measured in uninfected kidneys to determine baseline levels for the experimental groups. This measurement was performed through RT-qPCR, and the data was normalized to either D+ WT (Figure 5A) or D+ *Cyp27b1* KO (Figure 5B). By showing the data two different ways, it better elucidated the differences between the groups with low *Cyp24a1* expression.

In both Figure 5A and 5B, the uninfected D+ WT control group had significantly (p-value <0.001) upregulated *Cyp24a1* expression in comparison to all three other groups (D- WT, D+ *Cyp27b1* KO, D- *Cyp27b1* KO) as expected. These three groups had very low *Cyp24a1* expression in relation to D+ WT (Figure 5A). In Figure 5B, D+ WT mice also had significantly upregulated *Cyp24a1* expression, but the D+ KO group appears to have slightly increased *Cyp24a1* expression in comparison to the other non-1,25$(OH)_2$D-producing groups. Only D+ WT mice were able to upregulate *Cyp24a1* expression, although D+ *Cyp27b1* KO mice expressed more *Cyp24a1*.than its D- counterpart.

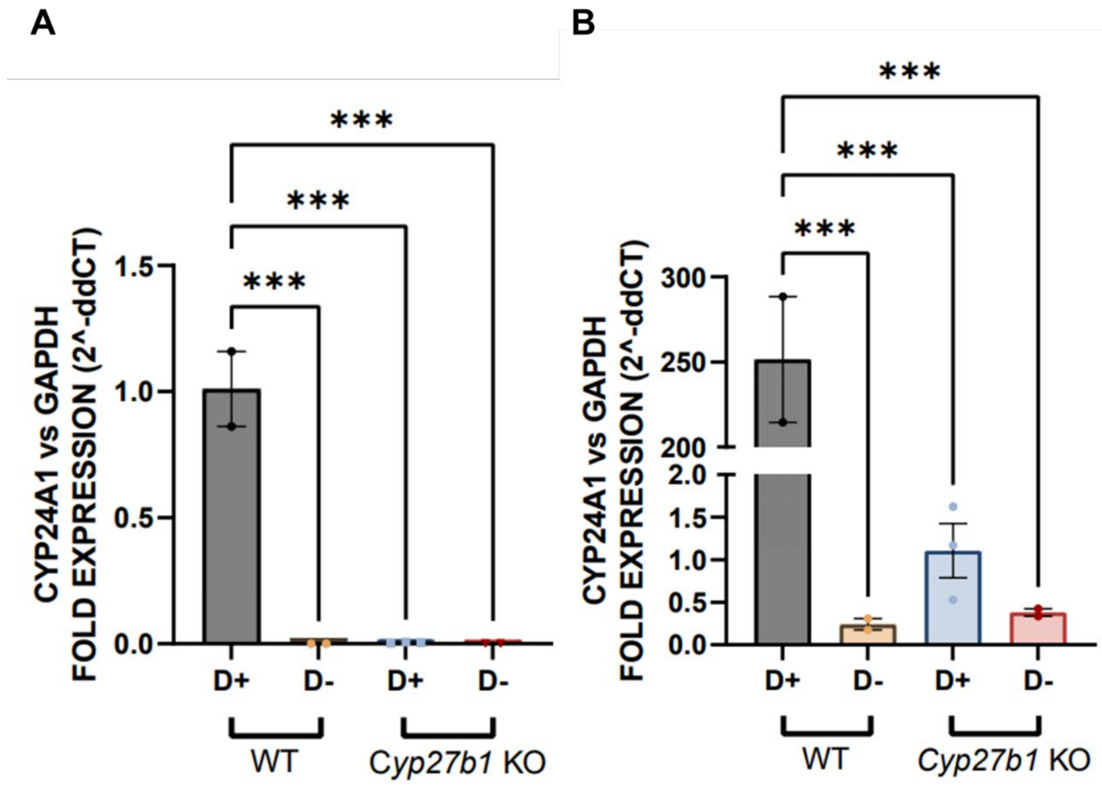

Figure 5. High *Cyp24a1* expression in uninfected D+ WT kidneys.

(A) *Cyp24a1* expression normalized to D+ WT kidney set at 1. *Cyp24a1* expression was greatest in D+ WT mice. (B) *Cyp24a1* expression normalized to D+ *Cyp27b1* KO kidney. *Cyp24a1* expression was greatest in D+ WT mice.
Statistical significance was determined through one-way ANOVA analysis. *** p-value <0.001.

