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Bioinformatic analysis of miRNA mechanisms in circadian rhythm using a zebrafish (Danio Rerio) model

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in the Department of Chemistry and Biochemistry

By
Layton Coursey

Under the mentorship of Dr. Rebecca Kocerha

Abstract

MicroRNAs are noncoding RNAs found in cells and the bloodstream, which help to maintain proper protein production and overall gene expression[1]. Typically, microRNAs bind to messenger RNA (mRNA) in the cell cytoplasm, acting as post-transcriptional regulators, and either degrade or repress mRNA[2]. Due to microRNAs playing a vital role in gene expression by repressing protein production of target genes, if they are underexpressed then the protein it regulates could be overexpressed as a result.

miRNAs have the potential to be biomarkers for numerous neurodegenerative diseases. Neurodegeneration can be seen in many forms such as Dementia, Alzheimer's (AD), Huntington's Disease (HD), Creutzfeldt-Jakob Disease (CJD), Vascular Dementia (VD), Dementia With Lewy Bodies (DLB), and Parkinson's Disease Dementia (PD). Early diagnosis of neurodegenerative disease is difficult due to the inability to analyze the diseased tissue. Tissue in the central nervous system cannot be biopsied without using invasive techniques. miRNAs originating from not easily accessible locations, such as neurons in the brain and spinal cord, have the ability to detect early biomarkers for dementia. With the analysis of miRNAs as biomarkers for dementia, early diagnosis of neurodegeneration may be facilitated.

Sleep deprivation (SD) is one of the many side effects, and causes, of neurodegeneration and it adversely affects the circadian physiology[3]. To investigate abnormal microRNA expression, *Danio rerio* (zebrafish) will be used as a model organism and will be exposed to altered circadian rhythms in order to mimic SD. Collection of brain tissue from zebrafish will be conducted to assess for dysregulation of miRNAs.

Thesis Mentor: Dr. Rebecca Kocerha

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Introduction

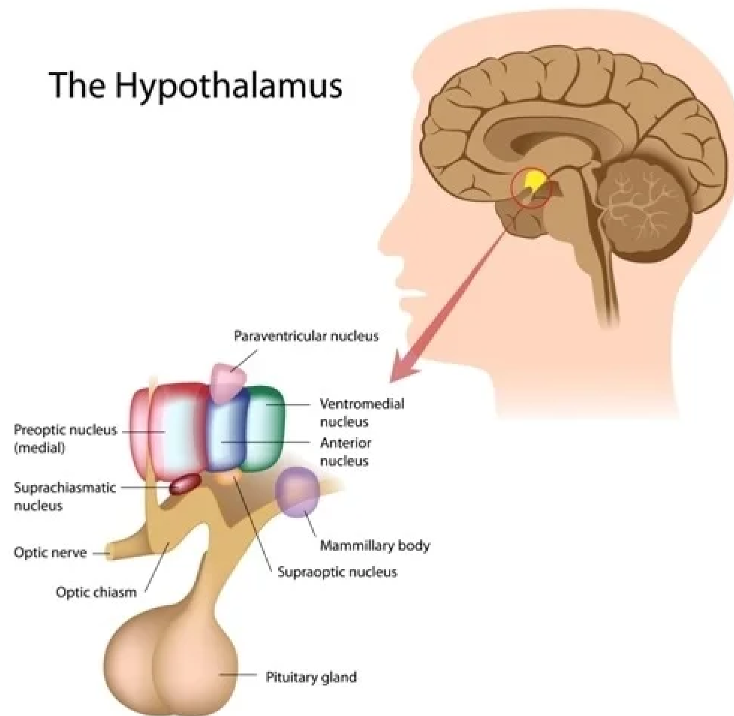
Neurodegenerative diseases affect millions of people worldwide and encompass a wide range of symptoms and ailments. The most common symptoms include gait disorder, abnormal movements, trouble swallowing, blood pressure fluctuation, abnormal heart function, decline in memory and cognitive abilities, frequent mood swings as well as issues sleeping, breathing, and talking[4]. Anomalous circadian rhythms are most commonly witnessed in those with AD, PD, and HD; disordered sleep/wake cycles, limited hormone release, and changes in antioxidant production seen as side effects from altered sleep[5].

