

Mitigating Cognitive Impairments in Parkinson's Disease: A Novel Approach

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ABSTRACT

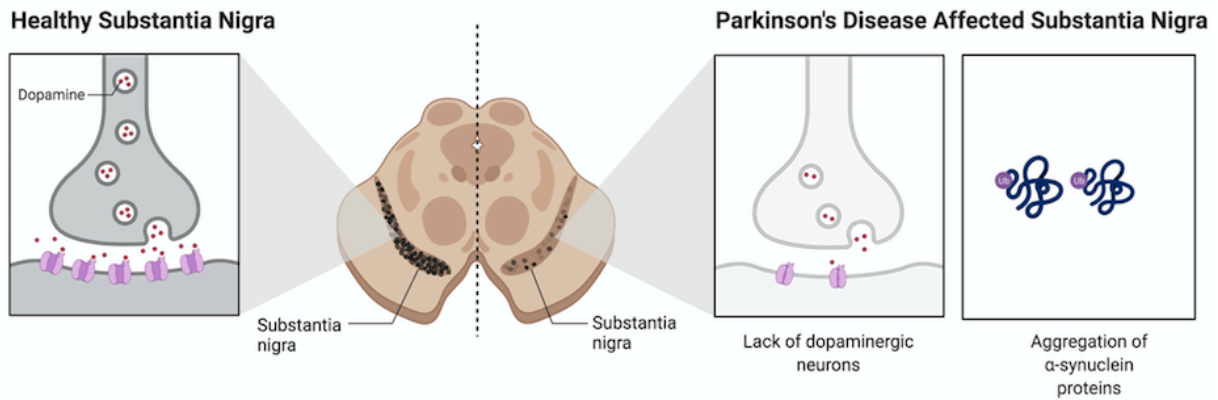
Parkinson's Disease (PD), a neurodegenerative disorder, is caused by the death of dopaminergic neurons in the substantia nigra. Caffeine exposure is suggested to mitigate the PD onset and progression by slowing the death of the dopaminergic neurons. Isolating the caffeine induced gene expression and the associated interaction pathways that target the dopaminergic death in the substantia nigra of a PD patient is vital for preventing or slowing the progression of the disease. Even though a number of genes have been identified to be related to PD, no direct correlation between the caffeine trigger and the associated pathways has been provided and explained. The Sodium Channel Subunit Beta-1 (SCN1B) gene and the novel voltage-gated sodium channel (VGSC) complex pathway triggered by it have not been adequately researched previously. This research study concludes that caffeine exposure (limits from data set) down-regulates the SCN1B gene, triggering a novel voltage-gated sodium channel complex pathway, blocking the sodium channel to negatively influence (reduce) cognitive impairment. This results in mitigating PD as a result of reduced neuronal damage in substantia nigra. GEO2R volcano plot analysis of microarray datasets identified that a caffeine trigger down-regulated differentially expressed gene, SCN1B. Results from String enriched network interactions suggested that SCN1B is identified to trigger 9 interactions (pathways) across different genes. Research conducted in vitro to determine the effect of various concentrations of caffeine on SHSY5Y cells with an overexpression of alpha-synuclein produced that high dosages may impose toxic effects, hence the dosing needs to be further studied. This research unleashes the potential to administer a regimented caffeine dosage to both proactively prevent and reactively slow down the progression of the PD.

INTRODUCTION

Neurodegenerative diseases are comprised of over 100 diseases that are characterized by damage of the nerve cells, affecting one's motor function, cognitive impairments, and ability to complete every day, common tasks. (Przedborski, Serge, 2003). Neurodegenerative diseases include Parkinson's Disease (PD), Alzheimer's Disease, Lewy Body Disease, and Huntington's Disease. The nerve cell damage occurs due to the aging and loss of function and activity in specific areas of the brain. The true cause of neurodegenerative diseases is unknown, although many underlying factors including environmental factors and genetics may be related.

Parkinson's Disease (PD), one of the most common neurodegenerative diseases, affects over 10 million people worldwide. Despite the striking impact it has on the population, it is widely misunderstood and complex. (Harvard Health, 2020). PD, a neurodegenerative disease distinguished by motor and cognitive impairments, is associated with the death of dopaminergic neurons in the substantia nigra. (Alexander G, 2004). The substantia nigra is responsible for processing motor functions, and it includes the substantia nigra pars compacta (SNpc) which houses the dopaminergic neurons. Dopaminergic neurons play a role in controlling motor function and work as a messenger for the brain. These neurons act as a messenger from one area of the basal ganglia to another. The basal ganglia, found inside the substantia nigra pars compacta, is responsible for motor functions and behavioral aspects. With the lack of dopaminergic neurons, the entire process is abbreviated.

Parkinson's Disease in the Substantia Nigra



Affected Substantia Nigra. (Adapted from Bio Render).

