

# Melatonin and Neurogenesis: A Comparative Study of the Efficacy of Melatonin, Its Precursors, and L-Dopa on Neural Stem Cell Metabolism in Human Adult Neurospheres

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MELATONIN AND NEUROGENESIS: A COMPARATIVE STUDY OF THE  
EFFICACY OF MELATONIN, ITS PRECURSORS, AND L-DOPA ON  
NEURAL STEM CELL METABOLISM IN HUMAN ADULT  
NEUROSPHERES

by

OMAR HERIBA

A thesis submitted in partial fulfillment of the requirements  
for the Honors in the Major Program in Biotechnology  
in the College of Medicine  
and in the Burnett Honors College  
at the University of Central Florida  
Orlando, Florida

Fall Term 2014

Thesis Chair: Kiminobu Sugaya, Ph.D.

## ABSTRACT

Human neurosphere stem cells offer promising potential for the treatment of neurodegenerative diseases. Their well characterized multi-potency of differentiating into neurons, astrocytes, and oligodendrocytes when exposed to the optimum exogenous growth factors make them an exciting area of study (38).

Finding novel endogenous methods of modulating stem cell metabolism will allow for the safer treatment of various brain disorders (34). In this experiment, melatonin, N-acetylserotonin, L-tryptophan, and L-DOPA are added in three different concentrations to neurospheres suspended in HNSC/GBM media with less than optimal concentrations of exogenous epidermal growth factor (EGF) and fibroblast growth factor (FGF).

The alamarBlue assay (resazurin) was chosen as the most suitable assay for measuring neurosphere metabolism. Metabolic neural stem cells would cause the greatest reduction of the oxidized alamarBlue reagent (resazurin→resorufin), which was detected by a fluorescent plate reader (39-41). The percent reduction in alamarBlue was calculated for all four molecules at three different concentrations and compared to controls without any molecule.

Our results illustrate that there was no statistically significant difference at  $p < 0.05$  between the biological molecules and the control group except for two exceptions (labeled with asterisks on figures 3 and 5) L-DOPA at a 40 micromolar concentration after 4 hours of incubation and melatonin at a 40 micromolar concentration after 52 hours of incubation.

## **DEDICATION**

For those who suffer from neurodegenerative diseases,

For science and all its potential for alleviating suffering,

And for my family, who have supported me in all my academic endeavors.

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Sugaya for assisting me during the course of my study. I would like to thank Dr. Singla and Dr. Yonetani for assisting me in the preparation of my proposal and thesis.

I would like to thank Mario Pita for acting as my mentor and assistant during our trials and tribulations over the course of this study.

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# INTRODUCTION

## Melatonin

In recent years melatonin has been hailed as a wonder hormone with many positive regulatory effects on the human body. Melatonin is produced in the pineal gland of the brain while melatonin receptors are found in the subventricular zone (SVZ). Although melatonin primarily serves as a circadian regulator in the body, it also influences reproductive habits, regulates body temperature, and helps prevent neuronal death. Melatonin carries out both hormonal and chemical functions with the former involving MT1 and MT2 receptors and the latter occurring at higher than physiological concentrations. It is believed that higher than normal dosages of the hormone trigger additional chemical effects (12).

Melatonin has even been shown to increase stem cell proliferation in embryonic murine brains pointing to its potential utility in treating humans. A study published in 2013 claims that melatonin may improve the function of mesenchymal stem cell proliferation after a cerebral ischemia (34). Luzindole acts as a competitive inhibitor of melatonin and has been shown to decrease the proliferation of the stem cells by separating Gi from adenylate cyclase, further clarifying the role of melatonin in contributing to the increased neural proliferation (35).

Melatonin has also been found to be a free radical scavenger and antioxidant which may serve as a *de facto* manner of reversing the effects of aging (4). A reduction in oxidative stresses takes place due to the up regulation of antioxidant enzymes including superoxide dismutase and

peroxidases and down regulating of pro-oxidant enzymes such as nitric oxide synthetase, lipoxygenase, and quinone reductase (21).

A study by Vanderbilt University (5) published in 2007 found that both synthetic and natural forms of melatonin are among the least toxic substances for humans with the only known negative side effects being increased drowsiness and slower reaction times. The long term effects of melatonin supplementation have not been fully ascertained in humans and the Food and Drug Administration has not yet approved over-the-counter forms of the hormone, despite the fact that it is widely sold by producers and consumed by the general public. Although more research is needed to establish the long-term negative effects of melatonin supplementation, the hormone appears to hold diverse therapeutic potential and minimal risk.

### **N-Acetylserotonin**

Serotonin is a neurotransmitter that is widely used in psychiatric and psychological treatments. Serotonin is normally present in highest concentrations in the peripheral nervous system with a total of fifteen serotonin receptors in the body. These receptors are known as 5-HT receptors and are located on the membranes of numerous types of cells, particularly those of nerve cells. Fourteen of the serotonin receptors are G protein coupled receptors with seven transmembrane subunits that activate secondary messenger molecules. 5-HT is the only exception, as it is a ligand-gated ion channel (31). Some of the lesser known effects of serotonin include changes in cardiovascular function, bowel movements, bladder control, and platelet aggregations. The majority of studies and clinical uses of serotonin involve its psychiatric and psychological effects (6).

A positron emission tomography study, conducted by Perreau-Linck and colleagues on humans, indicated that there is a positive correlation between levels of happiness and serotonin levels and crucially, a negative correlation between sadness and serotonin levels. According to the researchers, the phenomenon that mood can directly alter brain metabolism is not new. Thus, serotonin levels not only influence mood but mood also influences serotonin levels (12). Serotonin levels can be elevated by bright lights and higher levels of exercise, which both serve as examples of natural alternative therapies to artificial drug-based supplementation.

N-acetylserotonin (NAS) is the immediate precursor molecule of melatonin. Previous studies have illustrated that neuronal progenitor cell proliferation was shown to increase in sleep deprived mice when NAS was administered to them (29). NAS is regulated in a circadian rhythm similar to other pineal gland molecules. When administered to adult mice, the N-acetylserotonin had an antidepressant effect and caused the progenitor cell proliferation to take place both in awake and sleeping mice.

## **L-Tryptophan**

Tryptophan is an amino acid that serves as a precursor molecule of serotonin. 5-Hydroxytryptophan is directly linked to serotonin and melatonin production in the body (35). A study published in 1978 claimed that 5-Hydroxytryptophan could reduce the intensity of epileptic seizures in mice. According to the source, blocking the serotonin receptors caused a reduction in audiogenic (AGS) seizures but caused no clear changes in the occurrences of pentylenetetrazol (PTS) seizures. The findings of the study claim that AGS decreases serotonin receptors and PTS increases the receptors i.e. an inversely correlated effect (34).

Despite these early findings regarding tryptophan's role in mediating seizures, the amino acid's effect on depression has been called into question from a study published in 2001. This long-term meta-analysis involving the review of 108 studies of 5-hydroxytryptophan and tryptophan on depression, found that only two of the studies were up to the reviewer's standard, with the reviewer claiming that "the evidence was of insufficient quality to be conclusive," and "because alternative antidepressants exist which have been proven to be effective and safe, the clinical usefulness of 5-hydroxytryptophan and tryptophan is limited at present (32)."

## **L-DOPA**

Dopamine is a monoamine that is sold under several trade names for injection to treat hypotension, bradycardia, shock, and heart attacks. Some of its commonly known physiological effects include: increased sodium excretion by the kidney, increased urine production, increased heart rate, and increased blood pressure. Dopamine acts via the sympathetic nervous system and at higher dosages has been found to cause vasoconstriction and negative side-effects (31).

