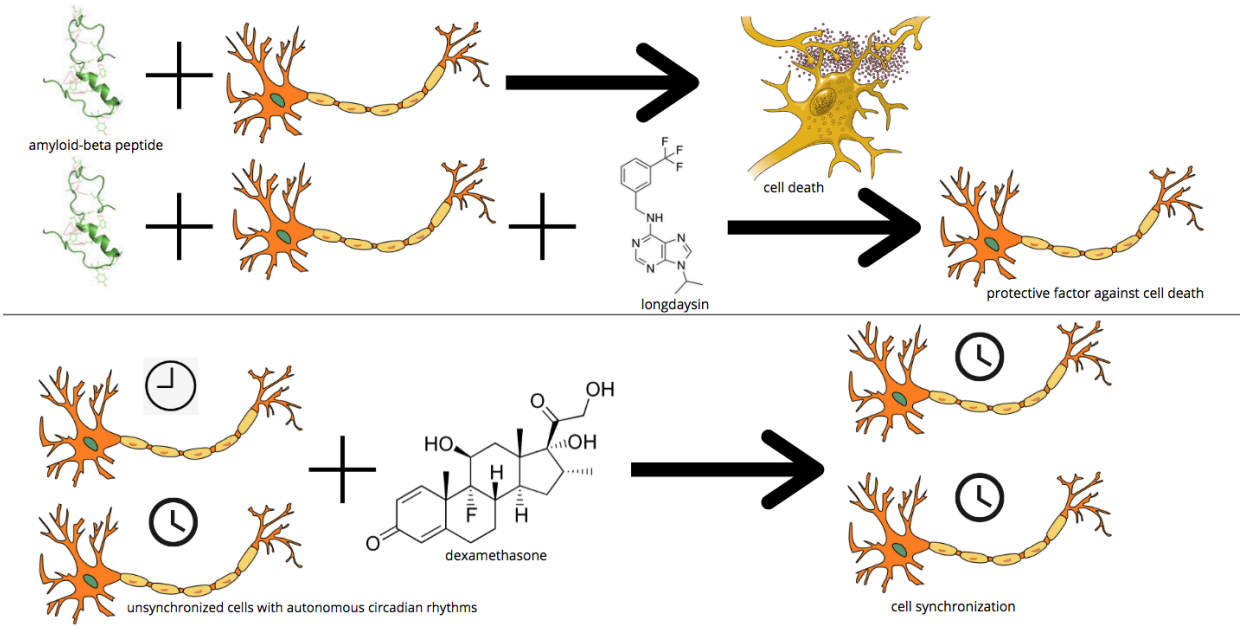
Novel Dexamethasone-based Neuronal Cell Synchronization and the Future of Circadian Rhythm-based Alzheimer's Disease Treatment

Abstract

Alzheimer's Disease (AD) is closely linked to circadian rhythm alterations. Not only does AD contribute to circadian rhythm alterations, but interruptions in the natural biological clock can have further deleterious effects on the progression of the disease. There is not yet knowledge on utilizing circadian rhythm modulators as a treatment for AD. In this experiment, longdaysin, a circadian clock lengthener, was tested as a possible treatment for AD-induced cell death. This paper aims to find a link between AD pathology and circadian rhythm, so that future AD treatments could focus specifically on circadian rhythm therapies. Specifically, the experiment examined how amyloid-beta peptide affects cell death of neurons and if longdaysin could reverse those effects. Cells were also treated with amyloid-beta peptide and longdaysin, and cell death was measured using the trypan blue exclusion method. The results indicated no significant difference in cell death rates or cell survival rates between all three groups- indicating that the treatments of amyloid-beta and amyloid-beta with longdaysin did not induce nor prevent cell death. A lack of significant difference in cell death between untreated and amyloid-beta treated cells was surprising; amyloid-beta peptide 1-40 is known to have a neurotoxic effect in mature neuron cells and is a key part of AD pathogenesis. To verify these results, an increased dosage or an increased treatment period should be conducted. An experiment in neuronal cell synchronization using dexamethasone was also conducted. Cell synchronization is a crucial step in measuring circadian rhythm gene expression in vitro. Each cell has its own autonomous circadian rhythm, which are each firing at different cycles and time intervals, making it impossible to measure gene expression. Cell synchronization effectiveness was measured using RT-PCR with the BMAL1 circadian gene. The results indicated dexamethasone as an effective circadian synchronizer in neuronal cell lines, using BMAL1 as an indicator.

