INVESTIGATING THE EFFECTS OF CAFFEINE ON NEURITE GROWTH AND DEVELOPMENT BY OBSERVING NEURONAL MORPHOLOGY

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Abstract
Caffeine is a psychoactive stimulant used in almost every part of the world. In Western society, more than three-quarters of the adult population drink enough coffee to affect the brain substantially. As the caffeine industry is growing, there has been an increasing amount of research on caffeine's effects on neuronal morphology. The current study investigates the cellular responses to caffeine treatment on primary rat cortical neurons by studying neurite outgrowth. Different levels of treatment were prepared in culture media and kept in a CO2 incubator. Paraformaldehyde was used to fix the cells, and bright-field, phase-contrast images will be taken, using ImageJ to assess neurons' size and morphology in control and treated cultures. Results were evaluated using Graphpad Software, a computer program that administers biostatistics and scientific graphs of data analysis. By observing direct dosage-dependent effects in rat neurons, the investigator found that caffeine increased neurite length, in which higher concentrations brought about significant results.

Keywords: caffeine, neuronal morphology, neurite outgrowth, phase-contrast images

Introduction

Cell culture methods have been the foundation of biology for centuries, in the way that scientists were able to grow and maintain cells under carefully controlled conditions. It is common practice for scientists to isolate cells from living tissue and conducting further experiments on them to gain mechanistic insight of the cells outside of their normal environment. The nervous system is composed of specialized cells, such as neurons and glial cells, and cell culture techniques have revealed that these cells have distinct behaviors that govern the brain's performance in managing a network that connects different regions of the brain. The structural makeup of neurons allows the brain to collect, store, and interpret information from the outside world. Brain cells across organisms are similar in that they form a network of connections between anatomical structures; however, each organism is still unique in a way that gives rise to a one-of-a-kind lifetime of experiences.

Cell culture studies support cells' survival and development outside their normal environment, allowing direct judgment of reactions to treatments and other stimuli. Cellular responses may include (but are not limited to): formation of synapses, proliferation and growth of normal structural processes, a delay in normal processes, or sometimes even death. Over 200 million neurons are found in the nervous system of the genus Rattus in adulthood, with 21 million just in the cerebral cortex (Korbo *et al*., 1990). This large amount is due to the high proliferation rate and increased plasticity that is natural to their development. For this reason, the cells collected for culture are usually from animals in the embryonic stage of gestation

or early postnatal period. Rats and rodents are popular models in scientific testing because their behavioral attributes closely mirror human characteristics, and therefore they can reproduce features of human conditions.

Caffeine is the most widely consumed drug in the world. This psychoactive stimulant is used in several different forms. However, it is typically found in beverages such as coffee, tea, or energy drinks and increases energy, productivity, and alertness. There are several known mechanistic actions that demonstrate the short- and long-term effects of caffeine. For instance, during the early stage of a cultured hypothalamus neuron, caffeine treatment alters the expression of the calcium response factor (CaRF) and downstream NMDA and GABA receptors, affecting the regulation of neurotransmitter receptors, nerve growth, and development (Chan *et al*., 2011). It has also been found that caffeine can enhance activity-dependent gene expression in developing cortical neurons, contributing to the neurological benefit observed in infants receiving caffeine treatment (Connolly and Kingsbury, 2010). A recent study identified novel genes that respond to acute caffeine stimulation, suggesting promotion of neuronal connections that might provide mechanistic insights into the enhancing effects of caffeine on memory and cognition (Yu *et al.,* 2017). Neonatal administration of caffeine may, in part, affect the dendritic morphology of the pyramidal cells of the prefrontal cortex, and this effect persists after puberty and may be implicated in several brain processes (Juarez-Mendez *et al*., 2006). The knowledge that caffeine intake among young adults and college students is substantial has led to a recent finding that adolescents are not likely to benefit from caffeine supplementation and may suffer ill effects from chronic ingestion of high doses (Curran & Marczinski, 2017). Given this, it is essential to examine both the benefits and drawbacks this drug has on neural growth and development using cell culture techniques.

Methods

Data availability was limited to what Saint Louis University provided to their biology students. The sample was taken from a lab where they preserved and dissected cells from rats' primary cortex. Single cortical cells were isolated and then plated with 50,000 to 100,000 cells per dish.
There are eight cell dishes in the present study, which the investigator preserved in a CO2 incubator (5% CO2, 95% air, 37° Celsius). All dishes were fed every three to four days to stay alive and were grown for a full week before the actual experiment took place. Additionally, the cells were still fed mid-way through the week when the treatment was applied during the investigation. The experimental research design includes Experiment Group A, Experiment Group B, and a control group. Experiment Group A is the short-term treatment, in which cells were treated with 10μM for three days and then sat in growth medium for the remainder of the week. Experiment Group B consists of the cells treated for the long-term treatment, where instead, cells were exposed to caffeine for a full seven-day treatment. All cells were plated simultaneously using the same growth mediums, except the two control group dishes that were simply treated with growth medium. Researchers assessed all cell dishes at the end of the week.

The following week of the experiment is where the analysis took place, in which cells were fixed with 4% Paraformaldehyde (PFA) and then rinsed with Phosphate-

buffered saline (PBS) 30 minutes later. We then had to coverslip them to take bright-field, phase-contrast microscopic images for every dish, which were then uploaded into Neuron J, a plugin software thru the ImageJ software the size and morphology of neurons in control and treated cultures. Graphpad Software was also used to conduct data analysis and to create 2D scientific graphs.