There are many ways that the human circadian rhythm can be altered; for example, working the night shift or traveling to different countries with differing time zones. The 24-hour cycles that include wake and sleep are referred to as circadian rhythms and are maintained by the suprachiasmatic nucleus (SCN) found in the hypothalamic nerve cells[6]. The SCN plays a vital role in behavioral adaptation by

controlling the sleep-wake cycle as well as human circadian rhythms by utilizing delayed negative feedback loops. Containing 50,000 neurons, the SCN can be separated into two sections; the vasoactive intestinal peptide (VIP) core that collects information from the retina and the arginine vasopressin (AVP) shell that collections information from the cortex, basal forebrain, and hypothalamus[7]. The SCN controls the homeostasis of the circadian clock by obtaining photic input from the intrinsically photosensitive retinal ganglion cells (ipRGCs). This information then goes through the retino-thalamic tract, using glutamatergic synapses, to its final destination of neurons in the SCN. Alternatively, the SCN can also obtain non-photic information from the brain to maintain the circadian clock. Serotonin (5-HT) receptors that are found within the SCN are utilized to balance phase shifts in response to environmental light.

Due to the SCN sending signals to the pineal gland, melatonin production and suppression can be controlled using the VIP to stimulate adenyl cyclase. Furthermore, when diurnal animals are exposed to environmental light, their melatonin production is decreased by the SCN for the animal to remain awake during the day. Alternatively, in the absence of light, melatonin production is increased and sleep is induced in the animal. Using the idea that environmental light can alter circadian rhythm in phase shifts, the experimental zebrafish will be exposed to abnormal light patterns to cause an altered sleep cycle, mimicking SD that is seen in those with neurodegeneration. Furthermore, by vigorously switching the zebrafish light/dark and sleep/wake cycles, circadian disruption will be induced to alter the SCN and cause neuroanatomical damage[8].

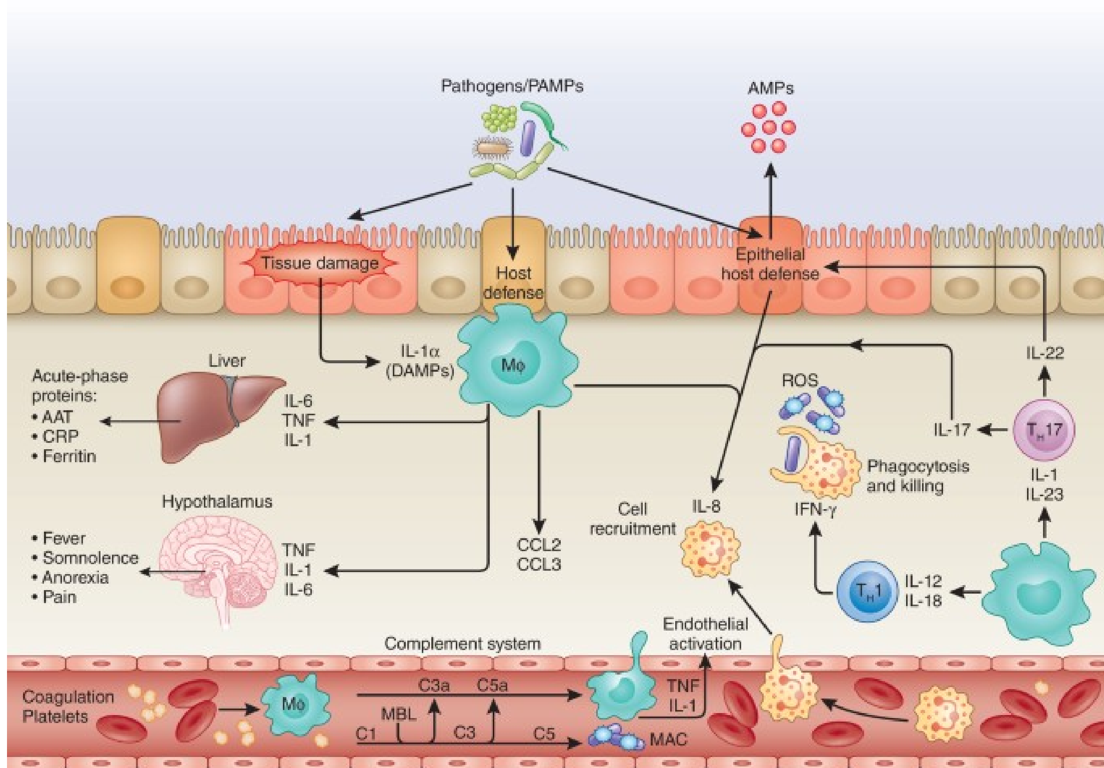
Figure 1. The image below shows the location of the SCN in the brain.
Image Credit: Mckenzie, Samuel. "How Does the Suprachiasmatic Nucleus (SCN) Control Circadian Rhythm?" *News*, 26 Feb. 2019, https://www.news-medical.net/health/How-Does-the-Suprachiasmatic-Nucleus-%28SCN%29-Control-Circadian-Rhythm.aspx.