Graphical Abstract



Introduction:

Alzheimer's Disease (AD) is a progressive neurodegenerative condition most prevalent in older adults, accounting for 70% of all dementia cases[1] and affecting an estimated 44 million people globally. The severity and onset of symptoms differs on a case-by-case basis, but most patients present with symptoms including memory loss, confusion, and behavioral changes. The disease begins in a mild stage, then progresses to moderate then severe Alzheimer's Disease, ultimately culminating in death an average 4 to 8 years after diagnosis.

Pathologically, brains affected with Alzheimer's exhibit neurofibrillary tangles (NFTs) and amyloid-beta plaques. These are masses of tau protein and amyloid-beta protein respectively, that accumulate in the brain, particularly in the hippocampus. In addition, some patients develop Lewy bodies, accumulations of alpha-synuclein proteins. While the exact mechanism of AD-induced cognitive decline is unknown, it is believed that these aggregates accumulate and cause apoptosis and atrophy in brain tissue and a decline of neuron and synapse firing. In the leading hypothesis, called the amyloid hypothesis, the accumulation of amyloid-beta (Aβ) peptides in large insoluble plaques is believed to be the driving cause of AD neuron death and neurological decline.

Of particular interest, AD patients are known to exhibit alterations in the sleep-wake cycle. These include difficulties sleeping and "sundowning," an umbrella term describing an exacerbation of confusion or agitation of AD patients in the late afternoon or nighttime, suggesting circadian rhythm disruptions. In some extreme cases of late stage AD, patients experience a complete reversal in regular sleep-wake cycles, sleeping primarily during the daytime and staying awake during the nighttime. Sleep disturbances also are considered a risk factor for the development of AD and may accelerate the progression of the disease. Some of the earliest onset symptoms of AD are sleep interruptions or irregularities, even before hallmark symptoms such as memory loss[2]. Amyloid-beta was found to have a direct disruptive effect on BMAL1 and PER2 (circadian clock gene) expression patterns and on CBP (CREB-binding protein, a circadian clock regulator)[3]. Amyloid-beta and tau are also known to inhibit orexin and adenosine A1R, neuropeptides that play an important role in sleep cycle regulation and sleep-wake systems, suggesting that these proteins directly contribute to sleep disruptions in AD patients[4]. These sleep disruptions create a vicious cycle in AD; sleep deprivation and sleep disturbances have been found to increase Aβ peptide production levels and amyloid beta

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[1] Kametani, Fuyuki, and Masato Hasegawa. "Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease." *Frontiers in neuroscience* vol. 12 25. 30 Jan. 2018, doi:10.3389/fnins.2018.00025

[2] Sterniczuk, Roxanne et al. "Sleep disturbance is associated with incident dementia and mortality." *Current Alzheimer research* vol. 10,7 (2013): 767-75. doi:10.2174/15672050113109990134

[3] Song, Hyundong et al. "Aβ-induced degradation of BMAL1 and CBP leads to circadian rhythm disruption in Alzheimer's disease." *Molecular neurodegeneration* vol. 10 13. 19 Mar. 2015, doi:10.1186/s13024-015-0007-x

[4] Liu, Z., Wang, F., Tang, M., Zhao, Y., Wang, X."Amyloid β and tau are involved in sleep disorder in Alzheimer's disease by orexin A and adenosine A(1) receptor ". International Journal of Molecular Medicine 43.1 (2019): 435-442.

plaque formation[5], while at the same time, amyloid-beta plaque accumulation causes a deterioration in sleep-wake cycles[6].