When it comes to the brain, dopamine is directly involved in modulating motor control, motivation, arousal, cognition, reward, lactation, sexual gratification, and nausea. There are 400,000 dopaminergic neurons in specific parts of the brain with the highest number of dopaminergic cells present in the Ventral Tegmental Area (VTA) of the midbrain (36). The dopaminergic "reward pathways" of the brain include the Ventral Tegmental Area, the Nucleus Accumbens, and the Prefrontal Cortex. The dopaminergic "motor pathways" of the brain include the substantia nigra and the striatum (31).

Dopaminergic cells are also found in the arcuate nucleus and periventricular nucleus of the hypothalamus. These dopaminergic cells influence the pituitary gland and inhibit the secretion of prolactin from the anterior pituitary. Because of this role, dopamine is also sometimes labeled as prolactin inhibiting factor/hormone. Dopaminergic cells are also found in the zona incerta. They are connected to the hypothalamus and control the release of gonadotropin-releasing hormone, which is important for reproductive development (25). Although the function of the dopaminergic cells located in the posterior hypothalamus that connect to the spinal cord are not fully understood, issues in this part of the brain have been linked to restless leg syndrome (28).

Dopaminergic cells also exist in the retina of the eye and are only active during the day. They are responsible for increasing the activity of cone cells and decreasing the activity of rod cells. This causes increases in contrast and sensitivity to color in bright light conditions but a reduced sensitivity in low light conditions. This particular function of dopamine may be related to the day/night effects created by melatonin (34).

## **Neural Stem Cells**

The cells used in this experiment will be neurosphere neural stem cells which have the capability to differentiate into neurons, astrocytes, and oligodendrocytes when stimulated by their exogenous environment. The process we are interested in measuring is the change in metabolism with the addition of melatonin, N-acetylserotonin, L-tryptophan, and L-DOPA; and ultimately the process by which the brain can repair its damaged cells through the self-renewal of cells. The brain is somewhat limited compared to other tissues and organs in its ability to repair

itself and this ability also diminishes with age. Finding novel methods of modulating neural stem cell metabolism will allow for the safer treatment of various brain disorders (34).

It is important to note that various growth factors are needed in order to coax the neurosphere stem cells into differentiation. These primarily include epidermal growth factor (EGF) and fibroblast growth factor (FGF) but may also include others. The neurosphere stem cells must also secrete their own growth factors in response to the culturing medium in order to ensure that neurogenesis takes place at optimal levels (35).

### **Parkinson's Disease**

When it comes to the brain, the neurons in the substantia nigra are especially sensitive to damage. If they are damaged, Parkinson like symptoms starts to affect the individual (20). Parkinson's Disease (PD) is the second most common neurodegenerative disease behind Alzheimer's Disease. It is estimated that 7 million people are afflicted globally, with 1 million of them residing in the United States. Parkinson's disease increases in prevalence based on increasing age, with 1 percent prevalence in those over 60 years of age and 4 percent prevalence in those over 80 years of age (16). There are numerous theories on what exactly triggers Parkinson's disease but many of those studies are inconclusive or contradictory including hypotheses stipulating that the disease is more common in men than women or that it is less prevalent among those with Asian or African ancestry (16).

## **Alzheimer's Disease**

Alzheimer's disease is the most common neurodegenerative disease. Like Parkinson's Disease, Alzheimer's disease increases in prevalence as the age of the individual increases. Every five years after the age of 65, the risk of acquiring the disease approximately doubles, increasing from 3 to as much as 69 individuals per thousand per year (27). Alzheimer's disease is believed to be more common in women, particularly those over the age of 85 (17,5). Alzheimer's disease is also more prevalent in industrialized countries (18).

## **Blood-Brain Barrier**

The blood brain barrier (BBB) is a physiological entity designed to protect the brain with a separation of blood circulation and the brain extracellular fluid (BECF). It is composed of tight junctions located along the capillaries of the central nervous system (23). Endothelial cells of the barrier limit the diffusion of microscopic, large, or hydrophilic molecules into the cerebrospinal fluid, and allow the diffusion of small hydrophobic molecules (29). When it comes to larger metabolic molecules such as glucose, active transport is used to push them across the barrier (21). The BBB ensures that the brain has a greater level of protection from infection, whilst also allowing essential nutrients to pass through and nourish the brain.

There are challenges associated with neurotransmitter monoamines and crossing the blood brain barrier. Researchers often categorize the blood brain barrier into what they term the "morphological barrier" and the "enzyme barrier". Hypertensive or hypertonic stimuli can be used to temporarily increase the permeability of the barrier. High concentrations of monoamines

(derived from tryptophan) can open the “morphological barrier”, but this is only an indirect result of an acute rise in systemic blood pressure. The increase in monoamine concentration causes changes in metabolism and blood pressure in the brain (20).

## **HYPOTHESIS**

We expect the L-DOPA to have the greatest effect on neurosphere neural stem cell metabolism due to the fact that it is a neurotransmitter with crucial reward centric functions in the central nervous system; particularly the brain. In addition, L-DOPA is administered as a treatment for Parkinson's disease and dopamine responsive dystonia, both of which involve damage to dopamine receptors in the brain. Melatonin's primary function is circadian regulation, and it is capable of crossing the blood brain barrier. It has been suggested that melatonin has widespread effects in the body including but not limited to regulating sleep, mood, and reproductive patterns. We expect melatonin to cause a greater effect on the neurosphere stem cells in comparison to its precursor molecules N-acetylserotonin and L-tryptophan.

## METHODS AND MATERIALS

### Experimental Design

In this experiment we are testing the effects of four biological compounds at three differing concentrations and at three incubation times as illustrated in figure 1, to measure the metabolic effects on the neural stem cells in suspension culture. We have three biological replicates for each treatment group ( $n=3$ ) and three technical replicates for each biological replicate. The data from the three technical replicates were averaged. There were two control groups: a media control which contained cell culture media and cells only, and the solvent control which included cell culture media, cells, and water.

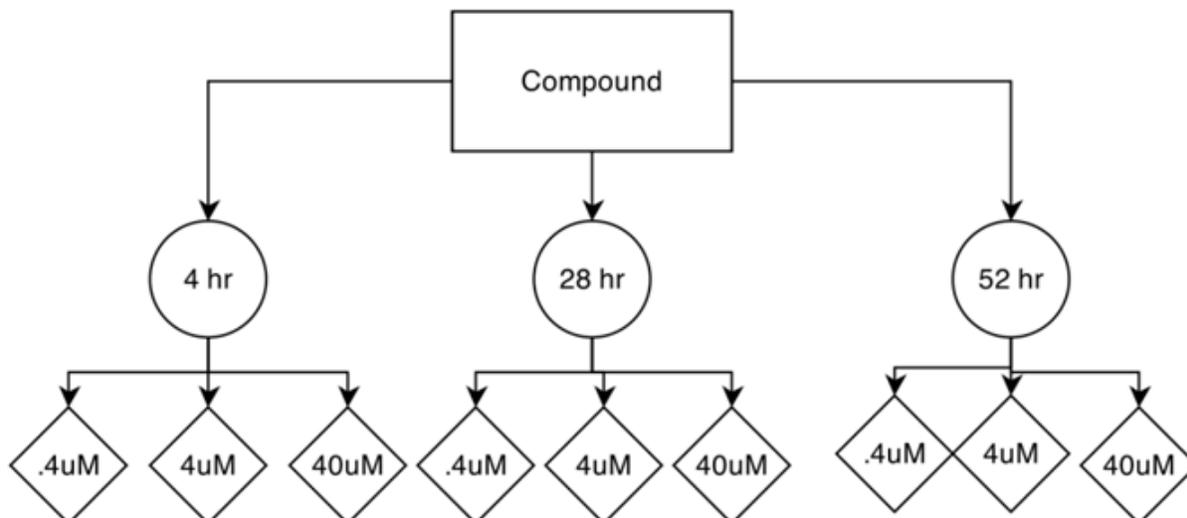


Figure 1 Schematic of Experimental Design

## **Culturing of Human Neural Stem Cells**

The HNSCs were (Gibco) maintained in a T-75 suspension flask (Corning) with 20 mL of HNSC/GBM Media containing DMEM/F-12 media (Gibco) with supplementary final concentrations of AB/AM [1%], B-27 [2%], epidermal growth factor [1 ug/uL], fibroblast growth factor [0.1 ug/ul], heparin [5000U/mL]. The neurospheres were cut consistently whenever they would exceed approximately 1 millimeter in diameter with a 50% media exchange every 48 hours. The flask of neurospheres was incubated at 37 degrees and 5% carbon dioxide.