Bioinformatics Results

There are numerous genes and microRNAs involved in the inflammatory pathway that correlates to neurodegeneration. As seen in Figure 2, inflammatory factors are associated with multiple parts of the body such as the liver and brain.

Figure 2. The figure below illustrates the inflammatory pathway and the genes associated. Image Credit: Netea, Mihai G, et al. “A Guiding Map for Inflammation.” *Nature News*, Nature Publishing Group, 19 July 2017, https://www.nature.com/articles/ni.3790.



A seed region plays a vital role in the process of miRNA binding to mRNA. It is seen as a conserved heptametrical sequence sitting in the position 2-7 in correspondence to the miRNA 5’ end[9]. Using the software TargetScan, seed regions in the human, mouse, and zebrafish were found and added into lists on the website Bioinformatics & Evolutionary Genomics. When the extensive list for each organism type was added, a Venn Diagram was made (see Figure 3). This software found 86 seed regions in common with all three organisms. These seed regions were then correlated to their microRNAs on Excel and an analysis was performed.

TargetScan was used again to find the microRNAs associated with the genes interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP) and

IL-6B. When analyzing chronic obstructive pulmonary disease (COPD), the systemic inflammatory factors IL-6, TNF-α, and CRP displayed repeatability and associations with each other with a high degree of statistical significance[10]. These inflammatory factors were grouped together based on this previous literature showing correlation. Each list of microRNAs was added into Bioinformatics and Evolutionary Genomics and a Venn Diagram was made (see Figure 4). With this, one microRNA was found to be in common; miRNA family miR-149-5p.

This process was repeated using genes IL-6, IL-8, IL-22, and IL-10 and a Venn Diagram was created (see Figure 5). These genes were grouped together to cross-examine all of the interleukin genes associated with inflammation. Using this data, the microRNAs associated with all four genes were miR-5692a and miR-4729. Lastly, the genes nucleotide-binding domain leucine-rich containing family, pyrin domain–containing-3 (NLRP3), Chemokine CCL3, and Chemokine CCL2 were analyzed and added to a Venn Diagram (see Figure 6). The human chemokines CCL3 and CCL2 are upregulated in both primary pancreatic ductal adenocarcinoma (PDA) carcinoma and PDA liver metastasis which promotes NLRP3-mediated T cell priming[11]. This cascade causes a direct effect on the immune checkpoint therapy response. Due to this relationship, these three genes were grouped together for analysis. The microRNAs associated with all three genes were found to be miR-5692a, miR-548e-5p, miR524-5p, and miR-520d-5p.

Lastly, to understand the correlation between three neurodegenerative diseases, my previously published article (*MicroRNA Networks in Cognition and Dementia*[12]) was utilized and the neurodegenerative diseases AD, DLB, and CJD were further analyzed. The microRNAs associated with each type of disease were found, using the published

article, to examine for correlation. A Venn Diagram was created to analyze the correlation between each disease; with this, the microRNA let-7i-5p was found in each type (see Figure 7).

Figure 3. The Venn Diagram below illustrates the shared microRNAs between human, mouse, and zebrafish.

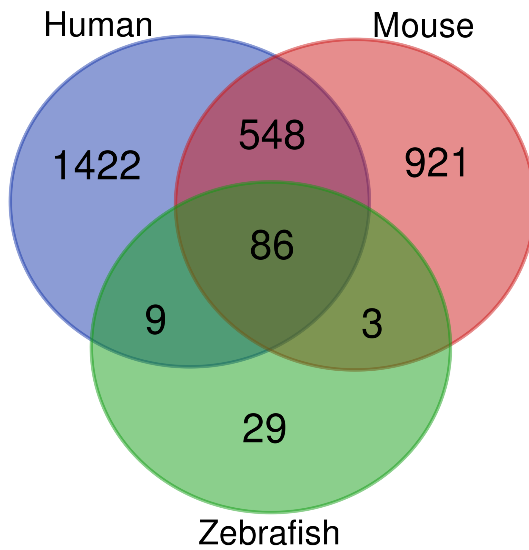


Figure 4. The Venn Diagram below illustrates the shared microRNAs between inflammatory genes IL6, IL6B, CRP, and TNFA.