As of now, there are no known cures for AD. Treatments are typically symptomatic or aim to improve cognition. For mild to moderate AD cases, patients are often prescribed cholinesterase inhibitors, aiming at easing behavioral and cognitive symptoms. In moderate to severe onset AD patients, N-methyl D-aspartate (NMDA) antagonists are administered, which improve symptoms by regulating glutamate production, which in large concentrations, can induce cell death. Recently, a disease-modifying drug was approved by the U.S. Food and Drug Administration (FDA), called aducanumab, an antibody targeting amyloid-beta proteins. Treatments specifically for AD-related sleep changes include antidepressants, antipsychotics, and benzodiazepines. However, drugs are often regarded as a last resort for sleep issues, as they have possible deleterious side effects on patients, such as stroke, confusion, suicidal thoughts, and sleep paralysis.

Recently, a substance called longdaysin was found to have the ability to slow down the biological clock. Specifically, longdaysin works as a casein kinase inhibitor. It has been touted as a possible treatment option for sleep disorders, by regulating sleep cycles and lengthening the circadian clock. In this experiment, we tested if treatment with longdaysin reduced amyloid-beta induced cell death, as an indicator if circadian clock disruptions are directly linked to the pathogenicity of Alzheimer's Disease. This experiment tested whether circadian rhythm-based treatments could be viable options for AD.

Cell synchronization is a crucial step in measuring circadian rhythm gene expression in vitro. Each cell has its own autonomous circadian rhythm, which are each firing at different cycles and time intervals, making it impossible to measure gene expression. Dexamethasone, a glucocorticoid, has been used to synchronize human skin fibroblasts[7] and human breast epithelial cell lines[8], however, has not been investigated in regard to neuronal cell line synchronization. In this experiment, we tested dexamethasone as a circadian clock synchronizer in mouse hypothalamic neuronal cell lines, using BMAL1 gene expression as an indicator.

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[5] Lucey, Brendan P et al. "Effect of sleep on overnight cerebrospinal fluid amyloid β kinetics." *Annals of neurology* vol. 83,1 (2018): 197-204. doi:10.1002/ana.25117

[6] Roh, Jee Hoon et al. "Disruption of the sleep-wake cycle and diurnal fluctuation of β-amyloid in mice with Alzheimer's disease pathology." *Science translational medicine* vol. 4,150 (2012): 150ra122. doi:10.1126/scitranslmed.3004291

[7] Schmitt, Karen et al. "Amyloid-β-Induced Changes in Molecular Clock Properties and Cellular Bioenergetics." *Frontiers in neuroscience* vol. 11 124. 17 Mar. 2017, doi:10.3389/fnins.2017.00124

[8] Ndikung, Johanna et al. "Restoring circadian synchrony in vitro facilitates physiological responses to environmental chemicals." *Environment international* vol. 134 (2020): 105265. doi:10.1016/j.envint.2019.105265

Materials/Methods

Cell Culture and Dosage

Mouse hypothalamic neuronal cell lines, mHypo3-39, were acquired from Cedarlane-CELLutions Biosystems Inc. They were cultured at 37°C with 5% CO_2 in DMEM. A total of 18 wells in two plates were plated 24 hours prior to initial dosing. In one plate, 3 wells remained undosed, while the other 6 were dosed with 10 uM amyloid-beta peptide. Rat Amyloid-beta peptide 1-40, in a stock solution with a concentration of 1 mg/mL, was given to 6 wells at 42.3 uL per well. These wells were placed in the incubator for 24 hours. Of these wells, after 24 hours, 3 were dosed with 100 uM longdaysin. Longdaysin, which came in 5 mg of powder, was made into a solution with 1.5 mL DMSO, and dosed to the three wells at 10 uL per well.

In the second plate, 3 wells remained untreated, while the other 6 wells were dosed with 1 uM dexamethasone. Dexamethasone, in a stock solution with a concentration of 40 mg/mL, was dosed at 9.8 uL per well. The treated wells were incubated for 1 hour, after which 3 of the dexamethasone-treated wells were combined into one sample for RNA extraction, whilst the remaining 3 dexamethasone-treated wells were aspirated with dexamethasone being replaced by media and incubated for 20 hours. After 20 hours, these three wells, along with the remaining three untreated wells in the plate, were extracted for RNA.