## **Preparation of Reagent Molecules**

The four reagent molecules including melatonin, N-acetylserotonin, L-tryptophan, and L-DOPA were acquired from Sigma-Aldrich. Melatonin had the lowest solubility value with solubility at room temperature of 0.1mg per mL of molecular grade water. Melatonin was suspended at a molarity below this saturation point and the remaining molecules were prepared to the same molarity by adding the required volume of molecular grade water.

The following represent the calculations for the preparations of all four reagents:

### *Melatonin*

- i) The molecular weight of melatonin is 232.28 grams/mole.
- ii)  $.00245 \text{ grams} / 232.28 \text{ grams/mole} = .00001055 \text{ moles of melatonin}$
- iii)  $\text{Molarity} = .00001055 \text{ moles melatonin} / .028 \text{ L solution}$

- iv) .0003767M Melatonin when we use .028L molecular water
- v) .028 L molecular water = 28.000mL molecular water stock solution
- vi) Serial dilution of stock solution by a factor of 10 and 100 with molecular water

*N-Acetylserotonin*

- i) The molecular weight of N-Acetylserotonin is 218.25 grams/mole.
- ii) .00213 grams/218.25 grams/mole=.00000976 moles of N-Acetylserotonin
- iii) .0003767 Molarity=.00000976 moles N-Acetylserotin/L solution
- iv) .02590921 L molecular water = 25.909 mL molecular water stock solution
- v) Serial dilution of stock solution by a factor of 10 and 100 with molecular water

*L-Tryptophan*

- i) The molecular weight of L-Tryptophan is 204.23 grams/mole.
- ii) .00230 grams/204.23 grams/mole=.00001126 moles of L-Tryptophan.
- iii) .0003767 Molarity=.00001126 moles of L-Tryptophan/L solution
- iv) .02989116 L molecular water = 29.891 mL molecular water stock solution
- v) Serial dilution of stock solution by a factor of 10 and 100 with molecular water

*L-DOPA*

- i) The molecular weight of L-DOPA is 200.21 grams/mole.
- ii) .00200 grams/200.21 grams/mole=.00000999 moles of L-DOPA.
- iii) .0003767 Molarity=.00000999 moles of L-DOPA/L solution

iv) .02651848 L molecular water = 26.518 mL molecular water stock solution

v) Serial dilution of stock solution by a factor of 10 and 100 with molecular water

Twelve 50 mL conical tubes were used to prepare the four stock solutions of the molecules, four 1/10 dilution solutions of the molecules, and four 1/100 dilution solutions of the molecules. In addition, a new set of twelve sterile 50 mL conical tubes were prepared and each of the twelve solutions were filter sterilized into these new tubes inside of the biological safety cabinet. This would eliminate the risk of contamination, including bacterial contamination of the cell culture that would compromise the AlamarBlue assay readings.

### **Preparation of Cell Suspension Plates**

Three 96 well suspension plates were chosen for this particular assay with the optimal volume of each well listed as cells suspended in 100 microliters of media. The total volume of media required to fill all the wells was 12.15 mL. Approximately 13 small neurospheres were accutased in 13 mL of media containing fibroblast growth factor and epidermal growth factor reduced to 25% concentrations relative to the baseline culturing of the neurospheres. 90 microliters of this [25% FGF/EGF] (epidermal growth factor [0.25 ug/uL], fibroblast growth factor [0.025 ug/ul]) HNSC/GBM media with accutased cells were micropipetted consistently into 135 wells. This would ensure that the proper concentration of cells would be in each well to ensure that the alamarBlue assay would produce valid data. Two controls were utilized in the procedure, with one containing the [25% FGF/EGF] HNSC/GBM media, neural stem cells, and

molecular grade water; the other containing [25% FGF/EGF] HNSC/GBM media, molecules, and molecular grade water.

### **AlamarBlue assay**

The alamarBlue cell viability reagent assay (ThermoScientific) was chosen for this experiment due to its suitability to measure HNSC metabolism with the EnVision 2104 Multilabel Reader (Perkin Elmer). AlamarBlue has been utilized reliably for the past 50 years to measure cell metabolism and health. PubMed currently holds approximately 200 publications citing alamarBlue and cancer research and approximately 1,000 articles citing alamarBlue and drug discovery. The widespread usage of alamarBlue is in part due to the diverse number of cell lines that can successfully be evaluated with the assay including but not limited to: bacteria, yeast, fungi, fibroblasts, immortalized cancer cells, lymphocytes, and primary neuronal cell cultures (44). AlamarBlue offers advantages in comparison to tetrazolium salts, due to the fact that tetrazolium salt assays involve the formation of formazan crystals which must be solubilized by DMSO and/or HCl/isopropanol which kills the sample cells (44).

AlamarBlue uses an active ingredient known as resazurin to measure the reducing environment of the living cell. It accomplishes this by acting as an intermediate electron acceptor in the electron transport chain of the mitochondria. It is water soluble, stable in cell media, non-toxic to cells, and permeable to cell membranes. These features of the assay allow for continuous monitoring of cells in culture, over an appropriate period of time (44).

The alamarBlue reagent utilized in this experiment was stored at 4 degrees with a complete absence of light in order to ensure maximum accuracy and effectiveness of the assay.

In addition, the reagent was added to the plates with minimal lighting from the biological safety cabinet and minimal ambient lighting from the laboratory itself. These precautions are crucial as the alamarBlue reagent degrades when exposed to light for a significant period of time.

### **PerkinElmer EnVision 2104 Multilabel Reader**

The EnVision 2104 Multilabel Reader (PerkinElmer) contains modular label-specific optical mirror modules, high energy flash lamps, and high speed detectors. It also contains a high energy time-resolved fluorescence laser excitation flash lamp that reduces the number of repeated flashes needed while measuring plates (37). The following were the parameters used for this particular assay:

<b>Operation Type</b>	Normal Measurement
<b>Excitation</b>	Top
<b>2nd Excitation</b>	Top
<b>Top Mirror</b>	*General (401)
<b>Excitation Filter</b>	*TAMRA 544 (117)
<b>Emission Filter</b>	*Photometric 595 (315)
<b>Measurement Height</b>	6.5mm
<b>Excitation Light</b>	2%
<b>2nd Excitation Light</b>	2%
<b>Detector Gain</b>	1
<b>2nd Detector Gain</b>	1
<b>Number of Flashes</b>	1

Figure 2 Measurement: alamarBlue lower gain(1)

HNSCs with [25%] FGF/EGF media only were assayed (AlamarBlue) immediately (0 hours) to assess background fluorescence of media, HNSCs, and 100% unreduced AlamarBlue reagent. These fluorescence values were compared to fluorescence values of media without cells but with the testing compounds (each compound separately). Furthermore, media without cells but with testing compounds were assessed at 24, 48, and 72 hours, to check for media color change over time\* as well as media-to-test-compound interaction. All of the fluorescence values from each discussed scenario remained in the 5,000-6,000 range of fluorescence units\*\*. Thus, we can reliably assume that the HNSCs themselves, the test compounds themselves, possible media-compound interaction, and possible media (with no cells) color change over time do not affect the fluorescence readings. We assume that the 5,000-6,000 values detected account for the phenol red pH color indicator in the media alone. Further fluorescence testing of AlamarBlue with water and media with no AlamarBlue can confirm that the media itself produces this background fluorescence.

\*This was done because the negative control value needed to be accurate for cells that were incubated for 24, 48, and 72 hours.