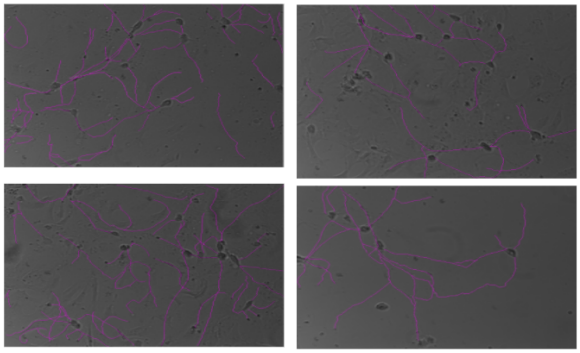


Figure 1. This graphic represents the microscopic, phase-contrast photos captured and then traced for measuring average neurite length per dish. Two images were taken from different fields within the same dish in an attempt to collect multiple representative images of the dish. The left column represents Dish 3 of the short-term treatment, and the right column shows Dish 7, illustrating the effects of caffeine on long-term treatment.

Results

A one-way ANOVA was conducted. The dependent variable must be a continuous level of measurement, so in this case, neurite length, and the independent variables must be categorical, which was the level of treatment. This test allowed us to determine whether there are any statistical differences between the means of the two experimental groups and the control group. This test assumes that the data has a normal distribution, so the analysis began by running a normality test to determine whether the sample data is within the same tolerance. The information we compiled passed three out of the four normality tests on Graphpad, which allowed us to conclude there was no significant departure from normality within the data.

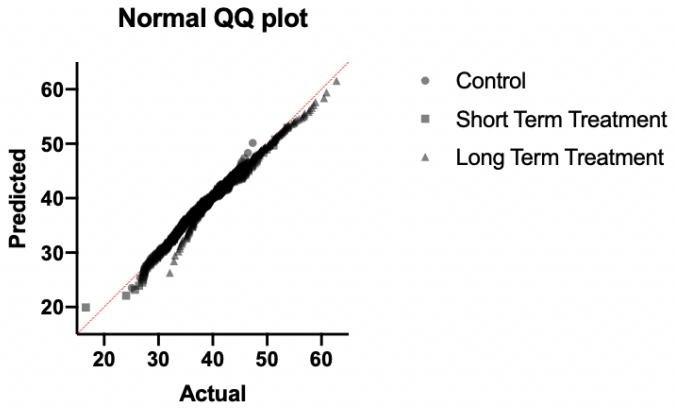


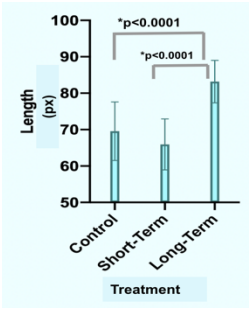
Figure 2. This graph shows the raw mean values compared to the sample values that would represent a normally distributed set of numbers with the same mean and standard deviation.

With a total of 793 values, the data came out to have a significant difference among the means, with a P-value that is less than 0.0001. There was also a significant difference among the standard deviations, according to Bartlett's test. A Tukey test was administered for post HOC analysis, which conducts independent comparisons to determine where the significant difference lies between the three groups. These multiple comparisons test showed significant differences between the control and long-term treatment (Experiment Group B), which had a mean difference of -7.107px. There was also a significant difference between the short-term (Experiment Group A) and long term

(Experiment Group B) treatments, with a mean difference of -7.501px.

	N	Mean	SD
Control	175	69.560	8.060
Short-Term	251	65.944	7.025
Long-Term	367	83.174	5.827

a)



b)

Figure 3. a) This table represents the sample size for each data set, actual calculated means, and standard deviations. b) This figure graphically represents the mean values per treatment group, with error bars reflecting standard deviation for each group, which shows the extent of variation for each group.

Conclusion & Discussion

This study found that seven days of 10µM caffeine treatment increased neurite length (by approximately 20% versus the control group). Three days of treatment did not significantly change neurite length. From this, we can conclude that the effect is time-dependent at this dose, and chronic or long-term exposure is different than short-term exposure. With that being said, chronic consumption of caffeine among humans may result in the formation of long, complex neuronal structures that, in turn, affect the standard arrangement and processes of the nervous system, especially during years of development and maturation. Though caffeine contributes to the enhancing effects of memory and cognition, the long-lasting effects of this drug can be harmful for the time being.

Future Implications

Regarding directions for a future study on this matter, it would be valuable to exclude the time-dependent component of the effect on neurite length, and instead compare samples treated with different doses of caffeine at the same time points to see how caffeine stimulation affects neurite length in a concentration-dependent manner, while also accounting for individual differences between neurons. Because our findings were due to caffeine treatment's acute effects, it would also be interesting to see if this stimulation is increased with prolonged exposure to caffeine. Suppose the activation of neuronal connections could be observed in a long-term treatment model. In that case, it may help explain the links between caffeine and improved cognitive performance, as well as suggested protective effects against Alzheimer's disease in epidemiological studies (Oñatibia-Astibia et al., 2017). Further, studying gene expression directly (either via in situ hybridization or a gene microarray technique) can help determine whether a genetic up- or down-regulation occurs in the replication of given caffeine treatment.

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