RNA Extraction

Of the 18 wells, 9 were extracted for RNA in order to determine the synchronizing effects of dexamethasone on mHypo3-39 cells. These 9 wells were split in three groups, with three wells each: dexamethasone-treated cells, extracted immediately after treatment; dexamethasone-treated cells extracted 20 hours after treatment; and untreated cells, extracted together with the 20-hour time point cells. The RNA extraction procedure was conducted as follows. Media was extracted from 3 wells, using a pipette, into a conical tube, where it was spun in a centrifuge at 1100 rpm for 5 minutes. Media was immediately aspirated, leaving only a cell pellet. In addition, 350 uL RLT buffer was placed into the aspirated wells, where they were scraped down with a cell lifter, and pipetted into an Eppendorf tube. The cell suspension was then transferred to the conical tube with the cell pellet and pipetted to mix and vortexed. The cell lysate was passed through a 20-gauge needle with a 1mL syringe 5 times, then transferred into an AllPrep DNA spin column in a 2mL collection tube and centrifuged at 10,000 rpm for 15 seconds. The spin column and flow through was then discarded, and 350 uL 70% ethanol was added to the collection tube and transferred into a RNeasy spin column in a 2 mL collection tube and spun at 10,000 rpm for 15 seconds. The same centrifugation process was then conducted with 700 uL RW1 buffer and 500 uL RPE buffer. 500 uL RPE buffer was added to the spin column and centrifuged at 10,000 rpm for 2 minutes. The spin column was then placed into a new 2 mL collection tube and centrifuged at 13,400 rpm for 1 minute. The spin column was placed into a new 1.5 mL collection tube, and 30 uL RNase-free water was added, and centrifuged for 1 minute at 10,000 rpm. RNA was quantified using a NanoDrop spectrophotometer. RNA concentrations were as follows: control RNA, 6.3 ng/uL; dexamethasone RNA extracted 0 hours after treatment, 8.8 ng/uL; and dexamethasone RNA extracted 20 hours after treatment, 19.4 ng/uL.

Reverse Transcription, Polymerase Chain Reaction, and Gel Electrophoresis

RNA primer reactions were prepared in three PCR tubes as follows; 14 uL of control RNA with 1 uL random primer; 10 uL of 0 hour extracted dexamethasone RNA with 4 uL nuclease-free water and 1 uL random primer; 4.54 uL of 20 hours extracted dexamethasone RNA with 9.46 uL nuclease-free water and 1 uL random primer. These three samples were held at 70°C for 5 minutes and chilled in ice immediately after. Reverse Transcription mix was prepared using 22 uL nuclease-free water, 16 uL GoScript 5x Reaction Buffer, 12 uL $MgCl_2$, 4

uL PCR Nucleotide Mix, 2 uL RNasin Ribonuclease Inhibitor, and 4 uL GoScript Reverse Transcriptase. Three new PCR tubes were prepared, with 15 uL of the Reverse Transcription mix and 5 uL of one of three RNA/Primer mixes, and placed in a thermocycler at 25°C for 5 minutes, 42°C for 1 hour, 70°C for 15 minutes, and held at 4°C.

The target gene was BMAL1, in order to indicate circadian rhythm synchronization. Two BMAL1 mouse primers were used for PCR.
Primer 1:
Forward: AATAGGTCAGGGACGGAGG
Reverse: GCATTCACTGCAGCCAACAA
Primer 2:
Forward: TGCGACATTTAGGGAAGGCA
Reverse: AGGGAACCGGAGAGTAGGTC

The PCR Reaction mix was made with 50 uL GoTaq Green, 2 uL of the forward primer and 2 uL of the reverse primer, and then vortexed and quick centrifuged. Three new PCR tubes were prepared, each with 9.5 uL nuclease-free water, 13.5 uL of the PCR Reaction mix, and 2 uL of one of three cDNA samples created from reverse transcription. These tubes were placed in a thermocycler at 94°C for 3 minutes. Then, it cycled at 94°C for 45 seconds, 56°C for 45 seconds, 72°C for 1 minute, and repeated 40 times. Then, it was held at 72°C for 7 minutes and then at 4°C.