The degeneration of these neurons and the low levels of dopamine lead to the impairments associated with Parkinson's Disease. (Sonne J, 2020). The loss of extreme levels of dopaminergic neurons induces motor impairments, consisting of bradykinesia, constant tremor, and lack of balance. (Mazzoni, Pietro, 2012). Generally, cognitive and motor impairments come hand in hand in regard to Parkinson's Disease. Cognitive impairments present in two ways: with dementia and without dementia. This varies in each patient; however, some impairments include decreased attention, long-term and/or short-term memory loss, and lack of facial recognition.

At the cellular level, clumping and overexpression of alpha-synuclein is another factor associated with Parkinson's Disease pathology. Alpha-synuclein is a protein found in all humans, however, in PD patients it is expressed in large amounts, in turn damaging the cells. The reason for the over-expression of alpha-synuclein is still unknown, another reason why there is difficulty in addressing this disease. (Stefanis, Leonidas, 2012)

Current treatment options are limited due to the lack of understanding of the etiology of the disease. The origin of this disease is largely unknown, alongside the fact that all patients have varying signs and symptoms. The current treatment options for PD, as well as for other neurodegenerative diseases, are used for symptom alleviation rather than slowing the progression of the disease. Although it has been helpful to relax the symptomatology, many patients opt out of these medications. The medications work to reinstitute the dopamine that is lacking in the brain. The most commonly used medication is levodopa. Although it provides temporary relief for the patient, this treatment poorly interacts with the disease as it continues to progress, and for some patients worsens the symptoms. (Hurtig, 1997). Relative to various other treatment options, levodopa remains the best option.

Caffeine is a commonly used, well-known stimulant. A key point that can contribute to finding an additional therapeutic option to be used alongside current treatments is determining how caffeine has an impact on PD patients at the cellular level. This *in silico* research focuses on the Sodium Channel Subunit-1 (SCN1B) gene expressed in caffeine treated rat brain, as well as Parkinson's Disease affected Substantia nigra pars compacta (SNpc).

In relation to Parkinson's Disease, caffeine is an antagonist that could potentially improve symptoms in PD patients. (Cieślak, Marek, 2008). There are currently clinical trial studies regarding the effect of caffeine, as an adjuvant treatment, on individuals with Parkinson's Disease, and there seemed to be a slight improvement in motor impairments. (Postuma, 2012).

However, there is a lack of research regarding the effect of caffeine at an *in vitro* level. This research reported here, looks at the effect of caffeine on cells that model Parkinson's Disease neurons through the over expression of alpha-synuclein.

This research unleashes the potential to administer caffeine dosage, as an adjuvant treatment, to decrease the cognitive impairment in PD patients.

MATERIALS AND METHODS

Dataset GSE59895

Gene Expression Microarray Dataset (GSE59895) compared caffeine treated *rattus norvegicus* rat brain to a control variable. The dosage of the caffeine administered to the *rattus norvegicus* is 93 mg/kg, with the solvent being water. The control variable was water, orally administered. Dataset was analyzed using GEO2R and differentially expressed genes (DEGs) were identified. Genes were determined to be statistically significant and biologically relevant if the absolute value of the log fold change values was greater than 1 and adjusted probability (p) values was less than 0.05.

Dataset GSE1157

Gene Expression Microarray Dataset (GSE1157) compared untreated Substantia Nigra pars compacta (SNpc) and Ventral Tegmental Area (VTA). Dataset was analyzed using GEO2R and differentially expressed genes were identified. Brackets for differential expression were determined by log fold change values being less than 1 and probability (p) values being less than 0.05.

GEO2R

The accession numbers presented above were inputted into GEO2R to determine the differentially expressed genes in each dataset. GEO2R presents the flexibility of inputting samples: control and variable groups, along with limitations such as p values. The analysis provided a table with the differentially expressed genes.

STRING.db

Common differentially expressed genes from the GEO2R analysis along with relevant genes noted in previously published work noting the correlation between caffeine and PD (Yamada-Fowler, N., & Söderkvist, P., 2015) were selected for further network analysis using STRING.db.

STRING.db produces a complete network highlighting various protein-protein interactions triggered by the inputted genes.

Cell Culture

The SHSY5Y (ATCC CRL-2266) cell line was transfected to overexpress alpha-synuclein using the SNCA plasmid. This exhibits a 2.1-fold increase in alpha-synuclein expression. The SHSY5Y cell line overexpressed with alpha-synuclein was cultured in Dulbecco's Modified Eagle Medium (DMEM) (#10569044, Thermofisher, Waltham, MA), along with 1% penicillin-streptomycin (#15140163, ThermoFisher, Waltham, MA) and 10% fetal bovine serum (FBS) (#97068-085, avantor, Radnor, PA). Cells were incubated at 37°C and 5% CO₂. Cells were trypsinized using 0.25% trypsin (#25200114, Thermofisher, Waltham, MA).

Cell Viability

50 mL from the flasks with the cultured SHSY5Y cells overexpressed with alpha-synuclein were pipetted into an Eppendorf tube. The viable and dead cells were measured using a VI cell (Beckman Coulter).

