The Effect of Caffeine on Migraine Headaches

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THE EFFECT OF CAFFEINE ON MIGRAINE HEADACHES

by

Deborah Shimshoni

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Sciences in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, Florida Summer Term 2016

Thesis Chair: Dr. Mohtashem Samsam
Acknowledgements

I would like to first thank Dr. Sugaya for allowing me to not only perform many experiments in his lab but also to thank him for allowing me to use his lab’s human neuronal stem cells, media, materials, incubators, and all that his lab had to offer. I also would like to extend a gracious thank you to the members of my thesis committee: Dr. Raheleh Ahangari, Dr. Michael Hampton, and Dr. Saleh Naser, who have been patient and have provided many insights throughout this endeavor. Finally, I would like to give my greatest acknowledgement and gratitude to Dr. Samsam, whom without this thesis would not be possible. I came to you with a question and thanks to you it has turned into a year and a half project that has shown me research like I have never seen. I am very lucky to have been given this opportunity and it was all made possible because of you. Thank you for your guidance, advice, and encouraging me all through this process.
ABSTRACT

As the most widely consumed drug around the globe, there is a vast array of contradicting research available on caffeine. One of the most debated and researched topics on caffeine is its effect on the brain. Meanwhile, the data on the neurological condition of migraine has information scattered throughout countless research articles and experiments.

Although neither migraine or caffeine are completely understood by the medical world, this analysis attempts to give a more coherent understanding of the relationship between the two. This is done by first understanding the known and theorized mechanisms of caffeine as well as the pathologies of migraine. Discussions on channelopathies, current migraine medications, and case studies will be presented.

After much background research, we hypothesized that caffeine could excite neurons at physiological concentrations to the point of activation. This was tested by targeting the transcription factor cFos using immunocytochemistry in vitro. The protein cFos was identified due to its rapid translation—just 15 minutes after stimuli—to indicate activation. In addition to a control culture, three different caffeine concentrations were tested on the neurons: 50 micromoles—average plasma level after 1-2 cups of coffee consumption, 100 micromoles—average plasma level after 5-6 cups of coffee also believed to be the therapeutic amount to defend against neurological diseases such as Alzheimers Disease, and 250 micromoles—the average plasma level considered to be toxic in humans. Indeed, we saw a 53.8% increase in cFos expression in the neurons as 100 micromolar of caffeine was added and exposed to the cell cultures for 24 hours.
In order to ensure the results obtained in this study were physiologically relevant in vivo, known toxic levels were tested for in vitro neurotoxicity. It was found in vitro that at the non toxic plasma concentrations of 50 micromolar and 100 micromolar of caffeine did not display cellular death as tested by Trypan Blue viability testing, Crystal Violet morphologies, and fleurojade immunochemistry that tests for degeneration. Each of these experiments identified a significant death increase as the toxic level of 250 micromoles of caffeine were utilized. This allowed us to theorize that the activation of neurons found in these experiments due to caffeine exposure would apply the same effect in vivo.
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INTRODUCTION

The idea that caffeine may have something to contribute to migraines is not a new idea. It is one that needs more research, but there are a few case studies and experiments that have been performed to try to analyze caffeine’s effect on migraine headaches. The intention of this report is to help bring light to the relationship between caffeine and migraines. With research that points to neuronal activation as the source of migraines, it is possible to hypothesize that caffeine’s stimulatory effects could possibly have a negative impact on migraines, depending on type of neuron and the neurotransmitters and receptors involved. On the opposite position, it could be argued that the indirect vasoconstriction effect of coffee could actually remedy the debilitating symptoms of a migraine onset. Could there be other mechanisms at work in respect to caffeine’s neuronal stimulation and complex vascular consequences that in some way interacts with a migraine? There has been a number of experimental and correlation studies that try to address this question.