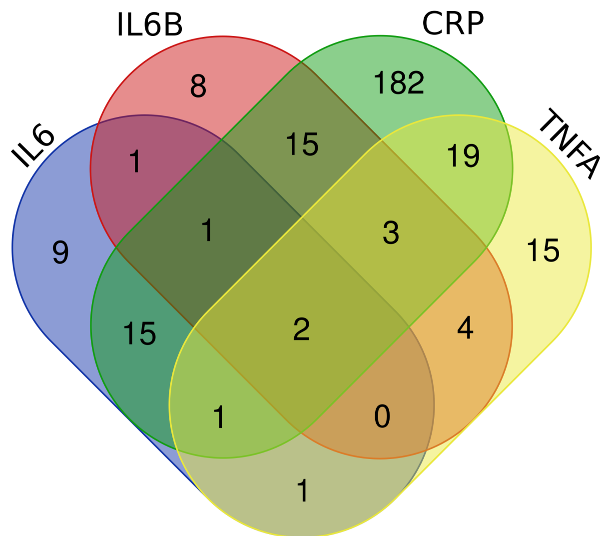


Figure 5. The Venn Diagram below illustrates the shared microRNAs between inflammatory genes IL6, IL8, IL22, and IL10.

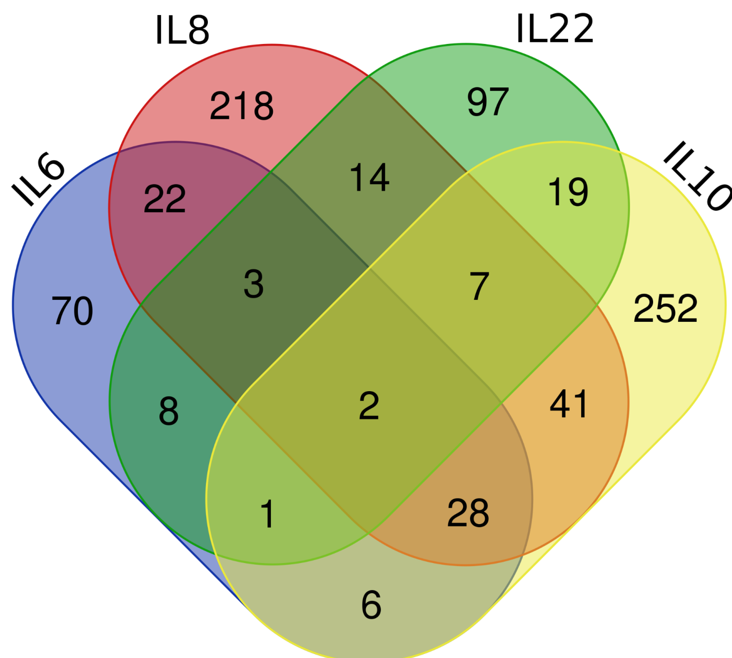


Figure 6. The Venn Diagram below illustrates the shared microRNAs between inflammatory genes NLRP3, CCL3, and CCL2.