Reagents

Caffeine (C0750-100G, Sigma-Aldrich) concentrations ranged from 0.005 mM to 50 mM. Caffeine powder was diluted in Dimethyl sulfoxide (DMSO). The resulting concentrations were prepared in 2mL Eppendorf tubes with a total of 6 concentrations, including the control variable of DMSO.

Cell Proliferation Assay

100 microliters of cells were pipetted into 30 wells (6 by 5) of a 96 well plate. Each concentration, including the control of DMSO, was pipetted at 1 microliter into 5 individual wells. Cell Titer Aqueous One Solution Reagent (G3582, Promega, Madison, WI) was added to each well. Cell proliferation and absorbance was measured after 48 hours at 490 nm using the Absorbance Microplate Reader (BioTek ELx808).

Preparation of Lysates

Cells were split into T25 flasks and treated with 4 concentrations of caffeine solution, including the control of DMSO. After 72-hour treatment, cells were trypsinized and 3 mLs of media were added into the flasks and transferred into centrifuge tubes. The cell viability was measured using a VI cell. Cells were centrifuged, supernatant was poured out, and the pellets were broken up. Phosphate buffer saline (10010023, Gibco, Waltham, Massachusetts, USA) was added to each

tube and centrifuged. The supernatant was removed, again, and then the appropriate amount of lysis buffer (9803S, Cell Signaling Technology, Danvers, MA) was added to the tubes and set to incubate on ice. The lysates were transferred to Eppendorf and centrifuged. The supernatant was removed and the lysate was stored at -80°C.

Indirect ELISA

100 microliters of lysates were pipetted into 20 wells on a 96 well plate and incubated overnight at 4°C. 24 hours later, the lysates were blotted and 200 microliters of 1X BSA Blocking Solution were added to each well and set to incubate. After incubation, the plate was blotted to remove the buffer. The primary antibody, alpha-synuclein (GR54029-6) was prepared in a blocking buffer with 1% BSA. The antibody solution was added at 100 microliters/well and incubated for 1 hour. The plate was blotted and washed 3 times with 300 microliters of wash buffer per well and the plate was blotted. The secondary antibody solution (31460 Invitrogen) was created and added at 100 microliter/well and set to incubate for 1 hour. After incubation, the plate was washed 3 times with 300 microliters of wash buffer per well and set to incubate for 5 minutes at the last wash. The substrate solution was created and after the plate was blotted, the solution was added at 100 microliters/well. The plate was incubated until it developed. The plate absorbance was read at 405 nm.

Statistical Analysis

Each assay was repeated 3 times unless otherwise stated. Each sample was repeated 5 times per assay. The T-test alpha was set at 0.05. All images were created by the author unless otherwise stated.

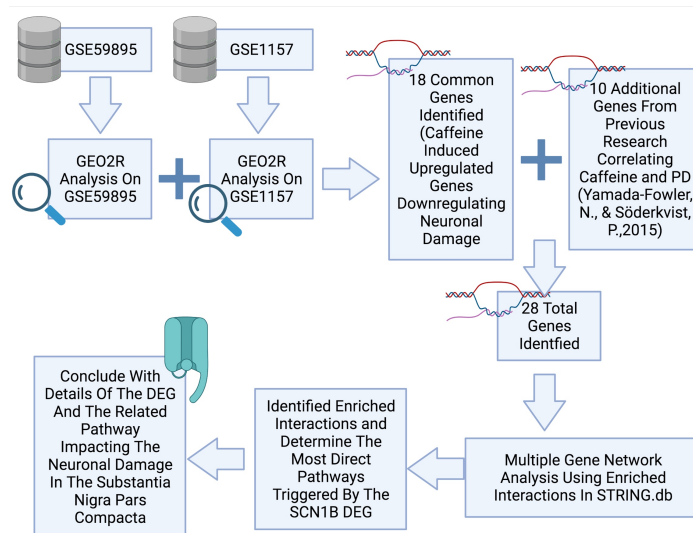


Figure 2. In Silico Methodology Flow Chart.

The materials and methods used for the in-silico research are detailed.

RESULTS AND DISCUSSION

Microarray GEO2R Data Analysis GSE59895: Exposure of Rat to a Variety of Toxicants

Microarray Dataset GSE59895 focuses on caffeine's effect on *rattus norvegicus*. The upregulated genes, in Figure 1, were key factors in the analysis of the dataset as they represent the upregulation of caffeine and its effect on PD. A total of 70 upregulated genes were noted following the analysis of this dataset.