Case Studies

Some research has directly identified caffeine as having a role or association with migraines. One study in particular called the Head Hunt Study—or the Nord-Trøndelag Health Survey—is the largest population based study done for migraine headaches (1). The results of caffeine’s effect on migraine was based on cross-sectional data from 50,483 out of 92,566 inhabitants—55%. The study analyzed participants from Norway that were 20 years old and older. The study broke down the variables of each participant by age, gender, migraine frequency, and caffeine consumption. The results demonstrate that migraine frequency is lowest at lowest consumption (0 mg per day) and interestingly enough also lowest at consumption
between mild and extreme (200-330 mg per day). Yet the migraine frequency peaks at 100 mg per day (42% frequency), drops to 200-330 mg (35% frequency) and steadily rises from 330mg a day to 500 mg per day (43%). It seems then that moderate caffeine intake could actually remedy headaches for those susceptible to migraine. However, closer look at the data and considering caffeine’s mechanisms of actions, there may be a less than ideal explanation for these results (1).

Another study performed in Baltimore, MD, Philadelphia, PA, and Atlanta, GA metropolitan areas concluded that dietary and medical caffeine consumption appeared as a modest risk factor for nonspecific chronic daily headache. Nonspecific in this case means migraine, tension, etc. High caffeine dietary consumption was in the upper quartile of risk factors and that chronic long term high caffeine exposure was more significant of a risk factor than just acute infrequent caffeine consumption (1).

A study was conducted at a headache clinic consisting of 36 children (2). These children had a mean age of 9.2 years. Each patient experiencing chronic headache consumed at least 1.5 L of caffeinated cola drinks daily. This amount of cola equates to nearly 2.5 small cups of coffee’s worth of caffeine. The children in the study limited their consumption to .2-.25L a day for two weeks. This is a decrease of 85% of caffeine daily. After two weeks, 33 out of the 36 subjects no longer experienced any form of headache. (2)

A study conducted in Japan attempted to gather commonalities of those who experience migraine headaches and dietary consumption (3). It was found that those with migraine also consume more fatty/oily foods, coffee, and tea than those with non-headache (3).

While conducting more research on this topic another surprising relationship was found. By expanding the research beyond the U.S.A, a direct correlation was found between caffeine consumption and migraine prevalence (4). In a report of caffeine consumption, the top caffeine
consuming countries were reported; it found the Netherlands to have twice the amount of caffeine consumption per capita (4).

Another report comparing Finland, Germany, Greece, Israel, Italy, Netherlands, Norway, Spain, and Sweden on migraine occurrence found some interesting statistics. For most missed days of school or work due to migraine the greatest occurrence was also the Netherlands (5). While the other countries’ coffee consumption stayed relatively equivalent at 1-1.25 cups a day, the Netherlands had a daily coffee consumption of nearly 2.5 cups (4). It may be pure coincidence that Netherlands have both the highest migraine occurrence and the highest coffee consumption. It could be because of the prevalence of migraines that people are drinking coffee to try to alleviate the pain. Although the corresponding results could just be coincidence, it could also be that the coffee is somehow aggravating or triggering the migraines.

Based on these studies alone, it may be worth considering that caffeine causes or at least contributes to migraine occurrence. However, this subject when researched has no direct answer. Caffeine has been praised for its recent research and findings of its potential neuroprotection against diseases such as Parkinson’s disease and Alzheimer’s disease (6). In the field of migraine and pain, caffeine may also have some benefits. Caffeine is an additive in many medications due to its faster absorption and administration efficiency. It is an additive in many non-steroid anti-inflammatory drugs (NSAIDs) for this reason. This is seen in Excedrin tension headache medication, where caffeine is a main ingredient. Not only does it allow for faster absorption of medication, but it is also an indirect vasoconstrictor. (7)

Before moving forward, it is important to have a full understanding of the mechanisms involved with caffeine. The problem is, the methodology and chemical interactions involved with caffeine
are still not completely known. This essay has pulled from many sources in an attempt to give a well-rounded picture of the known components and reactions of caffeine in the body.