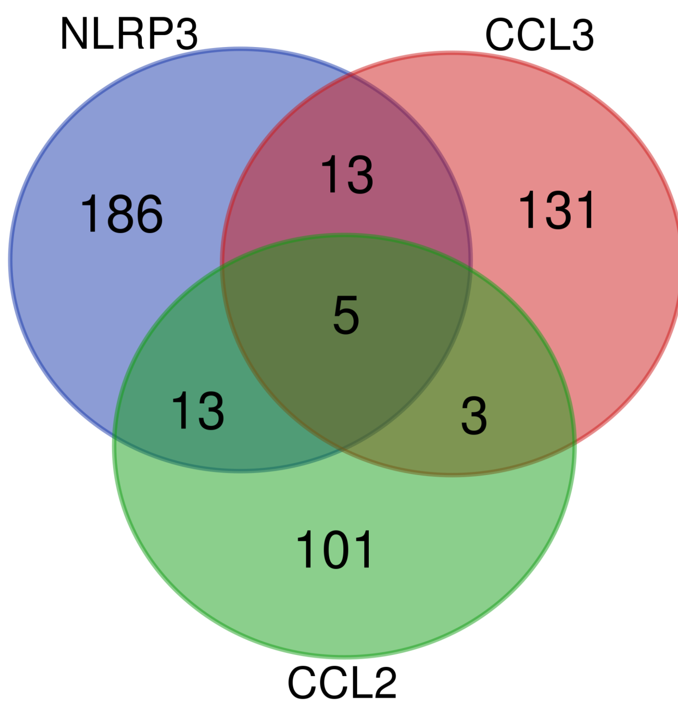


Figure 7. The Venn Diagram below illustrates the shared microRNAs between the neurodegenerative diseases AD, DLB, and CJD.

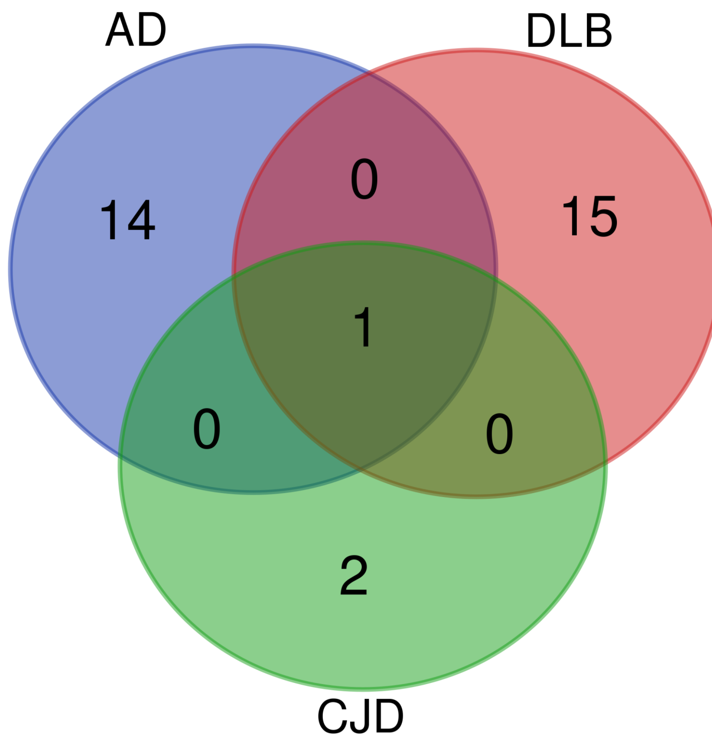
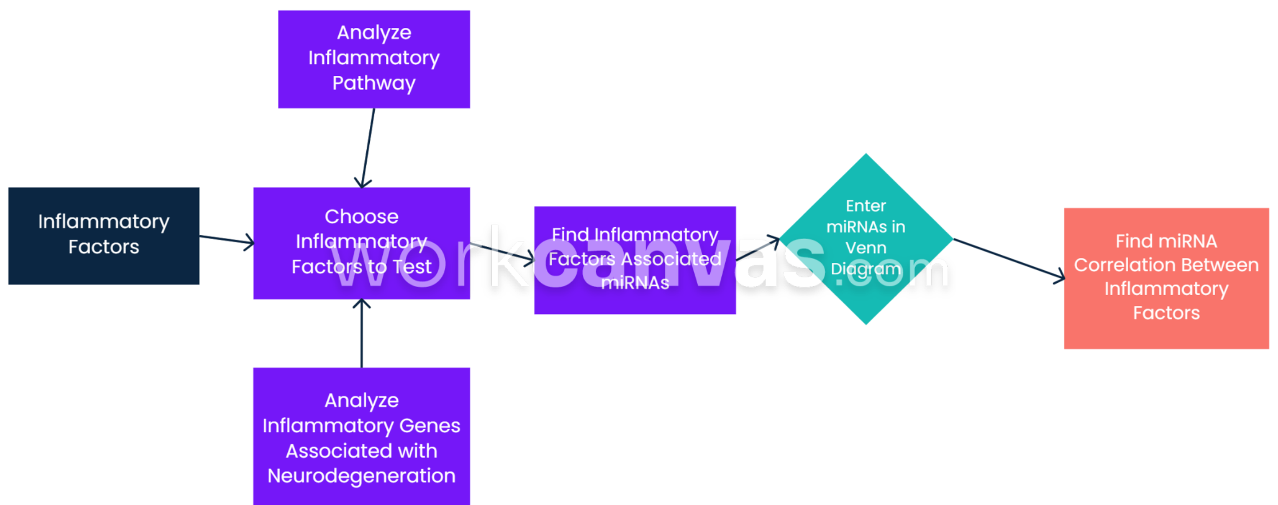