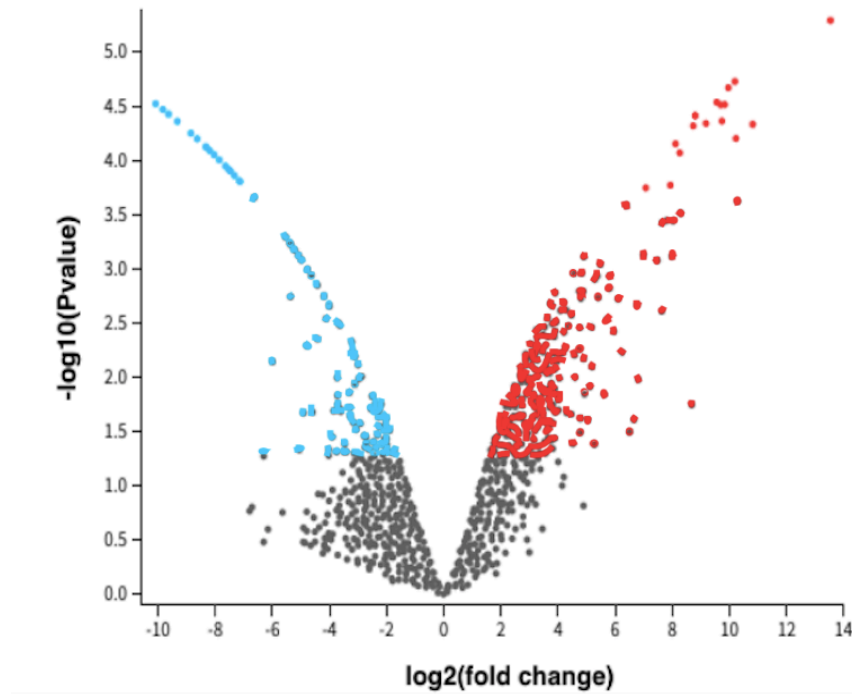


Figure 3. Volcano Plot: DEGs of Rattus Norvegicus Substantia Nigra Treated with Caffeine and Water.

The figure above, analyzed through GEO2R, is a volcano plot representing the GEO series GSE59895 dataset. The data was retrieved from a microarray dataset of *rattus norvegicus*. 1 of the samples was the control and 3 of the samples were treated with caffeine. All data was collected after 1 day of exposure. In the graphic above, the y-axis displays the adjusted p value while the x-axis displays the log fold change. The p value (less than 0.05) determines statistical significance and the log fold change (greater than 1) determines overexpression of a gene when compared to the control. The genes with a positive log fold change (greater than 1) are upregulated (red) and the genes with a negative log fold change (below -1) are downregulated (blue). The points that lie higher on the volcano plot are more statistically significant due to a lower p value.

Microarray GEO2R Data Analysis GSE1157: NINDS Rat Neuron Parkinson's Substantia Nigra Pars Compacta (SNpc) and Ventral Tegmental Area (VTA)

Microarray dataset GSE1157 focuses on the effect of untreated Parkinson's Disease on the Substantia Nigra pars compacta and the Ventral Tegmental Area. The SNpc, a region of the substantia nigra responsible for dopamine production, is currently more prevalent in Parkinson's Disease research as the dopaminergic neurons in the substantia nigra are more susceptible to neuronal damage than in the VTA. Downregulated genes, in figure 2, correlate with a decrease in dopaminergic death. There were a total of 177 DEGs.

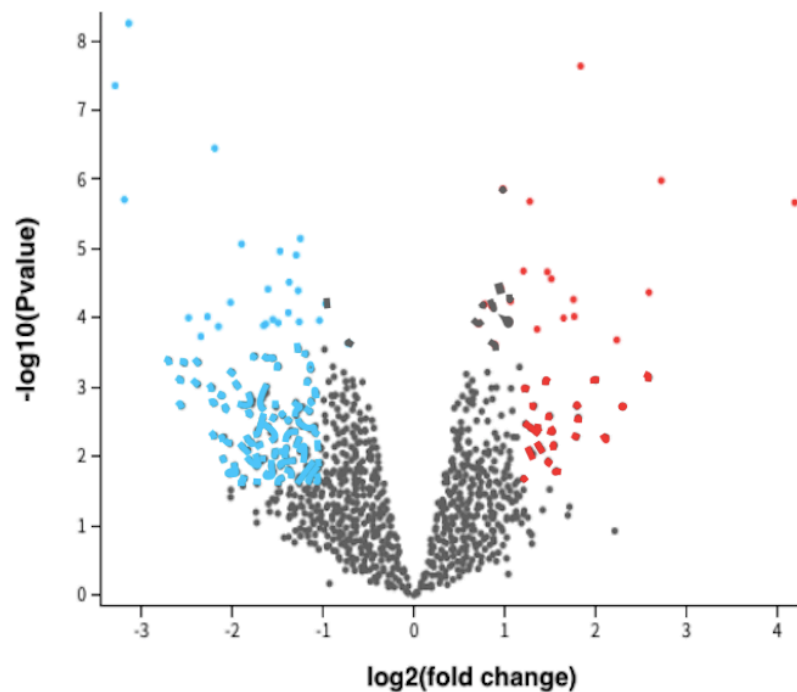


Figure 4. Volcano Plot: DEGs of Untreated SNC vs VTA. The volcano plot above was analyzed through GEO2R representing the GEO series GSE1157 dataset. The data from a microarray dataset was retrieved from a *rattus norvegicus*. 8 samples were of untreated SNC and 7 samples represented untreated VTA. The y-axis, in the volcano plot, displays the adjusted p value. The p value (less than 0.05) determines if the gene is statistically significant. The log fold change (x-axis) determines overexpression of a gene when compared to the control. The red genes with a log fold change greater than 1 are upregulated and the blue genes with a log fold change below -1 are downregulated.