A DNA gel box was prepared with 0.5x TBE buffer. 15 uL of each of 3 PCR samples (control, dexamethasone at 0 hours, dexamethasone at 20 hours) with primer 1 and with primer 2 were loaded, along with 3 uL DNA ladder. Gel was run at 100 uL for 30 minutes and photographed under a Doc-It system.

Cell Counting

Forty-eight hours after treatment with amyloid-beta peptide and 24 hours after treatment with longdaysin, cells were counted using trypan blue exclusion. Media was aspirated and 1000 uL HBSS was used to rinse away remaining media, and then discarded. 200 uL trypsin was added to each well and incubated for 5 minutes. Cells and trypsin were placed into Eppendorf tubes, and wells were washed with 200 uL DMEM, then added into the same Eppendorf tubes. Tubes were centrifuged for 1 minute at 1000 rpm. Supernatant was removed from the tube, and cell pellets were resuspended as follows. The control cell pellets were resuspended in 100 uL media, the amyloid-beta cell pellets in 40 uL, and amyloid-beta and longdaysin cell pellets in 100 uL media. These suspensions were mixed by pipetting.

10 uL trypan blue was prepared in an Eppendorf tube. 10 uL of cell suspension was added to the tube and mixed. 10 uL of the trypan blue cell suspension mixture was pipetted into one chamber of a hemocytometer. Live and dead cells were counted under a microscope using a 10x objective, counting cells within a square or on the left/bottom boundary lines.