Figure 8. The flow chart below shows the steps used to analyze the inflammatory factors and their associated miRNAs.



Procedure of Zebrafish Dissection

To analyze the effects of sleep deficiency on zebrafish, a group of control fish and a group of experimental fish were created. Each group of fish (2) were placed in a saltwater tank inside of a black box with lights on the inside. All fish had a 96 hour acclimation period in the tanks with lights on at 06:00 and off at 20:00. After the first 96 hours in the tank, the control fish continued to have the lights on at 06:00 and off at 20:00 while the experimental fish were exposed to longer periods of light throughout the time period of 20:00 to 06:00. This pattern of altered light was conducted for a period of seven days. On the eighth day, the fish were removed from the tank and they were dissected.

Each fish was removed separately and placed in an anesthesia solution consisting of 180 mL water and 20 mL tricaine. The time it took for the fish to be anesthetized was determined by pinching the pectoral fin. When the fish showed no reaction to the pinching, they were removed from the solution and put on a dissection tray. Once on the dissection tray, the fish head was removed and placed in 50 mL of cerebral spinal fluid (CSF) solution that was on ice under the microscope. The CSF solution was created using concentrations seen in Table One**.** A small sponge was immersed in CSF solution and placed in the same 50 mL CSF solution on ice. The head of the fish remained in the sponge for the entire dissection of the brain. Once the brain was removed, it was placed in a separate tray to dissect the olfactory bulbs, telencephalon, and optic tectum. The dissection time was recorded for each fish. Each part of the brain was placed in a labeled 0.5 mL microcentrifuge tube which was immediately dropped in liquid nitrogen to be flash frozen. The samples were stored at -80 ℃. The pectoral fin was then placed under a microscope to determine the sex of the fish.

Table 1. The table below shows the light schedule for the altered light (experimental) fish over a 12 day period. This was constructed by Carolyn Cohen and Dr. Robert Mans.

Day of Week	Time Lights On	Time Lights Off	Protocol Type
Monday	06:00	20:00	Acclimate
Tuesday	06:00	20:00	Acclimate
Wednesday	06:00	Thursday 14:00	Acclimate/Protocol 1
Thursday	22:00	Friday 14:00	Protocol 1
Friday	22:00	Saturday 14:00	Protocol 1
Saturday	22:00	Sunday 14:00	Protocol 1
Sunday	22:00	Monday 14:00	Protocol 1
Monday	22:00	Tuesday 10:00	Protocol 1/2
Tuesday	15:00	Wednesday 10:00	Protocol 2
Wednesday	15:00	Thursday 10:00	Protocol 2
Thursday	15:00	Friday 10:00	Protocol 2
Friday	15:00	Dissection time	Protocol 2

Figure 9. The image below shows the dissected zebrafish brain with the labeled olfactory bulbs, telencephalon, and optic tectum.