\*\*These units are arbitrary and dependent on our assay reader and its exposure/gain settings, though the scale is consistent and relevant for the purposes of this project.

## DATA ANALYSIS

In this experiment three incubation times were analyzed on three separate cell plate cultures. The three incubation times were 4 hours, 28 hours, and 52 hours of exposure to the biological molecules and reduction of alamarBlue. There are two independent variables, the biological molecules and each of their three concentrations. A two-way analysis of variance was utilized and we determined that there was no statistically significant interaction between the independent variables. A global F-test determined that there were statistically significant differences among the biological molecules and the control group. Bonferroni post-hoc t-tests were performed in which each biological molecule at the three differing concentrations (n=3) were individually compared to the control group (n=12).

There was no statistically significant difference at  $p < 0.05$  between the biological molecules and the control group except for two exceptions (labeled with asterisks on figures 3 and 5) L-DOPA at a 40 micromolar concentration after 4 hours of incubation and Melatonin at a 40 micromolar concentration after 52 hours of incubation. Despite this, certain trends in the data are evident and are consistent with these two exceptions pointing to possible further avenues of study. Specifically, the melatonin appears to cause the least reduction in alamarBlue compared to the control while the L-DOPA causes the greatest reduction in alamarBlue compared to the control.

## RESULTS

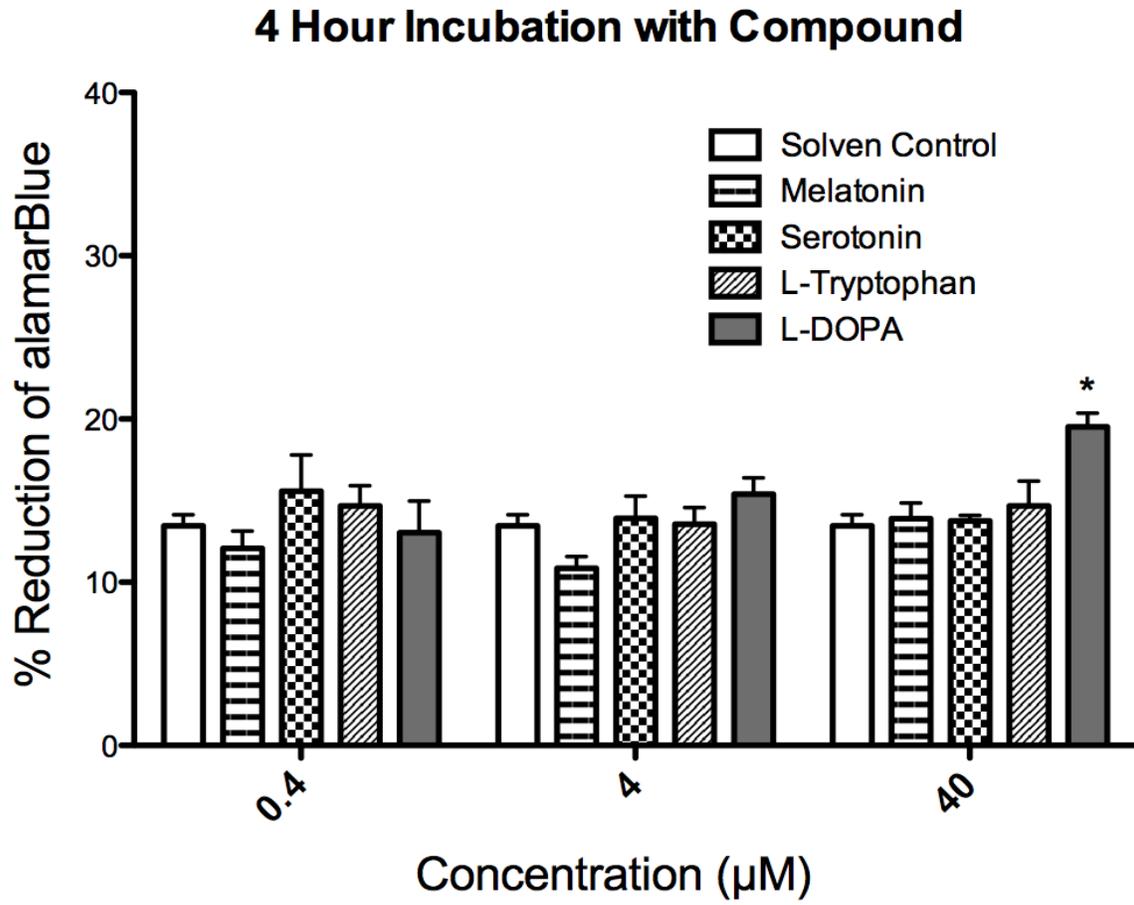


Figure 3 The Percent Reduction of alamarBlue after 4 Hour Incubation

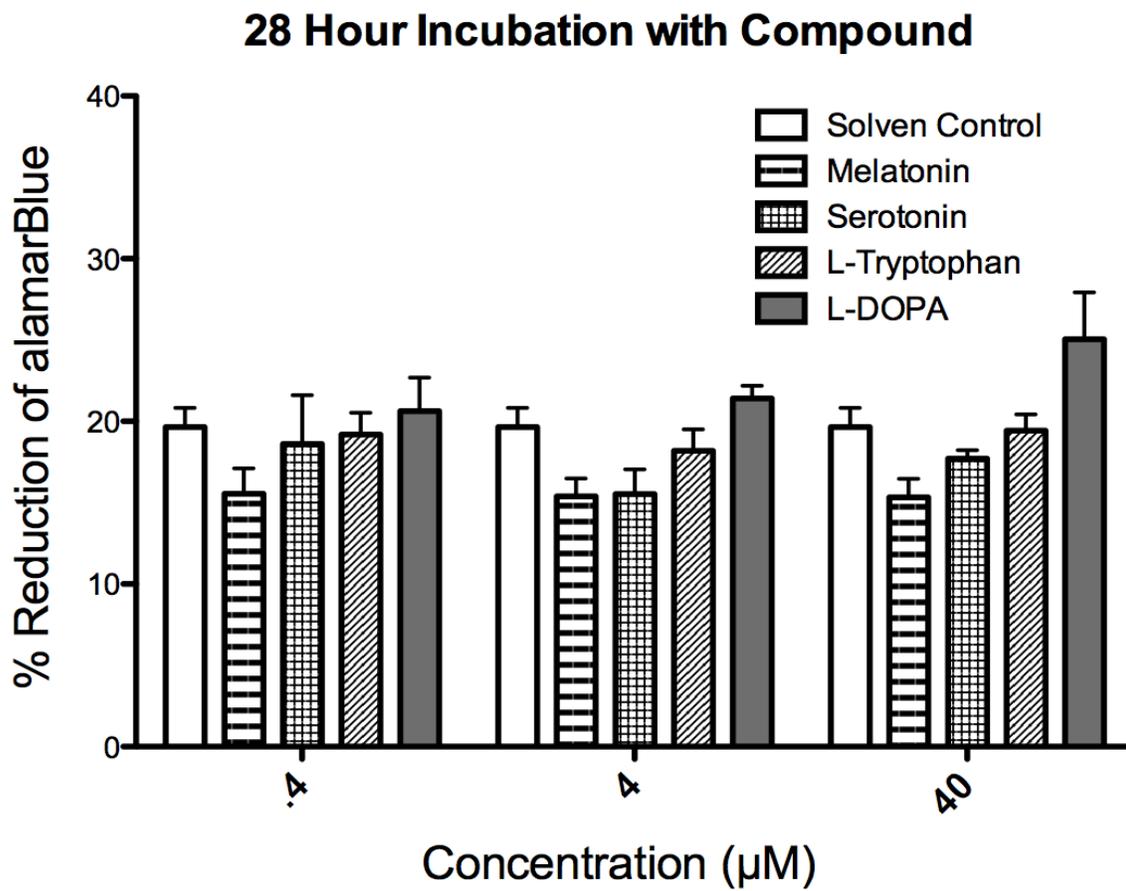


Figure 4 The Percent Reduction of alamarBlue after 28 Hour Incubation

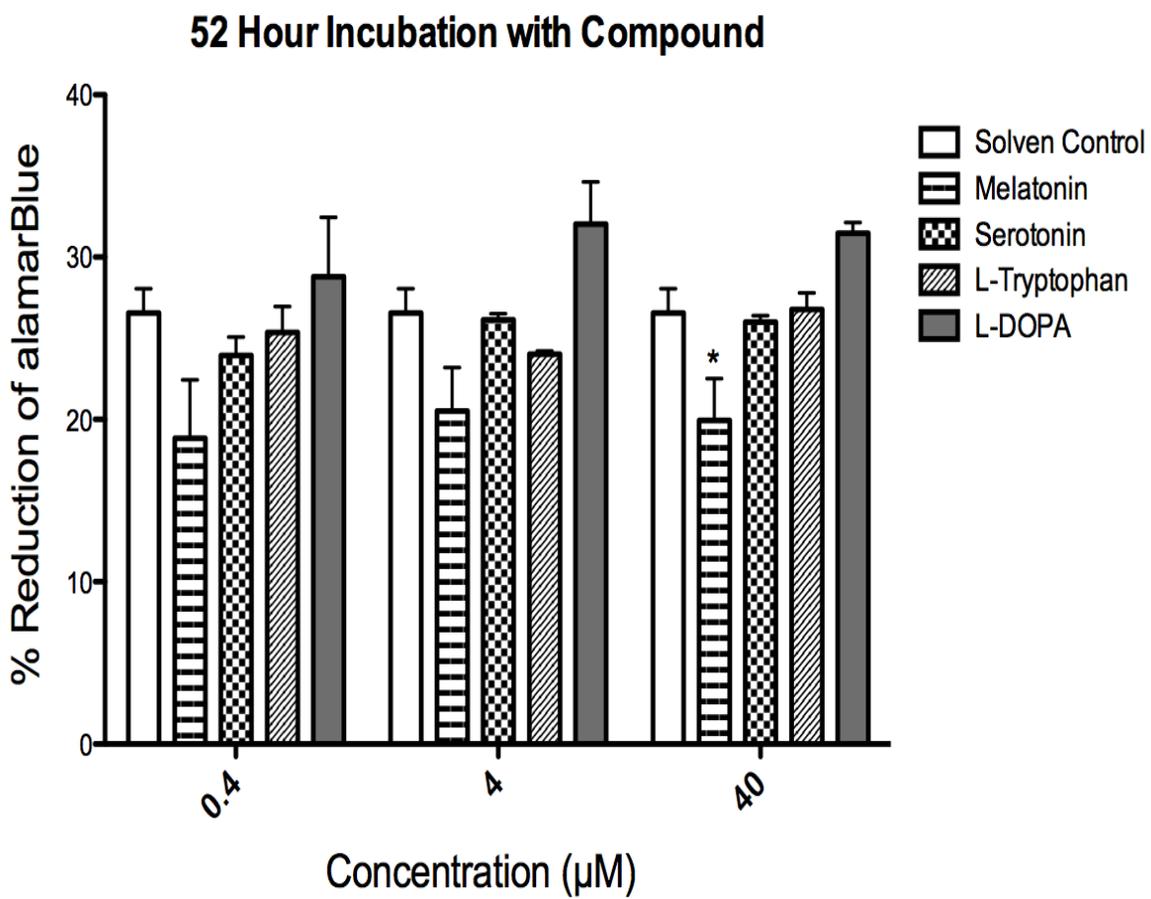


Figure 5 The Percent Reduction of AlamarBlue after 52 Hour Incubation

## Neurosphere Morphology

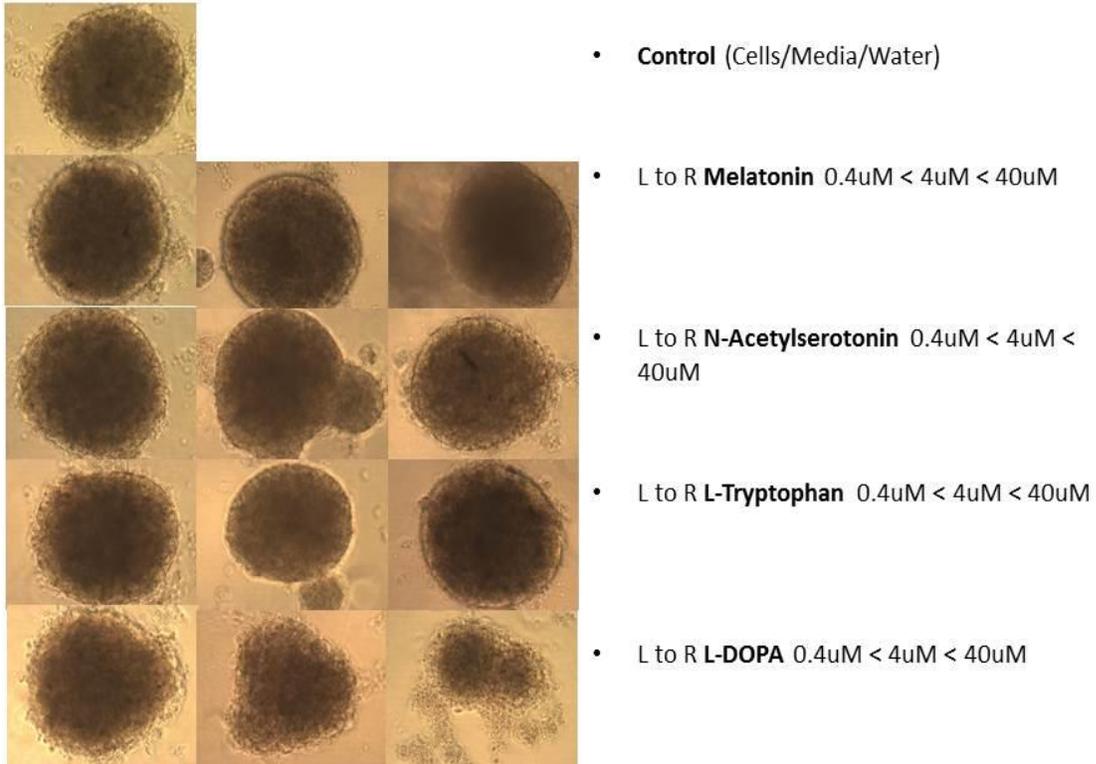


Figure 6 Neurosphere Morphology at Seven day Incubation/48 hour alamarBlue Incubation