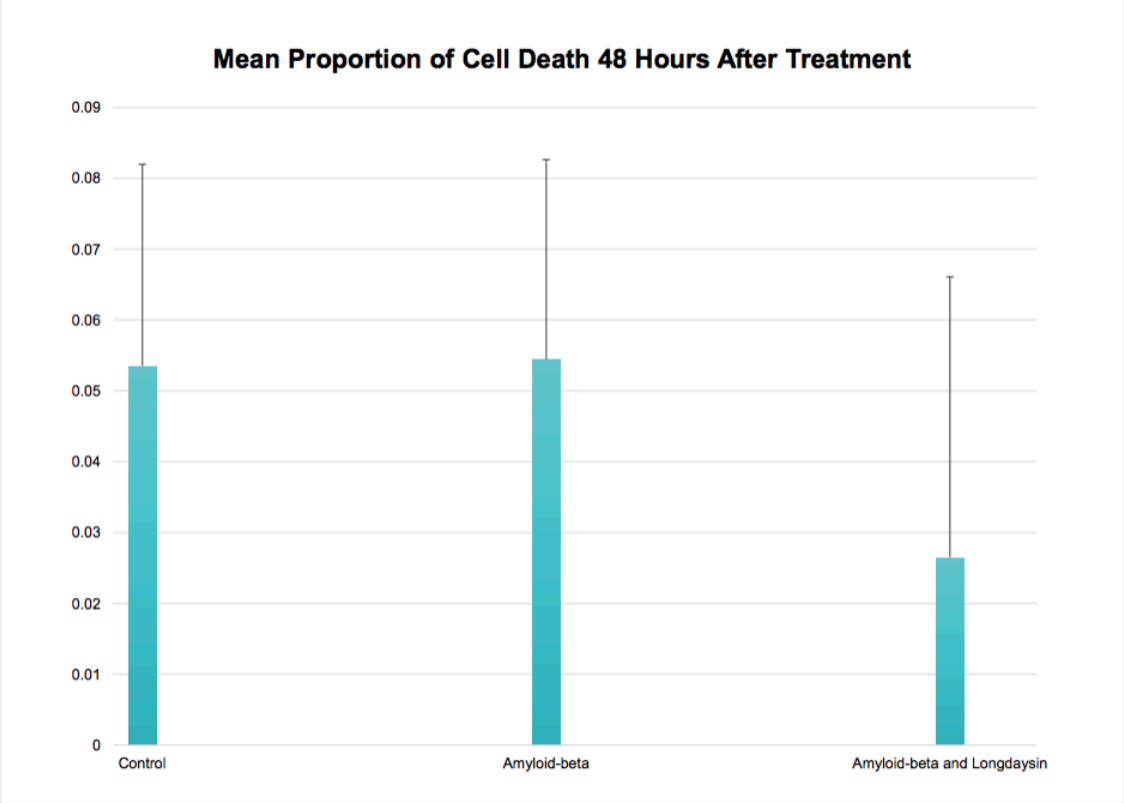
Image Acquisition

Images were taken of both plates prior to dosing, dexamethasone-treated cells 24 hours after dosage, amyloid-beta treated cells 24 hours after dosage, and amyloid-beta treated and amyloid-beta with longdaysin treated cells 48 hours after a-beta dosage, and 24 hours after longdaysin dosage. Images were taken using a screen-capture function on an Olympus CKX41 microscope on a 4x objective.

Results

Cell Counting

Figure 1



Error bars denote standard deviation

Table 1

Cell viability counts at 48 hours after treatment (24 hours after Longdaysin)

		Alive	Dead
Control	1	46,500	3000
	2	88,000	2000
	3	41,500	3500
Amyloid-beta			
	1	33,600	3200
	2	36,600	1400
	3	14,600	600
Amyloid-beta and Longdaysin			
	1	65,500	500
	2	58,000	4500
	3	72,500	0