Next, the effect of H1N1 infection on *Cyp24a1* expression was determined in D+ WT and D+ *Cyp27b*1 KO mice. RT-qPCR was once again used to determine RNA levels of WT and *Cyp27b1* KO mice at D0 (uninfected) or D14 (14 days post-infection). In Figure 6A, the results were normalized to the WT D0 control. While there appears to be greater *Cyp24a1* expression in the WT D14 group, this result is not significant. Figure 6B shows the data normalized to the *Cyp27b1* KO D0 control. This graph shows that the WT D14 group once again has the greatest *Cyp24a1* expression, although both WT groups appear to have greater *Cyp24a1* expression than

their *Cyp27b1* KO counterparts, but both findings are not significant. The low sample number for the WT D0 control may be preventing statistical significance.

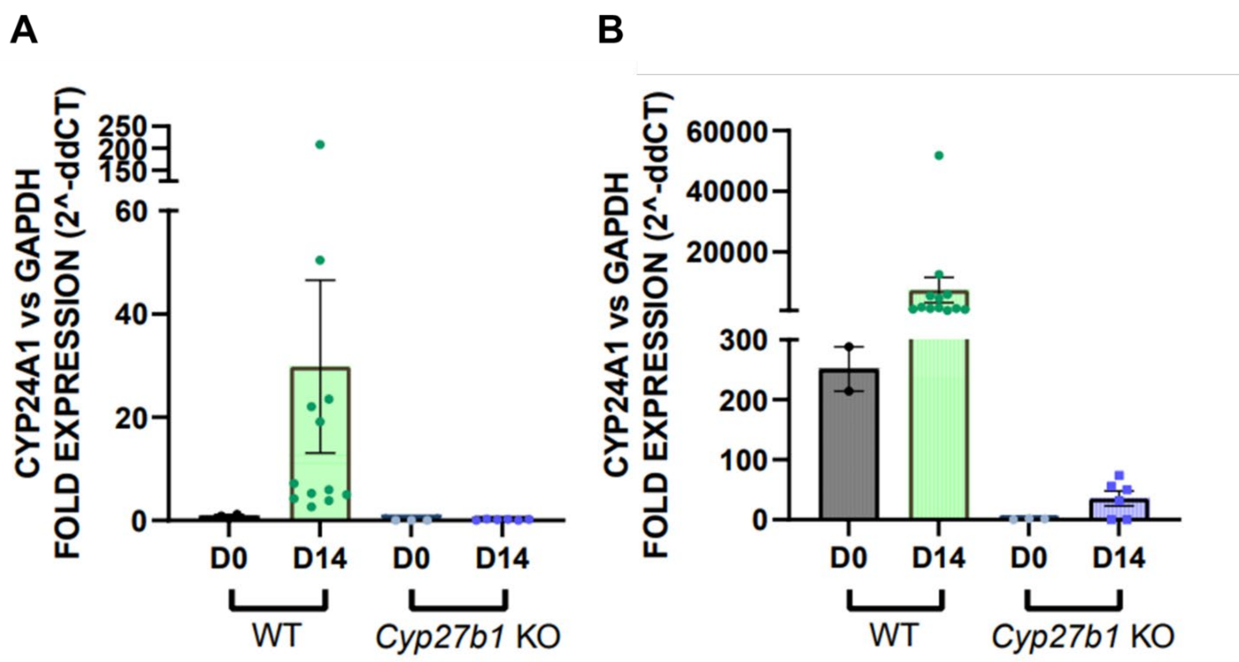

Figure 6. *Cyp24a1* expression in infected and uninfected kidneys.

(A) *Cyp24a1* expression normalized to uninfected D+ WT kidney set at 1. WT D14 appeared to have greater *Cyp24a1* expression than its D0 counterpart. With only 2 uninfected kidney samples in the WT D0 group, statistics were not consequential. (B) The same data was normalized to D0 *Cyp27b1* KO kidney values. WT kidney appeared to express more *Cyp24a1* than *Cyp27b1* KO kidney. Infection for 14 days seemed to increase *Cyp24a1* in WT and *Cyp27b1* KO kidney compared to their D0 respective controls.
Statistical significance was analyzed via one-way ANOVA, two-way ANOVA, and unpaired *t*-tests.