**Caffeine**

In humans, the half-life of caffeine is 2.5 to 4.5 hours (8). The major known relation of caffeine’s effect on the brain is the competitive inhibition of adenosine at its corresponding adenosine receptors. In particular, caffeine binds most efficiently to A₁ and A₂a. Adenosine is a molecule that when bound to its receptors has a regulatory effect on the neurological activity within the brain. Adenosine’s purpose is meant to balance the energy consumption and rate of substrate supply. Its homeostatic methods include inhibiting excitatory neurons to reduce firing rate. This is a main effect of the binding of the adenosine acting on its A₁ receptors. (9) A₁ receptors are inhibitory, Gi-coupled receptors that are widespread throughout the brain. A₁ inhibits Caᵥ2 channels that control the calcium intake at the pre and post synaptic terminals of excitatory and inhibitory synapses. One molecule in particular that is susceptible to inhibition via adenosine bound to A₁ receptors is glutamate. In the presence of adenosine, glutamate is diminished at the excitatory synapse by this mechanism. Adenosine inhibits glutamatergic afferents onto hypocretin/orexin neurons. It does this by preventing the entrance of calcium into presynaptic voltage-gated Ca²⁺ channels. In addition, the A₁ receptor bound to adenosine also decreases the calcium entry into N-type channels in the hippocampus. It has also been shown that mesocortical cholinergic neurons are tonically inhibited by adenosine (9).
Figure 1: Adenosine Targets

What does this excitatory inhibition mean? Throughout the day, adenosine levels rise naturally in the brain. Adenosine is created by the enzyme AMP-selective 5'-nucleotidase. The rate that adenosine is created by this pathway is dependent on the amount of AMP (Adenosine Monophosphate) available. Adenosine’s rate of formation is thus dependent on ATP production and breakdown—creating AMP through ATP usage. The two enzymes responsible for the formation of adenosine are adenosine deaminase and more significantly adenosine kinase. As the production and breakdown of ATP continues on throughout the day, there is an accumulation of AMP. ATP releases energy in the form of losing two phosphate molecules. The resulting molecule is AMP. AMP is broken down, and thus leads to a buildup of adenosine. These higher amounts of adenosine are responsible to the gradual fatigue that starts low at the beginning of the day and increases the longer one remains awake. The ATP is being broken down faster (ATP \rightarrow \text{AMP}) than there is of substrate (ATP) available. The adenosine signals to the firing neurons such as GABA (Gamma Amino Butyric Acid) releasing glutamate at the synapses to slow energy utilization. This homeostasis allows for the brain to catch up on ATP production.
Figure 2: The breakdown of ATP resulting in AMP.

Figure 3: Trimethylxanthine; analog of adenine

When the A₁ and A₂a receptors are inhibited, the regulatory actions of adenosine do not take place. There is no inhibition taking place at the pre and post synapses allowing glutamate to continually fire. The Ca_{2} channels are no longer being regulated by adenosine allowing increased calcium intake---increasing positivity of the membrane thus increased ease of depolarization to the action potential threshold---and excitation in the cells. These actions allow for the desired effect caused by a caffeine ‘boost’.
The body cannot operate like this permanently, however, and in response to constant caffeine consumption builds a tolerance against the caffeine. There is some debate as to the mechanism behind caffeine tolerance though it can be agreed that tolerance is built (11). A widely recognized thought is that adenosine receptors increase to combat the competitive inhibition of caffeine. This has been the basis for the understanding of caffeine withdrawal headache which is discussed later in this report. As seems to be the case with much of the evidence found on caffeine, there is research that conflicts this theory. In an experiment performed on mice to measure tolerant mice and control mice brains, there was no difference in the affinity or the amount of adenosine receptors in the cerebral cortex (10).

**Headache and Migraines**

Headache is the manifestation of several diseases such as infections, tumors, and vascular disorders. Primary headaches include tension headache, migraine, and cluster headache. Migraine is generally divided into two categories: 1. Migraine without aura (common migraine about 70%) and migraine with aura (classic migraine, about 30%). However, to the migraineur
the distinction is clear; migraines can be debilitating and last from 4 to 72 hours (12). Many times there is a sense of aura categorized typically by a visual disturbance or sensitivity that some refer to as the “Alice in Wonderland” syndrome. There have also been reported cases of vomiting and out of body sensations leading up to a migraine (14). In most cases, the pain from the trigeminal ganglion is a signature prevalent and distinguishing characteristic in migraines. Although many types of migraines exist, migraines are usually categorized by pain throughout the head with associated vascular dilation. More specifically this pain from migraines is due to inflammation, dilation, and activation of nociceptive afferents in the meningeal and dural vasculature (15). Because of this relationship, it was widely accepted by the scientific community up until recently that the migraines stemmed from vasodilation (16). It is now believed that these changes in vasculature are secondary to the root cause of migraines. More and more evidence is pointing to excitatory effects being the initial causation. In fact, positron emission tomography suggests that neuronal hyper excitability and brain stem activation is what triggers migraine (17).