Parkinson's Disease Gene Expression Correlation

The differentially expressed genes that were noted in Figure 1 and 2 were further analyzed to determine the commonalities between both datasets. In essence, the common genes between the genes that constitute upregulation of caffeine in Figure 1 and the genes that depict

downregulation of dopaminergic death in Figure 2 were deemed important. 18 common genes were noted in Table 1.

Table 2 presents previously published research (Yamada-Fowler, N., & Söderkvist, P., 2015) to strengthen the foundation for this research study. It proposes 10 genes that were considered relevant in the correlation between caffeine and PD.

Genes	Genes
SCN1B	Mpst
Pc	Gipc1
Crhr1	Me1
Pgam1	I15
Sds	Drd4
Ret	Uchl1
Cd47	Sub1
Slc3a2	Smarca4
Got2	Ykt6



Genes
SNCA
MAPT
NOS1
NOS3
CYP1A2
NAT2
ESR1
ESR2
GRIN2A
ADORA2A

Table 1. Common DEG's found from a GEO2R analysis of the Dataset

GSE1157 and Dataset GSE59895. The genes listed above were common differentially expressed genes that were collected from the two analyzed datasets. These genes are caffeine induced upregulated genes that downregulated the dopaminergic death in the substantia

Table 2. Genes found from previously published work nothing the correlation between caffeine and PD. (Yamada-Fowler, N., & Soderkvist, P., 2015). The genes listed above are from previously published research regarding correlation of caffeine and PD. This allows for a foundation to be added to the new research.

Gene Protein Network Pathways Using STRING.db

18 DEGs identified from GEO2R analysis were selected for further STRING.db network, alongside 10 non DEG's, found from previous research (Yamada-Fowler, N., & Söderkvist, P., 2015), that were also present in datasets were added in this analysis. Shown in figure 4, the main gene that initiates major pathways is the SCN1B gene, with a previously researched

correlation, presents pathways of interest. The outcome of this analysis was a total of 9 interactions stemming from SCN1B.

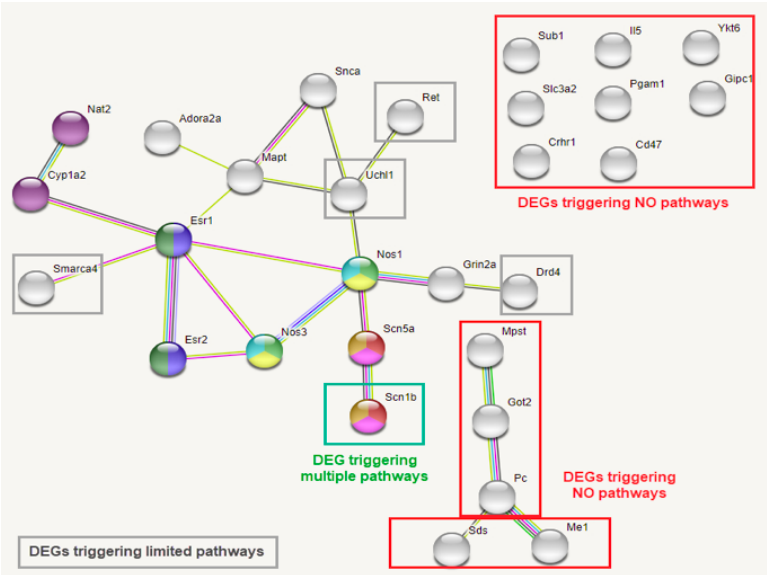


Figure 5. STRING.db Network Analysis: Interactions Triggered by DEGs. The above visualization is a protein-protein interaction network of differentially expressed genes produced through STRING.db. The nodes represent the inputted differentially expressed genes and the connecting lines represent the interactions or pathways. The SCN1B gene is the starting DEG that was determined from the volcano plots.

STRING.db Protein-Protein Interactions

The highlighted interactions and pathways in Table 3 are those that stem directly from the SCN1B gene in Figure 4. Table 4 explains the importance of each pathway in relation to this research. The pathway considered most relevant was the Voltage Gated Sodium Channel. This complex is directly connected from the SCN1B gene to remaining DEG’s. The understanding of this gene and its interaction through the VGSC would further allow the understanding of the other 8 pathways that are connected with this association.