The Effect of H1N1 influenza Infection on Vitamin D Metabolism in the Lungs

Before investigating vitamin D metabolism in the lungs, levels of *Cyp24a1* expression in the kidneys and lungs were compared. In Figure 7, *Cyp24a1* expression in D+ WT kidneys and lungs were normalized to the kidney control RT-qPCR. The kidney expresses significantly more *Cyp24a1* than the lungs (Figure 7). As kidneys are the main location for vitamin D metabolism,

high expression of *Cyp24a1* was expected. However, the lungs express measurable *Cyp24a1* levels in this control group.

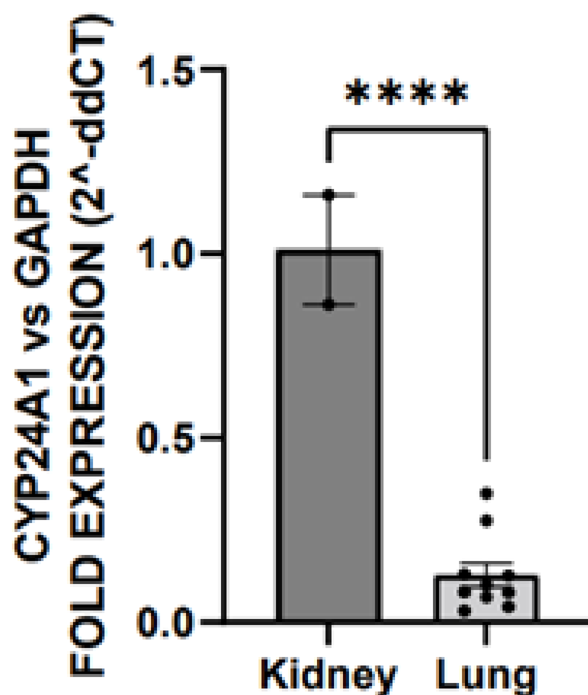

Figure 7. *Cyp24a1* expression in uninfected D+ WT mice.

The average WT kidney values were set at 1. Kidneys express significantly more *Cyp24a1* than the lungs.
Statistical significance was determined through one-way ANOVA analysis. **** p-value <0.0001.

After establishing the baseline levels of *Cyp24a1* expression in D+ WT lungs, the effect of H1N1 infection on *Cyp24a1* expression was determined in lungs at D3, D6, and D9 (Figure 8) through RT-qPCR. In comparison to the uninfected control, there was significant upregulation of *Cyp24a1* expression at D6. The *Cyp24a1* expression then decreases from D6 to D9.

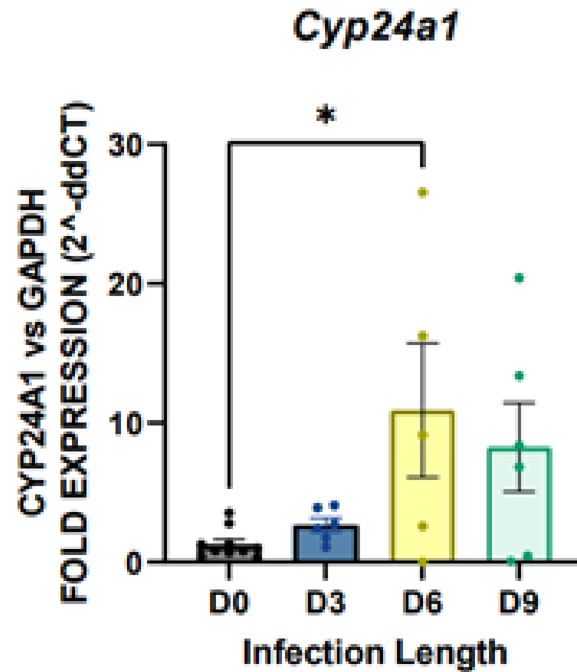

Figure 8. The effect of H1N1 infection on *Cyp24a1* in the lung.

The D0 control data was set to 1. All infection timepoints showed increased expression, and expression peaked significantly at D6.
Statistical significance was determined through one-way ANOVA. * p-value <0.05.