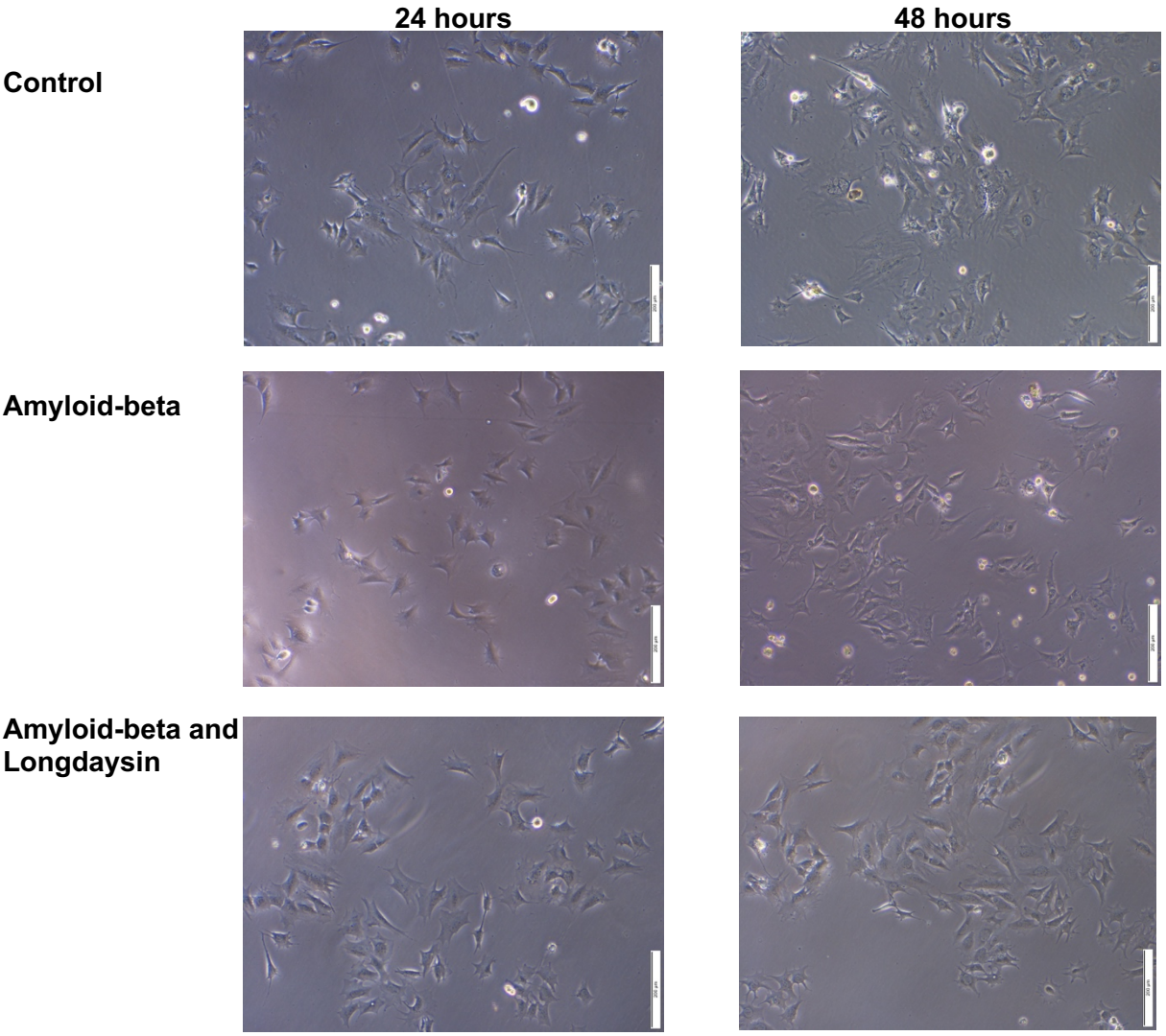
Table 2

Cell death rates at 48 hours after treatment (24 hours after Longdaysin)

	Control	Amyloid-beta	Amyloid-beta and Longdaysin
1	0.06060606061	0.08695652174	0.007575757576
2	0.02222222222	0.03684210526	0.072
3	0.07777777778	0.03947368421	0
Mean	0.05353535354	0.05442410374	0.02652525253
Standard Deviation	0.02844470271	0.02820460898	0.03956403062

Figure 2

Images showing neuronal cells at 24 hours and 48 hours post treatment



BMAL1 Gene Expression

Figure 4

Gel electrophoresis imaging, with target wells highlighted

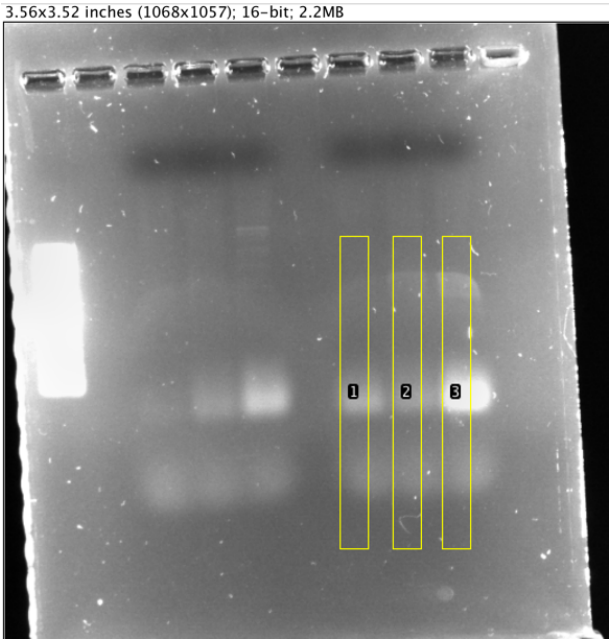
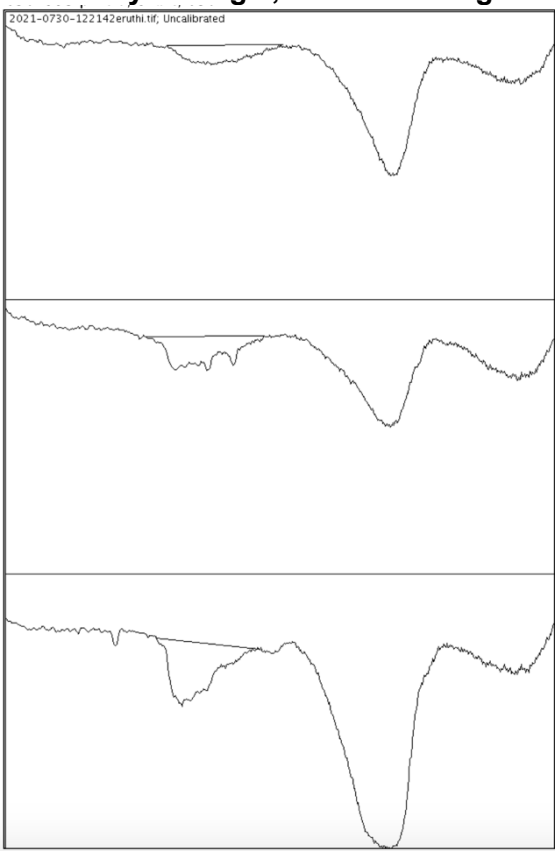