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0086047	membrane depolarization during Purkinje myocyte cell acti...	2 of 2	2.89	0.00011
GO:0060011	Sertoli cell proliferation	2 of 4	2.59	0.00022
GO:0009403	toxin biosynthetic process	2 of 4	2.59	0.00022
GO:0031284	positive regulation of guanylate cyclase activity	2 of 5	2.5	0.00028
GO:0071000	response to magnetism	2 of 6	2.42	0.00037
GO:0006809	nitric oxide biosynthetic process	2 of 6	2.42	0.00037
Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0086062	voltage-gated sodium channel activity involved in Purkinje ...	2 of 2	2.89	0.00018
GO:0030284	estrogen receptor activity	2 of 3	2.72	0.00025
GO:0004517	nitric-oxide synthase activity	2 of 3	2.72	0.00025
GO:0003958	NADPH-hemoprotein reductase activity	2 of 6	2.42	0.00051
GO:0010181	FMN binding	2 of 10	2.2	0.00095
Cellular Component (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0001518	voltage-gated sodium channel complex	2 of 13	2.08	0.00078
KEGG Pathways				
pathway	description	count in network	strength	false discovery rate
mo00232	Caffeine metabolism	2 of 6	2.42	0.00059

Table 3. PD Pathways Triggered by DEGs. The pathways mentioned were retrieved from the STRING.db Network Analysis. The pathways/interactions highlighted in red are significant to this research because they are directly connected to the SCN1B gene.

Interaction	Description	Gene	Analysis
Molecular Function	Voltage-Gated Sodium Channel Activity Involved in Purkinje Myocyte Action Potential	SCN1B, SCN5A	This action potential is linked with causing tremor which is associated with the motor impairments of Parkinson's Disease. (Brown et al., 2020)
Molecular Function	Estrogen Receptor Activity	ESR1, ESR2	Estrogen receptors may assist in the survival of neurons. (Baraka et al., 2011)
Molecular Function	NADPH-Hemoprotein Reductase Activity	NOS1, NOS2, NOS3	The clumping of the alpha-synuclein in the substantia nigra can be partially attributed to NADPH-Hemoprotein Reductase. (Belarbi et al., 2017)
Cellular Component	Voltage-Gated Sodium Channel (VGSC) Complex	SCN1B, SCN5A	The sodium channels have been related to the cognitive impairments that arise as a symptom to some with PD. (Wang et al., 2019)
KEGG Pathways	Caffeine Metabolism	CYP1A2, NAT2	Low levels of caffeine metabolites are diagnostic biomarkers in patients with PD showing motor impairment symptoms. (Fujimaki et al., 2018)
Biological Process	Membrane Depolarization During Purkinje Myocyte Cell Action Potential	SCN1B, SCN5A	There has been no recent research regarding this correlation.
Biological Process	Positive Regulation of Guanylate Cyclase Activity	NOS1, NOS2, NOS3	The cGMP pathway, synthesized by Guanylate Cyclase, has a negative impact on the degeneration of neurons. (Chalimoniuk et al., 2004)
Biological Process	Nitric Oxide Biosynthetic Process	NOS1, NOS2, NOS3	The level of nitric oxide produced by cells poses as a toxin towards dopaminergic neurons. (Hunot et al., 1996)
Biological Process	Sertoli Cell Proliferation	ESR1, ESR2	Sertoli cell proliferation progresses dopaminergic neuronal growth by providing neurotrophic support. (Kuan & Barker, 2005)

Table 4. Protein-Protein Interactions originating from SCN1B. The interactions noted above originate from the DEG of focus, SCN1B and discuss their role in Parkinson's Disease as well as the genes that are correlated. The results from the STRING.db network interactions suggested that SCN1B triggers 9 pathways across various different genes. The red text notes the pathway that will be focused on in the conclusion.

Caffeine and Cell Proliferation

Although the in-silico data presented in figure 5 highlights the positive correlation and significance of the VGSC between caffeine and Parkinson's Disease, it is necessary to determine the optimal dosages at which caffeine can actively work as a medication, while not providing toxic effects. As shown in figure 6, the cell proliferation, relative to the control, stays relatively leveled at the middle concentrations, in the provided range, yet decreases at lower and higher concentrations. Hence, it becomes antiproliferative at a certain concentration imposing a caution among users who may be consuming caffeine at high concentrations or dosages.