Cyp27b1 Expression in Lungs

After investigating *Cyp24a1* expression, *Cyp27b1* was the next gene studied under the effect of H1N1 infection (Figure 9). The RT-qPCR expression of each experimental group, D3, D6, and D9 was normalized to the uninfected control (D0). There was no significant upregulation of *Cyp27b1* as a result of infection, although there appears to be a slight trend towards increasing *Cyp27b1* expression as infection progresses.

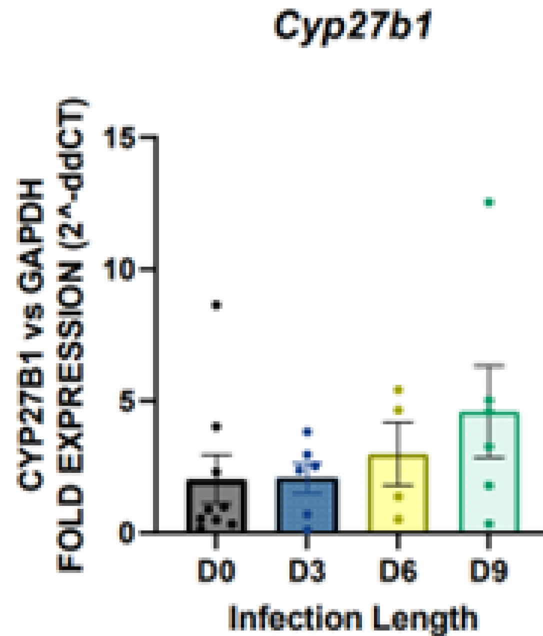

Figure 9. The effect of H1N1 infection on *Cyp27b1* in the lung.

The D0 control data was set to 1. There is no significant upregulation of *Cyp27b1* during infection, although the *Cyp27b1* expression appears to increase throughout infection. Statistical significance was determined through one-way ANOVA.

Vdr Expression in Lungs

Last, the effect of H1N1 infection on *Vdr* expression was explored in the lungs (Figure 10). Once again, RT-qPCR was used to measure expression, and the D0 group was set as the control. The infection timepoints were D3, D6, and D9. There was no significant upregulation of *Vdr* over the course of infection, although there appears to be a small increase in *Vdr* expression at D6.

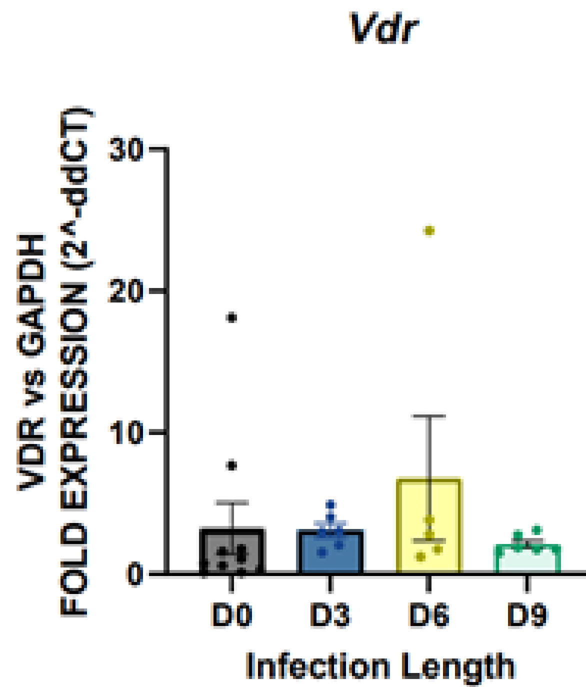

Figure 10. The effect of H1N1 infection on *Vdr* in the lung.

The D0 control data was set to 1. No significant upregulation of *Vdr* is seen. Statistical significance was determined through one-way ANOVA.

Chapter 4

DISCUSSION

Vitamin D has long been suggested to play a role in the immune response to respiratory infection, especially influenza. Previous studies by the Cantorna lab have shown the clinical significance of vitamin D to the H1N1 infection response[26]. However, this study hoped to answer the biochemical questions of the vitamin D immunological response by investigating the expression of three key genes to vitamin D metabolism and response.