### Cell Densities over the Course of 75 Hours

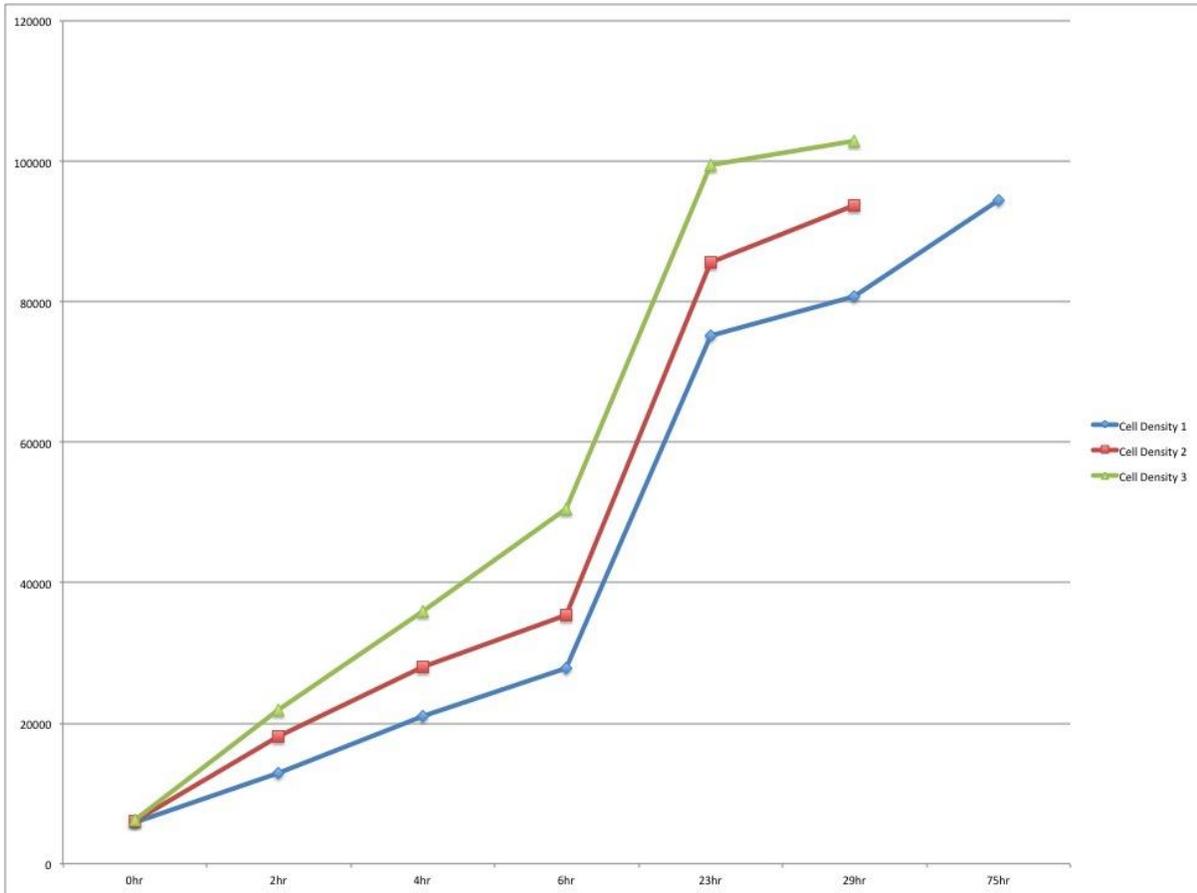


Figure 7 Cell Densities as a function of 75 Hour Incubation

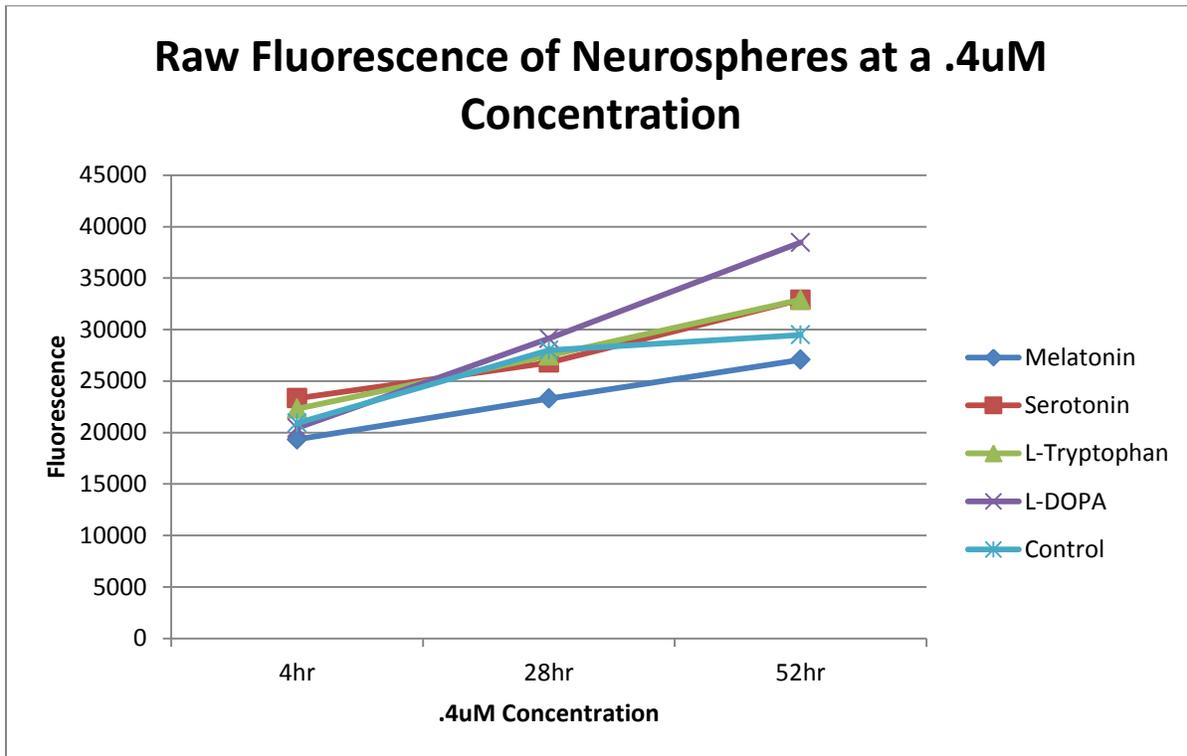


Figure 8 Fluorescence of Neurospheres exposed to .4uM of Molecules over 52 hours

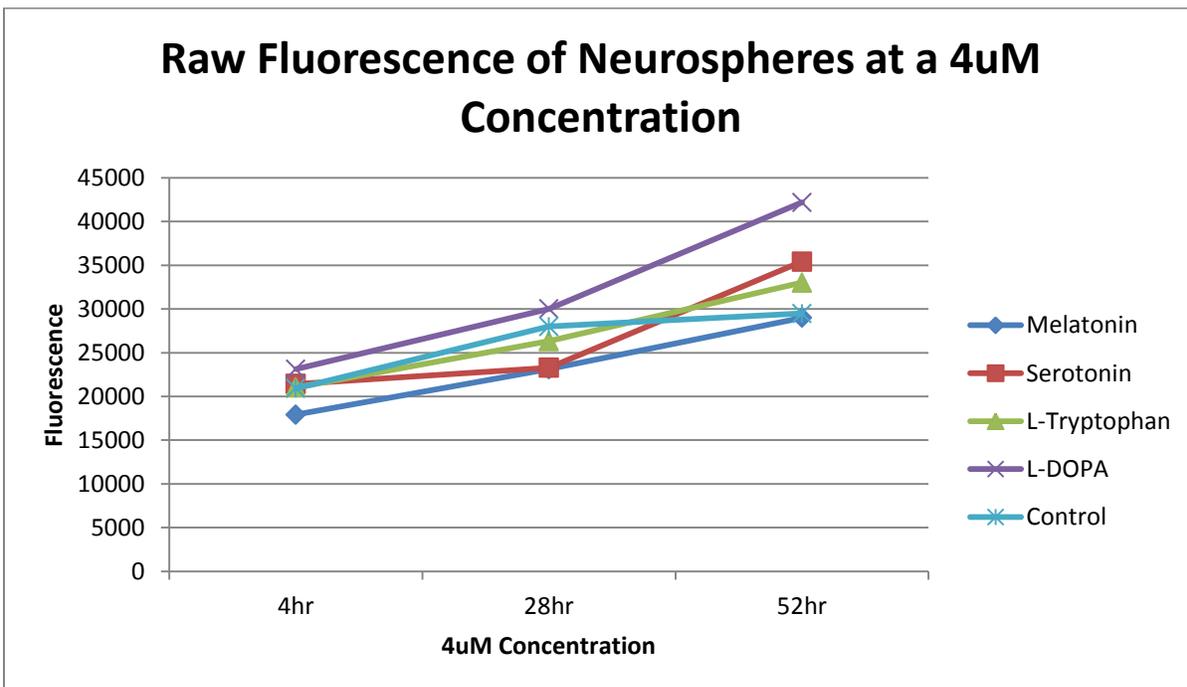


Figure 9 Fluorescence of Neurospheres exposed to 4uM of Molecules over 52 hours

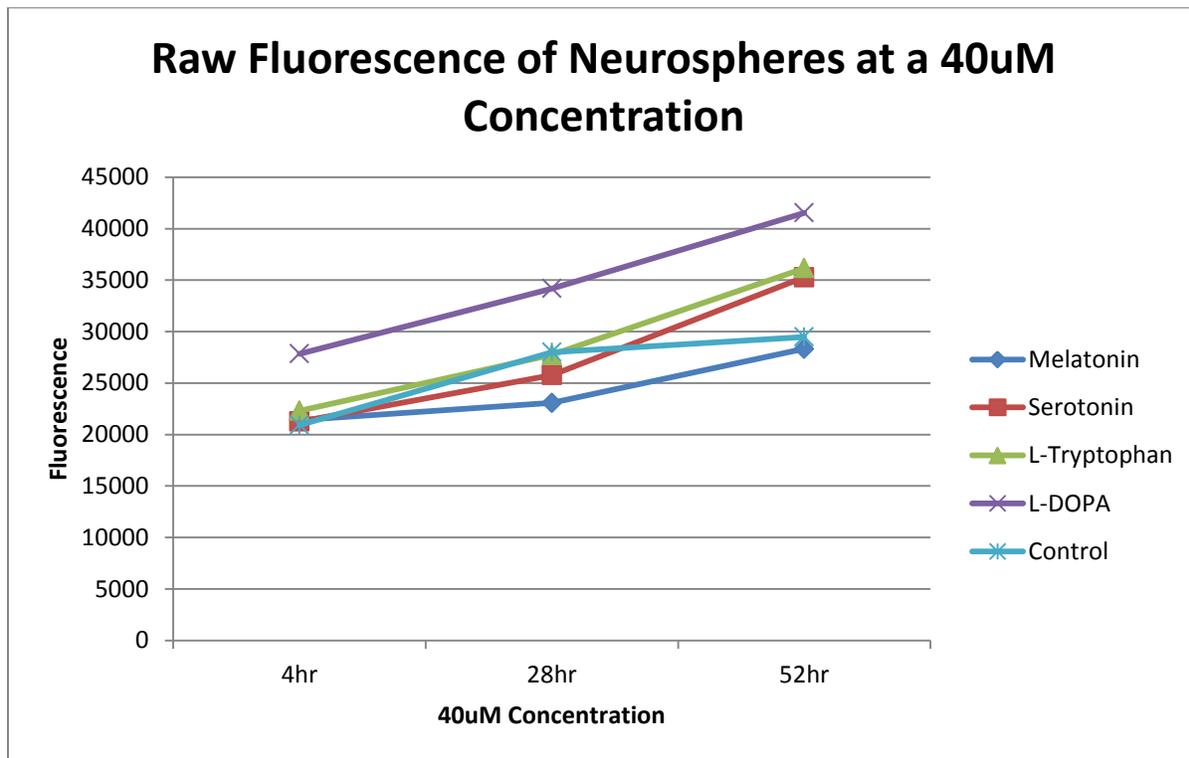


Figure 10 Fluorescence of Neurospheres exposed to 40uM of Molecules over 52 hours

## DISCUSSION

### 4 Hour Incubation

According to the data present in figure 3 after 4 hours of incubation, at 0.4 micromolar concentration, melatonin and L-DOPA cause a lesser reduction in alamarBlue than the control. N-acetylserotonin and L-tryptophan have a greater reduction in alamarBlue than the control. At a 4.0 micromolar concentration, melatonin still has a lesser reduction in alamarBlue than the control with the N-acetylserotonin, L-tryptophan, and L-DOPA all experiencing the equivalent reduction or a slightly greater reduction in alamarBlue when compared to the control. At 40 micromolar concentration, all of the compounds cause a greater reduction in alamarBlue when compared to the control. Up until this point in the results no clear trends are evident in the data. At the 40 micromolar concentration the effect appears to be most pronounced for L-DOPA. In this particular case, the L-DOPA caused a statistically significant change in comparison to the control according to the results of the Bonferroni post-hoc t-test at  $p < 0.05$ . Figures 8-10 illustrate that the differences in raw fluorescence values at 4 hours were the least compared to lengthier incubation times.

### 28 Hour Incubation

According to the data present in figure 4 after 28 hours of incubation, at 0.4 micromolar concentration, melatonin causes the least reduction in alamarBlue compared to the control. N-acetylserotonin and L-tryptophan cause slightly lesser reduction in alamarBlue compared to the

control. L-DOPA causes a greater reduction in alamarBlue compared to the control. At a 4.0 micromolar concentration, melatonin still experiences the least reduction in alamarBlue compared to the control. N-acetylserotonin and L-tryptophan cause a slightly lesser reduction in alamarBlue when compared to the control. L-DOPA once again has the greatest reduction in alamarBlue when compared to the control. At a 40 micromolar concentration, melatonin causes the least reduction in alamarBlue when compared to the control. L-DOPA causes a greater reduction in alamarBlue in comparison to the control. At this incubation time, the trends for melatonin and L-DOPA are more distinct. Figures 8-10 illustrate how there were greater differences in fluorescence values between the different compounds compared to the 4 hour incubation time.