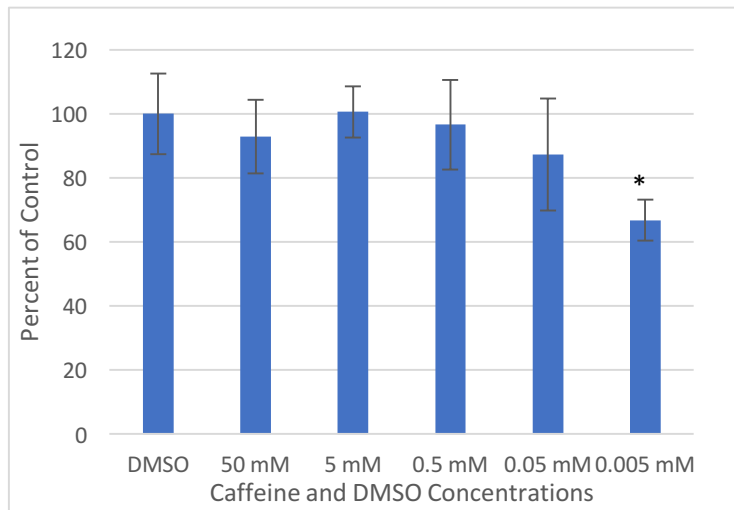


Figure 6. The Effect of Caffeine on Cell Proliferation. SHSY5Y cell line

overexpressed with alpha-synuclein were treated with varying doses of caffeine solutions ranging from 0.005 mM to 50 mM. A cell Proliferation Assay was performed to determine the cell proliferation of the cells following treatment with caffeine. The bars are representative of the standard deviation.

** represents $p < 0.01$ in comparison to the control of DMSO.

Cells and Alpha-Synuclein Levels

Further research must be conducted to determine the optimal and appropriate concentrations at which caffeine can lead to decreased cell death, or in the case of the indirect ELISA in figure 7, a change in alpha-synuclein levels. Caffeine seems to become toxic at a dosage that has no effect on alpha-synuclein levels. Figure 7 includes 3 caffeine concentrations along with the control of DMSO. The three concentrations presented were the most relevant as displayed in Figure 6. The remaining concentrations: 0.05 mM and 0.005 mM are relatively smaller dosages, hence producing no effect in the viability of the cells.

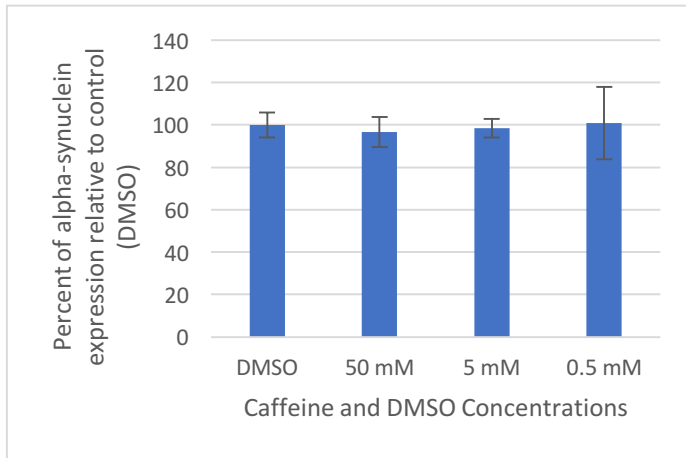


Figure 7. The Effect of Caffeine on Alpha-Synuclein Expression. An indirect ELISA was performed to measure the alpha-synuclein levels following the administration of three caffeine concentrations to the SH-SY5Y cells with an overexpression of alpha-synuclein. Lysates from 72 hours after treatment were used. The bars represent the standard deviation.

As this indirect ELISA, in figure 7, was in vitro data, it is important to note that these dosages do not correspond to those that were reported in in silico and in vivo literature. 93 mg/kg (GSE59895) seems to be an optimal concentration at which there is a positive correlation and effect on the Parkinson's Disease cells. However, in vivo concentrations cannot accurately be converted into in silico dosages due to the inevitable effect of factors, such as the difference in ranges and the various factors that are considered for each type of study. Hence, further research needs to be conducted in this area to determine appropriate concentrations that won't produce toxic effects, yet will positively affect alpha-synuclein levels and Parkinson's Disease symptomatology.

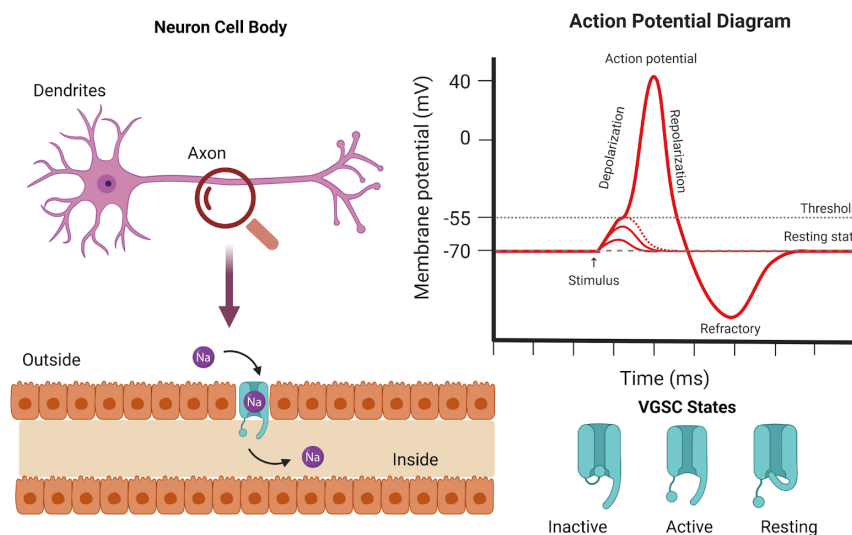


Figure 8. Neuron Cell Body Depicting VGSC States and the Firing Action Potential. (Created by Author)

The cognitive and motor impairments characterized by Parkinson's Disease have been noted to relate back to the action potential of neurons. When the dopaminergic neurons begin to die, the action potential is no longer firing as it did before. The action potential of a neuron is shown in figure 8.