The uninfected kidney results showed that the D+ *Cyp27b1* WT mice, the only mice expected to be able to produce 1,25$(OH)_2$D, had significant expression of *Cyp24a1.* The other groups, the D+ *Cyp27b1* KO, D- WT, and D- *Cyp27b1* KO, expected to be deficient in 1,25$(OH)_2$D, had comparable low levels of *Cyp24a1*. This data agrees with what is known from previous literature that *Cyp27b1* KO status significantly reduces the expression of *Cyp24a1*, although the study done here does not show that providing a vitamin D sufficient diet can upregulate *Cyp24a1*[27]. Given the concurrence of these results with previous studies, the *Cyp27b1* KO model is effective, and the *Cyp24a1* gene is sensitive to 1,25$(OH)_2$D availability.

The effects of vitamin D status during infection are not definite, as the results show no significant differences, although there seems to be a trend towards infection-induced *Cyp24a1* upregulation. These results indicate that 1,25$(OH)_2$D and vitamin D metabolism may be more active under vitamin D sufficient status due to H1N1 infection. As infection effects are usually studied in the lungs, previous literature has not focused on exploring the effects of H1N1 infection on *Cyp24a1* expression in the renal system. However, the low expression of *Cyp24a1*, and therefore vitamin D metabolism, by *Cyp27b1* KO groups during infection supports that vitamin D deficiency leads to greater susceptibility to infection as shown previously by the

Cantorna lab[26]. While there are no significant conclusions to support with this data, an increase in sample size for the uninfected control group (*n*=2 currently), would likely yield a significant result. In all, the kidney data from these experiments support the sensitivity of *Cyp24a1* expression and potential H1N1 infection susceptibility to vitamin D availability.

Despite its prevalence throughout other cells in the body, including the lungs, *Cyp24a1* is largely regarded as a kidney enzyme[10]. While *Cyp24a1* is a sensitive and highly regulated target gene in the kidneys, a comparison was done between kidney and lung expression of the gene to demonstrate the comparative expression levels and ensure lung studies would be feasible. The uninfected vitamin D sufficient controls of each tissue showed that kidneys had significantly greater expression of *Cyp24a1* relative to the lungs. However, the data did show that *Cyp24a1* was induced in the lungs, and the lungs demonstrated an adequate level of expression which would allow for further investigation. This comparison agrees with the well-known prevalence and locations of expression of the *Cyp24a1* gene.

The lung studies further investigated the role of infection on vitamin D sufficient mice's ability to express vitamin D-related genes. As it is known that the influenza immune response in mice peaks at days 6 to 9, the significant upregulation of *Cyp24a1* at day 6, then gradual decline, was expected. However, the *Cyp27b1* data reveals no differences in expression throughout infection, and the *Vdr* data only shows a small, insignificant increase in expression coinciding with day 6. A limitation of this data, however, is that by using whole lungs as the sample for study, the immune cells cannot be differentiated from the other lung and epithelial cells. Therefore, if vitamin D metabolism is meaningfully different by cell type, this difference may be indistinguishable in the data collected, which may contribute to inconclusive results.

Previous literature has investigated the regulation of these genes in a variety of infections, including similar influenzae. H9N2 is another influenza A virus, like the H1N1 studied here[28]. In the lungs of H9N2-infected mice, a previous study showed that *Cyp24a1* mRNA and protein expression was unaffected at several timepoints following infection[29]. In contrast, the study also found that H9N2 infection upregulated both *Cyp27b1* and *Vdr* at day 7. Their study concluded that this may indicate that the negative feedback loop of *Cyp24a1* degrading $1,25(OH)_2D$ is not highly active, allowing for $1,25(OH)_2D$ accumulation and amplified vitamin D signaling. These results are in direct opposition to the findings produced here with H1N1 infection. This data showed that *Cyp24a1* is more expressed during infection, and the other vitamin D genes are unaffected. This would indicate that the negative feedback loop is more active in this H1N1 study as a result of vitamin D metabolism. While it may be that vitamin D metabolism and signaling act according to the specific infection, both H9N2 and H1N1 are influenza A viruses that have flu pandemic potential. Their similarity was exhibited by a study which saw a nucleoprotein-based vaccine being effective against both viruses[30]. Therefore, as the different results cannot immediately be reconciled with differences in the virus, further investigation into the H1N1 results or a repeat of these experiments with a new influenzae virus subtype may be warranted.