## **52 Hour Incubation**

According to the data present in figure 5 after 52 hours of incubation, the trend is similar at a 0.4 micromolar concentration with melatonin causing a much lesser reduction in alamarBlue compared to the control and L-DOPA causing a much greater reduction in alamarBlue compared to the control. The trend is similar at the 4.0 micomolar concentration with melatonin causing the least reduction in alamarBlue and L-DOPA causing the greatest reduction in alamarBlue by an even greater margin. At a concentration of 40 micromolar, the trends are just as distinct with melatonin causing the least reduction in alamarBlue and L-DOPA causing the greatest reduction in alamarBlue in comparison to the control. It is important to note that at the 40 micromolar concentration, melatonin's lesser reduction in comparison to the control is statistically significant

according to the results of the Bonferroni post-hoc t-test at  $p < 0.05$ . Figures 8-10 illustrate how there were the greatest differences in fluorescence values between the different compounds compared to the 4 hour incubation and 28 hour incubation times.

## **CONCLUSION**

The data illustrates a trend of a decrease in reduction of alamarBlue with exposure to melatonin in comparison to the control. In addition, there is a trend of an increase in the reduction of alamarBlue with exposure to L-DOPA in comparison to the control. These two molecules appear to have caused the greatest changes in alamarBlue reduction in comparison to the control. The N-acetylserotonin and L-tryptophan did not follow as clear a trend as the other two molecules when it came to the reduction in alamarBlue in comparison to the control. However, the L-tryptophan appeared to cause a greater reduction in alamarBlue in comparison to the N-acetylserotonin in several instances. The normal morphology of the neurospheres exposed to melatonin coupled by the lesser reduction of alamarblue indicates that melatonin may act as a repressor of neural stem cell metabolism. Our results point to a less clear conclusion when it comes to N-acetylserotonin and L-Tryptophan. The abnormal morphology of the neurospheres exposed to L-DOPA coupled with the greatest reduction in alamarBlue indicates that L-DOPA causes elevated metabolic levels in the neural stem cells and possibly the initiation of apoptotic pathways.

### **L-DOPA**

According to a study from Japan published in 2003, L-DOPA and Dopamine were found to be neurotoxic when used for long-term treatment regimens due to an excess oxidation that promoted apoptosis of neuronal cells (42). According to the study, dopamine and L-DOPA contain quinone residues that irreversibly damage proteins through the formation of 5-cysteinyln-

catechols, which are cytotoxic to neuronal cells. These findings of promotion of neuronal apoptosis are consistent with the neurosphere morphology after exposure to L-DOPA as illustrated in figure 6. In particular, as the concentration of L-DOPA was increased by factors of ten, the morphology of the neurospheres further deteriorated, further suggesting that the molecule was pushing the neurospheres further into apoptotic stages.