Cortical spreading depression (CSD) is the aura mentioned earlier that precedes migraines. Although aura is not present in all migraines, the basic changes activated in the brain caused by CSD are still thought to occur. It is a wave of depolarization across the cerebral cortex. The brain stem becomes activated and begins to sensitize the trigeminovascular system. The CSD involves changes in cortical activity, neuronal activation, and finally change in blood flow (15). Because of the vasculature and electrical changes happening in an almost simultaneous manner, it has been difficult to determine exactly which happens first and the appropriate causation. In the most recent findings, it has been theorized that intense neural excitation and activation causes a period of rapid firings followed by neural suppression and
increased blood flow. Although it is accepted by many that there is a sensitization of the nociceptors in migraines, the exact mechanisms and players are still cause for debate in the scientific community. There has been much research conducted with mast cells, endothelial cells, glial cells, and more. Different chemicals and molecules have also been investigated and are taking a front role in identifying possible major correlations such as nitric oxide, calcium, glutamate, CGRP, and many more (18).

Medications are available to help deal with the symptoms of migraine as well as preventative medications focus on different signaling pathways such as NSAIDs, serotonin receptor agonist, nitric oxide inhibitors, calcium channel blockers, antihypertensive and even antiepileptic medications that are taken to prevent seizures, and recently have added antagonists of CGRP and its receptors (18). Genetic predisposition is also a foremost indicator of falling victim to migraines. Familial hemiplegic migraine is a dysfunction of the ion channels and is now the basis of experimentation of knockout mice with the same mutation. This dysfunction may affect the development of CSD, creating a lower threshold for depolarization. The mutation further supports the hypothesis naming neuronal changes and activation as the main culprit of migraine occurrences (16).
VASCULATURE

It is believed that during migraine attacks, some vasoactive and inflammatory substances including CGRP dilate the cranial vessels, leading to the headache phase of migraines (18). Many times when people get a headache, they turn to caffeine for immediate relief. In medicines such as NSAIDs, caffeine is added for increased efficiency. When a habitual caffeine drinker misses their timely dose of caffeine, they may experience what is known as a withdrawal headache. These are all recognized values of caffeine held in the eye of the general public. What is the science behind it, and why the controversy?

Caffeine is an indirect vasoconstrictor. It reduces cerebral blood flow by 20-30%. This reduction of oxygen coupled with the increased metabolism that is characteristic of caffeine use could lead one to theorize that there must be an increase of glucose uptake to supply the fast acting anaerobic metabolism needed in a lowered oxidative state (19). Caffeine’s main mechanism of action is through the blocking of adenosine receptors. When caffeine is consumed, up to 80% of it is metabolized into paraxanthine, an adenosine analog, which has a higher affinity for adenosine receptors than that of just caffeine in its tri-methyl-xanthine un-metabolized form. Adenosine’s effects once bound to its receptors are highly dependent on location. In the spinal cord and within the brain, it has an analgesic effect. However, in the periphery, bound adenosine causes vasodilation. In fact, adenosine concentration in the head and neck is increased up to 68% during a migraine, theoretically contributing to the peripheral pain felt in those with migraine (21). By blocking the adenosine, the vasodilation will be lessened and caffeine becomes an analgesic for migraine. In fact, it has been demonstrated that NSAIDs such as ibuprofen and Advil when coupled with caffeine provide a greater relief from headache than just the anti inflammatory taken on its own. This relief is highest within 3-6 hours of
consumption, tying perfectly with caffeine’s metabolism timespan (20). When these medicines are paired with caffeine, less NSAIDs are needed due to the increased medicinal metabolism as well as caffeine’s own constriction, thus causing less possible adverse side effects from NSAIDs which can occur when taken in excess (21). Adenosine typically does cause vasodilation throughout the body. When blocked, adenosine plasma levels rise causing a release of catecholamines into circulation, renin secretion from the body, and increased peripheral resistance due to the increase in sympathetic tone throughout. This is why it may be advised for those with higher blood pressure to avoid caffeine. For instance, an increase in systolic as well as diastolic pressure is observed following acute caffeine consumption (21).