Another infection study investigated VDR expression in H1N1-infected mammalian cells[31]. As the experiment utilized cultured cells, the infection results were much quicker, and H1N1 infection was shown to upregulate VDR greatly by 48 hours post-infection. This result is in concordance with the previous H9N2 study. Although this study used mammalian cells and not a mouse model, these results suggest further experiments or samples should be added to scrutinize the *Vdr* results garnered through the mouse H1N1 studies.

While the results shown in this thesis are in contradiction to some of the previous literature, the major findings agree with previous studies from the lab. The susceptibility of vitamin D deficient mice to severe H1N1 infection coincides with lower baseline *Cyp24a1* expression levels, indicating that vitamin D response is not highly active within the immune system under these conditions[26]. Upregulation of *Cyp24a1* in the local lung tissue during infection indicates the greater presence of 1,25$(OH)_2$D, initiating the negative feedback loop and suggesting the presence of a vitamin D immune response. However, these results are not enough to fully elucidate the biochemistry behind the vitamin D response during infection.

In terms of future studies, mRNA expression of H1N1 M gene and proinflammatory cytokines throughout H1N1 infection will be investigated. The H1N1 M gene expression is a measure of the viral replication within the tissue. This data could then be correlated with the vitamin D gene response data to understand the relationship between peak vitamin D response and viral influenza replication. After further investigation into the vitamin D response, it will then be useful to examine the downstream effects of this response and how this signaling pathway is actually working to fight infection. To understand this, measuring the levels of proinflammatory cytokines will be beneficial to elucidate the mechanisms of action or protection against infection.

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EDUCATION

The Pennsylvania State University, University Park, PA 2020 – 2024
Schreyer Honors College | Eberly College of Science
Bachelor of Science in Biochemistry and Molecular Biology
Minor in Political Science

Relevant Coursework: Introduction to Human Physiology, Introductory Microbiology Laboratory, Genetic Analysis, General Biochemistry, Principles of Immunology, Biochemistry Protein and Enzyme Laboratory, Organic Chemistry, Developmental Biology

HONORS AND GRANTS

Dean's List (2020—Present)
Academic Excellence Scholarship (2020—Present)
Biochemistry and Molecular Biology Summer Undergraduate Research Fellowship Award (2022)

RESEARCH EXPERIENCE

Pennsylvania State University, Department of Veterinary and Biomedical Sciences
Undergraduate Researcher January 2023 – Present
Advisor: Dr. Margherita Cantorna
Investigated the role of Vitamin D in H1N1 influenza immune response in mice. Harvested kidneys and lungs for RNA isolation and reverse transcription to cDNA. Quantified vitamin D metabolism through RT-qPCR of relevant genes.

Pennsylvania State University College of Medicine, Schreyer MD/PhD Summer Exposure Program
Research and Clinical Intern May – August 2023
Advisor: Dr. Hyun Jin Kwun
Examined the biological pathways and mechanisms of Merkel Cell Polyomavirus (MCPyV). Transduced and transfected cells to acquire desired protein expressions. Ran qPCR to determine experimental effects on Large T antigen expression. Produced western blots to investigate effects on protein levels. Observed fluorescence through immunofluorescence and other assays.

Pennsylvania State University College of Medicine, Schreyer MD/PhD Summer Exposure Program
Research and Clinical Intern May – August 2022
Advisors: Dr. Todd Schell and Dr. Catherine Paules
Explored the efficacy of novel anti-cancer drug through experimentation on cultured murine bladder cancer cells. Developed schedule and planned experiments for testing of drug. Cultured cells and administered drug. Analyzed cells and data with flow cytometry via FlowJo software. Engaged in clinical experience through observation of Dr. Paules and interaction with her bone marrow transplant patients.

Pennsylvania State University, Department of Biochemistry and Molecular Biology
Undergraduate Researcher January 2021 – August 2022
Advisor: Dr. Melissa Rolls
Examined Class IV sensory dendrites of *Drosophila* larvae under fluorescence microscopy. Severed dendrites using UV pulse laser to later quantify degree of regeneration. Surveyed gene deficiencies for dendrite regeneration efficacy. Imaged adult *Drosophila* neurons for dendrite regeneration.

PRESENTATIONS

Penn State College of Medicine Undergraduate Research Symposium, Hershey, PA, August 2023. Kozloski, S; Pham, A.M; Kwun, H.J. "Investigating the regulation of ribosomal biogenesis by the Merkel Cell Polyomavirus Large Tumor Antigen" (poster).