Figure 5

Plot analysis of gel, with areas of gene expression interest sectioned off



The well labelled 1 represented BMAL1 expression in dexamethasone treated cells extracted at 0 hours, well 2 represented BMAL1 expression in dexamethasone treated cells extracted at 20 hours, and well 3 represented BMAL1 expression in untreated control cells. Primer 1 did not display results so bands from primer 2 were analyzed instead.

Gel electrophoresis results were analyzed with densitometry using ImageJ software. Dips in the plot represent areas of higher density- and going from left to right in the plot represents going from top to bottom in the gel. The primer had a length of 592- the areas of gene expression interest were sectioned off in the plot and quantified using the ImageJ plot analyzer.

Table 3

Area of sections of gene expression levels of interest

	Area
1 (Dexamethasone-treated, extracted at 0 hours)	1387.397
2 (Dexamethasone-treated, extracted at 20 hours)	2627.246
3 (untreated control)	3833.368

Area designated pixels in the image and the density of the bands; higher density indicated greater gene expression levels.

Discussion

Cell Death

A two-tailed unpaired t-test was conducted based on these results. The results indicated no significant difference in cell death rates or cell survival rates between all three groups- indicating that the treatments of amyloid-beta and amyloid-beta with longdaysin did not induce nor prevent cell death. A lack of significant difference in cell death between untreated and amyloid-beta treated cells was surprising; amyloid-beta peptide 1-40 is known to have a neurotoxic effect in mature neuron cells and is a key part of AD pathogenesis. To verify these results, an increased dosage or an increased treatment period should be conducted.

However, the magnitude of the difference between longdaysin treated cells and amyloid-beta/control cells was sizeable and in the expected direction. Longdaysin treatment exhibited a lower mean proportion of cell death compared to both control and amyloid-beta treated groups. This indicates a possible protective factor of longdaysin against cell death- not necessarily against amyloid-beta peptide, as amyloid-beta peptide did not have a significant effect on cell death rates in this experiment. To further investigate, longdaysin dosage time could be altered- rather than waiting 24 hours after amyloid-beta treatment, it could be immediately dosed. In addition, longdaysin treatment could be allowed to sit longer in the cells before cell counting.

Gene Expression

The levels of gene expression recorded were consistent with a synchronized circadian rhythm. BMAL1 endogenous expression levels are known to be at a minimum at 0 hours and peak at 18 hours[8], consistent with our findings of a lower level of expression at 0 hours and an increased level at 20 hours. To confirm these results, we recommend further analysis at a wider

range of timepoints, and to possibly verify the extent to which dexamethasone-induced cell synchronization lasts.

In the future, these two experiments could be combined, and using synchronization of cells using dexamethasone, and measuring circadian rhythm through gene expression, circadian rhythm alterations with amyloid-beta and from longdaysin could be measured.

Determining if circadian rhythm-based treatments for AD are viable in reducing cell death could open up new possible medications or treatments for the disease and could signify a direct link between circadian rhythm alteration and AD pathology and disease-causing mechanisms.

Supplemental Figures

Supplemental Table 1

Cell Survival Rates at 48 hours after treatment (24 hours after Longdaysin)

	Control	Amyloid-beta	Amyloid-beta and Longdaysin
1	0.9393939394	0.9130434783	0.9924242424
2	0.9777777778	0.9631578947	0.928
3	0.9222222222	0.9605263158	1
Mean	0.9464646465	0.9455758963	0.9734747475
Standard Deviation	0.02844470271	0.02820460898	0.03956403062

Supplemental Table 2

Calculated p-values of differences between mean proportions of cell death

Control vs Amyloid-beta	0.9711872654
Amyloid-beta vs Amyloid-beta and Longdaysin	0.3762574694
Control vs Amyloid-beta and Longdaysin	0.3913822435