Caffeine is a remarkable drug. Although it indirectly causes vasoconstriction, it can directly cause vasodilation as well (21). This vasodilation is seen in the endothelial cells and vascular smooth muscle cells. Ryanodine receptors of the endothelial cells and vascular smooth muscles cells are stimulated by caffeine, Ca^{2+}, and adenosine. The receptors are found on the endoplasmic reticulum. When stimulated, Ca^{2+} is released from the reticulum to the cytosol. Those in the cytosol can then bind to calmodulin- a self inhibitory enzyme that needs calcium to bind to remove the inhibition and become activated. Because caffeine also lowers the threshold of “Calcium-Induced-Calcium-Release” from the endoplasmic reticulum and releases calcium into the cytosol, the calcium released stimulates the reticulum to release more. As the intracellular concentration rises, calmodulin catalyzes the pathway for endothelial nitric oxide synthase (eNOS) development. ENOS is the enzyme that catalyzes the formation of Nitric Oxide, which plays an important role in vascular tone regulation. This increase of nitric oxide has a direct vasodilation effect on endothelium. The nitric oxide then leaves the endothelial cell and enters the vascular smooth muscle cell stimulating further vasodilation (21).
Vasodilation is a source of pain in migraine headaches since it may lead to plasma protein extravasion and edema/inflammation in the surrounding tissue. That disturbs the sensory nerves in the meninges (54). Therefore the vasoconstriction caused by caffeine is in the peripheral vasculature in the neck and head can lead to some pain relief. We planned to see the effect of caffeine on endothelial cell cultures but could not do the experiment due to lack of time and resources.
TOLERANCE

What is the cause for the massive headache felt by those habitual caffeine consumers that do not consume caffeine within 24 hours? It is widely accepted and observed that a tolerance based on the consumption pattern of caffeine is generated. A common theory is that in order for the body to compete with the inhibitory effect of caffeine, there is a consequent up regulation of adenosine receptors. More receptors are placed outside of the cell membrane for the unbound adenosine to bind to (21). This forces the caffeine user to increase their caffeine consumption to obtain the same desired outcome by binding to an augmented number of adenosine receptors. When the constant flow of caffeine into the body is suddenly stopped for an extended period time—approximately 12 to 24 hours—there is an insufficient amount of caffeine to offset the magnitude of the adenosine receptors (23). More free adenosine substrates bind to its receptor causing substantial drowsiness and increased vasodilation in the periphery arterioles. This causes the characteristic throbbing withdrawal headache from increased intracranial pressure produced by excessive vasodilation. As the caffeine levels remain low or absent, the body begins to reabsorb these receptors to reach homeostasis of original adenosine receptor concentration usually within a week. This theory has been challenged, however, by an experiment that sought to test the adenosine affinity and concentration bound to receptors once caffeine was abruptly removed from habitual consumption. The results of the study found no change in the number of binding sites or affinity for adenosine in control and caffeine-tolerant rats (22). This evidence would support the hypothesis that an alternative mechanism of tolerance must exist given the theory that receptor concentration would not necessarily lessen the effects of a competitive antagonist. However, there has yet to be a viable alternative to this theory.

Due to the buildup of adenosine by continuous blockade by caffeine, it could be reasonable to
assume that adenosine would begin to compete with the inhibitor. It has been suggested in drug studies that as the concentration build up of the original substrate begins to overpower the inhibitor (caffeine), that the target substrate will have a greater chance at binding. This would cause more inhibitor to be needed to continue its blocking effect (55). This hypothesis would allow the tolerance sensation to make sense without needing an increase of receptor uptake.