Penn State College of Medicine Undergraduate Research Symposium, Hershey, PA, August 2022. Kozloski, S; Neighbors, J.D; Hohl, R.J; Sharma, A.K; Degraff, D.J; Schell, T.D. "Schweinfurthins Induce Growth Inhibition of Murine Bladder Cancer Cells and Promote Surface Calreticulin Translocation" (poster).

Penn State Undergraduate Research Exhibition, Penn State University, University Park, PA, April 2022. Bernard, A; McKay, M; Kozloski, S; Hertzler, J.I; Rolls, M. "Using Drosophila to Elucidate the Pathways of Dendrite Regeneration and Growth Using New Methods" (poster).

Penn State Undergraduate Research Exhibition, Penn State University, University Park, PA, April 2021. Simonovitch, S; Bernard, A; McKay, M; Kozloski, S; Hertzler, J.I; Rolls, M. "Using deficiencies to screen for dendrite regeneration genes in class IV neurons of Drosophila" (poster).

TEACHING EXPERIENCE

Organic Chemistry I+II
Learning Assistant January 2021 – December 2023
Professor: Dr. Joseph Houck
Interacted with 200+ organic chemistry students to aid in development of their learning. Attended all class sessions to assist the professor in the teaching of content and answering questions. Hosted learning communities and office hours as an extracurricular source of assistance to students.

WORK EXPERIENCE

Pennsylvania State University, Department of Chemistry
Organic Chemistry Grader January – December 2022
Communicated with professor and other graders to grade quizzes and exams fairly and constructively. Managed schedules to ensure finished grading by the due date. Worked with students to understand the grading system and how to acclimate to the tests.

CVS Pharmacy
Pharmacy Technician May – August 2021
Processed 1000+ prescriptions. Interacted with and advised patients on their prescriptions in-person and over the phone. Collaborated with the pharmacy team to ensure all prescriptions were filled. Covered the register and drive-throughs to efficiently deliver prescriptions to patients.

The Rolls Lab
Research Assistant August 2021 – December 2021
Maintained the laboratory necessities for experiments on *Drosophila*. Created food and maintained fly stocks to ensure upkeep of all organisms and resources. Cooperated with other research assistants to ensure all necessary maintenance was completed.

LEADERSHIP/SERVICE

Schreyer Honors College Student Council
Secretary May 2022 – Present
Facilitated communication with the organization via e-mail, websites, and social media. Organized and tracked membership throughout the organization. Promoted organization events through social media and e-mail. Managed a Public Relations Chair who aided in creation of social media posts.

University Park Undergraduate Association
At-Large Representative January – March 2024
Represented 40,000+ undergraduate students in the student government. Head-sponsored a resolution, as a member of the Academic Affairs Committee, advocating for menstrual equity. Voted on key resolutions and bills passed by the UPUA affecting campus-wide events and initiatives.

Schreyer Honors College Student Council
Service Director May 2021 – May 2022
Coordinated service-based initiatives and events for honors college scholars and organization members. Planned events in collaboration with other organizations such as the Student Farm and the Lion's Pantry. Established events such as food drives, card writings, and volunteer opportunities for members. Led Service Committee dedicated to expanding service opportunities in the organization.

Mid-State Literacy Council
ESL Tutor Volunteer February – December 2023
Tutored one-on-one in English as a second language. Established long-term language goals with the student. Created schedule and curriculum to help student achieve language goals. Improved tutor-tutee relationship and tutee's language skills over the course of 15+ meetings.

Hershey Medical Center Volunteer
Emergency Department June – August 2023
Guided visiting family members and guests to patient beds. Comforted visitors and patients waiting in reception, providing drinks or other necessities. Performed maintenance through replacing oxygen tanks and disposing of soiled linens. Ran errands to ease the workload of healthcare professionals.
Cancer Ward Comfort Cart June – August 2023
Visited patient rooms in the cancer ward with the comfort cart providing free leisure or care items such as snacks, toiletries, games, etc. Formed connections with patients through friendly conversations. Served all patients with their requests if possible.

MEMBERSHIP

Schreyer Honors College Student Council (2020—Present)
Campus Orchestra (January 2022—Present)
Volé Dance Company (January 2022—December 2023)

TECHNICAL SKILLS

Computational: PRISM GraphPad, Excel, Word, FlowJo, SnapGene