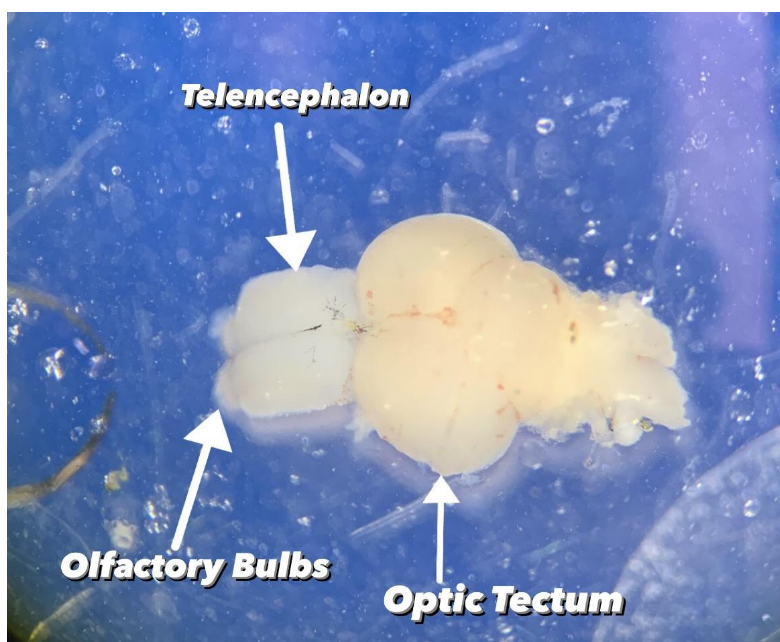


Figure 10. The image below shows the light box the fish were kept in. The set of experimental fish were in one box and the controls were in another.

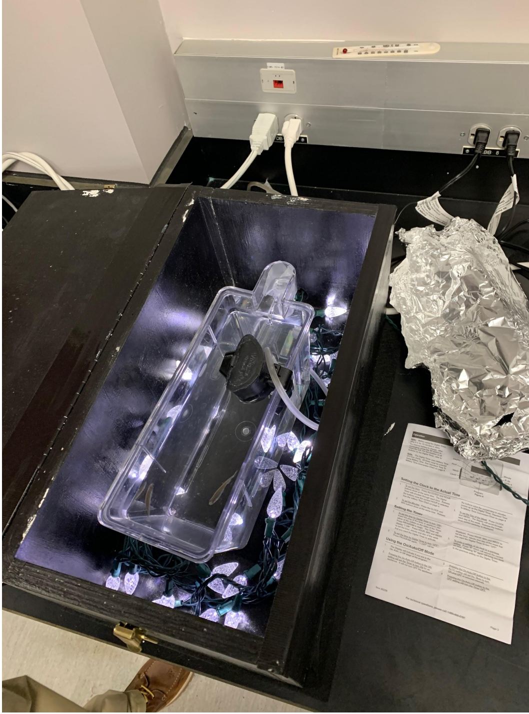


Figure 11. The image below shows the pectoral fin of the zebrafish for identification of sex. The image below represents a female zebrafish.

Table 2. The table below shows the compound used to create Cerebral Spinal Fluid (CSF) concentration from Dr. Robert Mans.

Component	Concentration (mM)	Mass for 1L (g)	Mass for 500 mL (g)	Catalog #	Price ($)
Sodium Chloride	120	7.01	3.505	S9888-500	37.00
Potassium Chloride	3.5	0.260	0.130	P3911-25G	33.00
Calcium Chloride Dihydrate	2.0	0.296	0.148	C5080-500G	65.00
Magnesium Sulfate Heptahydrate	1.3	0.32	0.16	M1880-500G	42.20
Magnesium Chloride Heptahydrate	1.3	0.32	0.16	M2670-100G	33.10
Sodium phosphate monobasic monohydrate	1.25	0.175	0.088	S9638-25G	34.00
Sodium Bicarbonate	26	2.18	1.09	S6014-500G	40.00
Glucose	11	2.178	1.089	G8270-KG	39.80

Conclusion/Future Studies

By altering the circadian rhythm of zebrafish and causing a disruption in the SCN portion of the brain, SD can be mimicked. Neurodegeneration in the form of Dementia, AD, HD, CJD, VD, DLB, and PD can cause many forms of ailments, including SD. Using the zebrafish with altered circadian rhythms, a miRNA analysis could be done on dissected brain sections to analyze the dysregulation of specific miRNAs of interest. The

information collected from Target Scan indicates that the following miRNAs may be of particular interest and could be targeted in a miRNA analysis: miRNA family miR-149-5p, miR-5692a, miR-4729, miR-5692a, miR-548e-5p, miR524-5p, miR-520d-5p, and miRNA let-7i-5p. By running an experimental analysis on the dissected brain tissue and targeting specific miRNAs, a set of miRNAs could be found that are consistently dysregulated. These miRNAs could then be further tested using human samples and then targeted in those who have a predisposition for neurodegeneration. Identifying miRNAs that are dysregulated in those with neurodegeneration could help in early diagnosis of the disease.

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