Voltage-Gated Sodium Channels (VGSC) are responsible for the depolarization in the action potential diagram. (Yu, Tucker, Levitan, Shepard, Canavier, 2014). Dopaminergic neurons in the SNpc tend to want a depolarization block. Depolarization block is when there is a pause in the action potential spike. Hence, the blocking of the VGSC would temporarily stop the depolarization, as the dopaminergic neurons prefer.

CONCLUSION

Caffeine exposure is suggested to mitigate the onset and progression of PD by slowing the death of the dopaminergic neurons. Previous studies suggest that dopamine depletion is responsible for motor disabilities and cognitive impairment in PD. Elucidating the caffeine induced gene expression and the associated pathways that target the cognitive impairment in the SNpc is vital for preventing or slowing the progression of PD. Caffeine exposure at 93 mg/kg downregulates the SCN1B gene, triggering a VGSC complex blockage. A VGSC blockage decreases the dopamine depletion, hence reducing cognitive impairments in PD patients. VGSC complex blockage is the pathway between the SCN1B gene and various other previously known caffeine induced upregulated genes. Downregulating of SCN1B triggers the VGSC complex blockage pathway, reducing the dopaminergic death due to reduced dopamine depletion in the SNpc.

Some limitations are imposed due to this research being in silico, including using the p value to determine differential expression, the results suggesting a correlating relationship, and the dataset being tested on *rattus norvegicus* brain which may differ from human brain. These genes and the corresponding pathways can be further researched to divulge more into the unknown aspects of PD, while improving current treatment options and unraveling new ones.

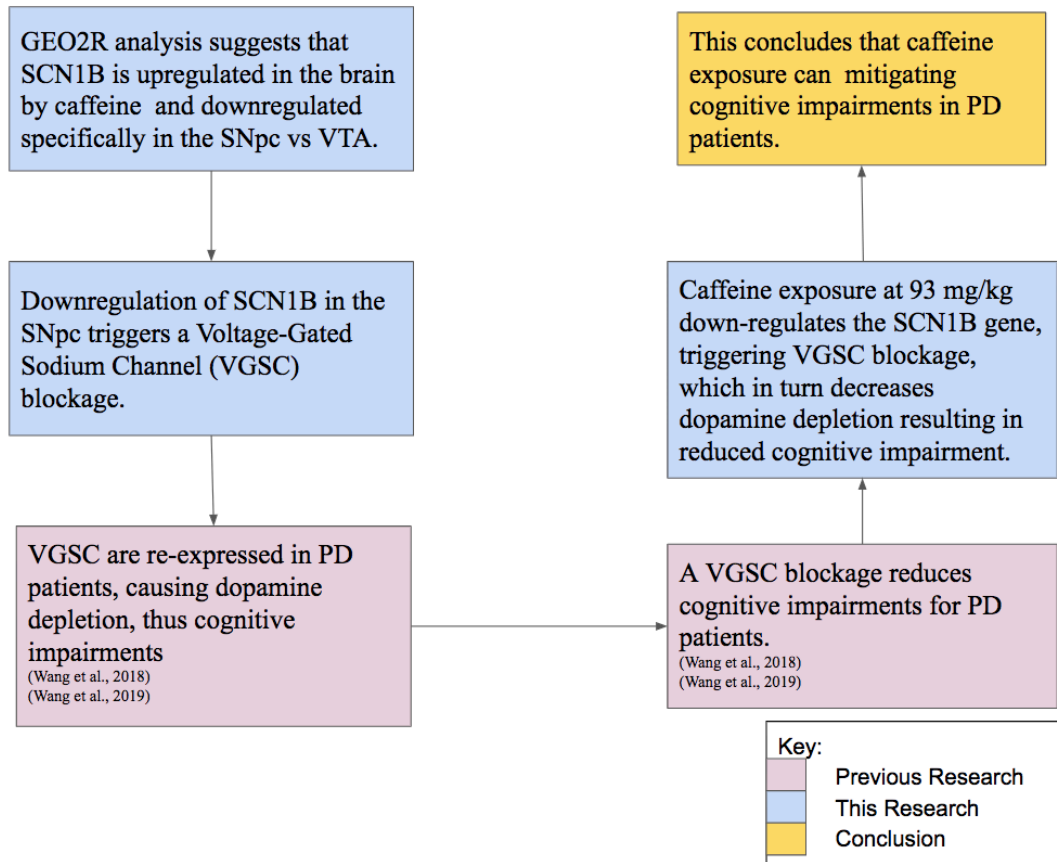


Figure 9. Caffeine and Voltage-Gated Sodium Channel Flow Chart

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