### **Melatonin**

Melatonin consistently causes the least reduction in alamarBlue according to the data and the values are also lower than the percent reduction in the control. This trend suggests that melatonin causes a reduction in metabolism in the neurospheres. According to a study conducted at the Shandong University School of Medicine, melatonin has been found to promote proliferation and differentiation of neural stem cells in hypoxic conditions (43). Proliferation was induced although the effect on differentiation was limited to neurons not astrocytes. Melatonin's induced proliferation of HNSCs is tied to the MT1 receptor and increased phosphorylation of ERK1/2. Melatonin's induced differentiation of HNSCs is tied to altered expression of genes (43). Our results with the decreased percent reduction in alamarBlue appear to contradict these trends, suggesting a decrease in metabolism although further analysis is needed to verify these trends. Higher and lower concentrations of melatonin in comparison to the experimental groups in this study should be further tested with the neurospheres to determine what if any relation this may have to the concentration of melatonin that is utilized.

### **N-Acetylserotonin**

According to a publication in Neuropsychopharmacology, adult neural stem cells were cultured expressing both isoforms of tryptophane-hydroxylase (TPH) and Serotonin (5-HT). When their TPH function was blocked by para-chlorophenylalanine (PCPA), the proliferation of the neural stem cells was reduced. Exogenous serotonin reduced this effect and induced greater proliferation. The results of the study illustrated that serotonin action on the adult neural stem cells was controlled primarily by the serotonin receptor subtype 5-HT1A and to a lesser extent the 5-HT2C receptor. This was determined by selectively antagonizing the receptors. Serotonin also caused an increase in the migration of the adult neural stem cells (45).

A large number of neural stem cells that maintain self-renewal and multipotency are found in the subventricular zone (SVZ), the subgranular zone (SGZ), and the subcallosal zone, a region between the hippocampus and the corpus callosum of the rodent brain (45-46). The serotonergic system forms early in brain development and forms one of the most comprehensive neural networks in the brain (47). It is believed that serotonin modulates neurogenesis in the hippocampus and recently hippocampal stem cells have been found to be the target of antidepressants although the exact mechanism or potential role that serotonin may have on the hippocampal progenitors or stem cells is still unclear and warrants further investigation (48). Our results for N-acetylserotonin do not appear to illustrate a specific trend and thus our conclusions are limited at the experimental groups of this study.

## **L-Tryptophan**

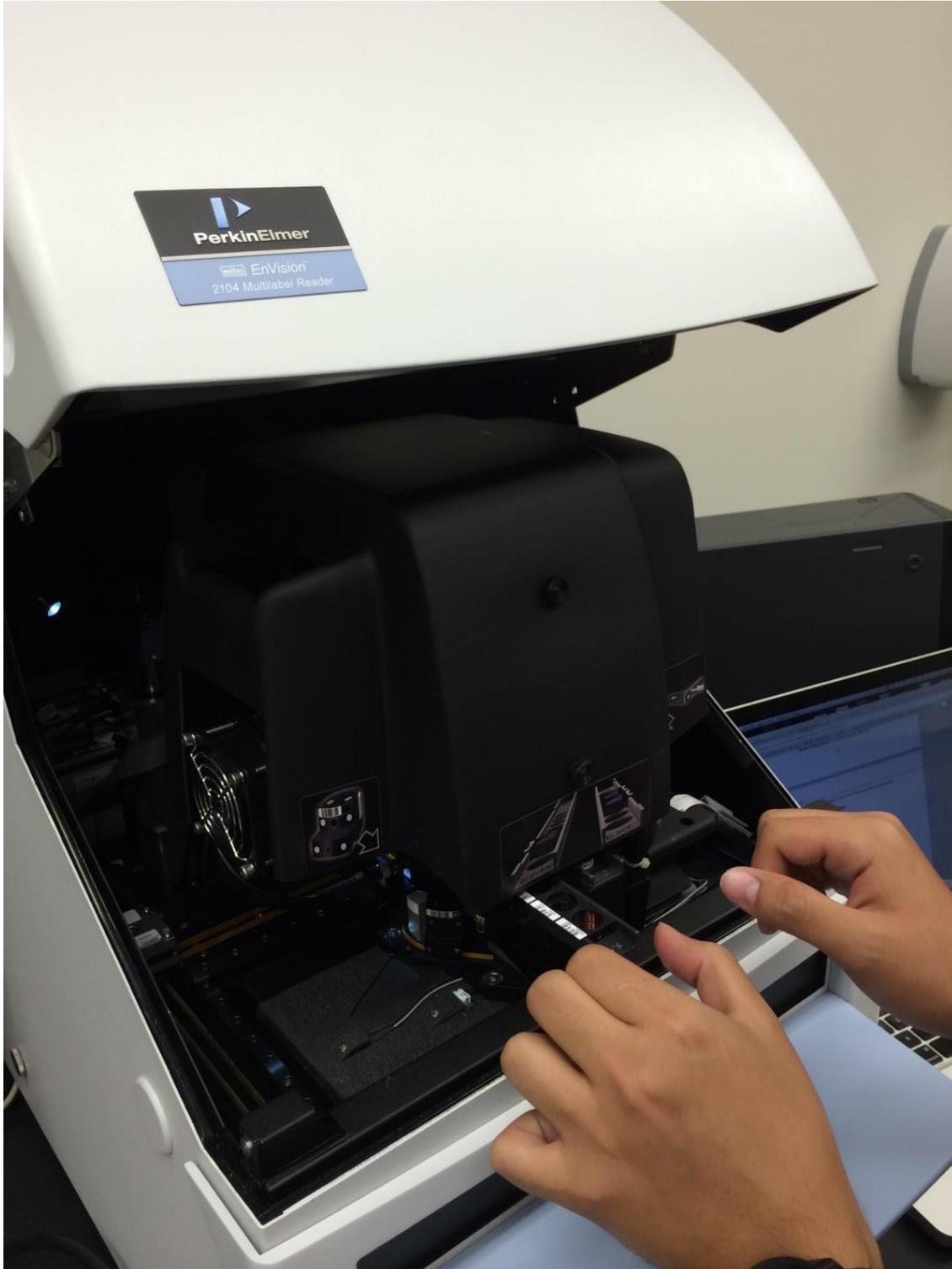
L-tryptophan is believed to influence anxiety related behavior similarly in relation to serotonin. Tryptophan metabolism is completed by 2,3 dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). Mice that were deficient in TDO showed increased levels of tryptophan, its metabolites 5-hydroxytryptophan (5-HTP), and serotonin (5-HT) in the hippocampus and midbrain. An increase in neurogenesis correlated with the increased tryptophan levels with these findings demonstrating that there is a direct link between tryptophan metabolism and neurogenesis (49). In a similar manner to N-acetylserotonin, the L-tryptophan at the tested concentrations does not follow a clear trend in influencing the percent reduction of alamarBlue by the neurospheres at the experimental groups of this study.

## **IMPLICATIONS AND FUTURE RESEARCH DIRECTIONS**

This study serves as a guide for further investigation of melatonin and its closely related precursor molecules and their effects on neurosphere stem cell metabolism. From the results, it is clear that melatonin and L-DOPA caused the greatest changes in metabolic levels of the neurosphere stem cells. Melatonin is currently not regulated by the FDA, although it has great treatment potential and has not been discovered to have toxic effects in humans, in contrast to L-DOPA which has been found to cause cytotoxicity in concentrated long-term dosages. In the future, melatonin's apparent lacks of toxicity as well as its diverse benefits, ensure that replacement therapy utilizing melatonin exposed tissue cultures such as neural stem cells could one day become a reality.

**APPENDIX: PERKIN ELMER ENVISION 2104 MULTILABEL READER**

## Perkin Elmer Envision 2104 Multilabel Reader



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