Yet when experimented on mice to test for the concentration of A₁ receptors in vivo, found that the Bmax (receptor concentration) to be increased 22% upon chronic caffeine consumption. This level rose for 7 days until it leveled out at a consistent 22% rise about the control for the following 7 days (40).
EXCITATION

The new theory of migraine’s foundation suggests it all begins with the excitation of neurons. The proposed mechanism of cortical hyperexcitability has long been known to be the cause of migraine with aura. In addition to increased excitability and responsiveness, the theory also suggests that there is an excitation-inhibition imbalance in the brains of those who experience migraines (31). However, in the last two decades, migraine is believed to be a brain disorder and a sensory dismodulation (18) (53). Which is triggered by activation of specific parts of the brain stem and hypothalamus (53).

Glutamate

More chemical evidence provides support to the theory of neural excitation in migraines. Glutamate is one of the main neurotransmitters (amino acid) responsible for excitation in synaptic neurotransmission (32). Glutamate as well as its receptors are often found in components related to pain in migraine (33). In those who suffer from migraine, glutamate levels are elevated in the patients’ cerebral spinal fluids (33). Furthermore, glutamate has been linked to the causation of cortical spreading depression. Glutamate is responsible for this excitation by metabotropic receptors—G coupled receptors causing release of intracellular second messengers and their subsequent signaling. In addition to the metabotropic receptors, ionotropic glutamate receptors allow for rapid signal transmission by allowing an influx of calcium in the cell (32).

This would also activate the intracellular signaling cascade responsible for migraine symptoms (32). It is for these reasons that some attention has been given to glutamate antagonists as possible migraine treatment. It is important to note that glutamate does have a regulatory role in neural function therefore the key in treatment is to inhibit without dysfunction. The specific ionotopic glutamate receptors AMPA and kainite were inhibited in a randomized
triple blind study with results that pointed to kainite inhibitors as most promising (32). They also
tested the drug “Memantine” that inhibited N-methyl-D-aspartate (NMDA) receptors also found
on ionic channels activated by glutamate. The results found that the Memantine also showed
promise, specifically to those who often experience aura prior to their migraines (32). Another
study was attempted to discover if memantine also had benefits in migraine prophylaxis with
migraineurs without aura. The results indicated that there were significant therapeutic benefits of
memantine in both groups; there was in overall decrease in frequency and intensity in migraines
without aura. The drug is currently only FDA approved for treatment for Alzheimer’s disease
(34). There are, however several limitations to blocking glutamate. It is necessary for function
and normal excitation in the brain and blocking such a neurotransmitter may have several
negative consequences. Unconsciousness has been one known consequences of this blockade
(18).

Caffeine consumption has been shown to increase glutamate release (35). In an
experiment measuring glutamate, GABA, adenosine deaminase and cFos in the posterior
hypothalamus in vivo of rats found increased levels of glutamate and the transcription factor
cFos. There was a 26% increase in glutamate concentration 30 minutes after caffeine
administration. These levels stayed elevated for at least 210 minutes—the total measurement
timespan. There was no increase in GABA; the molecule designated to inhibit glutamate.
Adenosine deaminase was also activated up to 60%. It is believed that adenosine deaminase not
only helps with immunity but also causes a release of excitatory amino acids along with
 glutamate release (35). Where in the control subject only 6.5% of the ADA positive neurons
were also cFos positive, in the caffeine administered group 66% of the ADA positive neurons
were cFos positive (35).
Adenosine’s main target by binding the A₁ receptor is the major excitatory neurotransmitter glutamate. Adenosine’s binding to glutamate forces the inhibition of its excitatory effect (36).

cFos

The protein cFos mentioned in the prior experiment is a transcription factor that is the result of an intracellular signaling cascade. It is one of the first transcription factors to be translated, approximately fifteen minutes after cell activation. Like its name suggests, this transcription factor signals for the nucleus to transcribe DNA into mRNA which can later be translated into a protein. This transcription factor is therefore used as evidence for activation in cells, particularly in relation to neural activity (37).

In a study trying to map the activation present in migraines by electrical stimulation of the trigeminal ganglion, it was found that cFos was increased in the stimulated trigeminal ganglion (38). The results also revealed that there were no cFos or subsequent activation of the brain stem nuclei typically associated as the migraine ‘generator’. It can be theorized then that the activation of the trigeminal ganglion is migraine pain specific but also a result of the neural activation elsewhere in the brain; not the cause of the migraine.

Although cFos indicates neural activation, could it have another purpose? Excitotoxicity is a condition in which neuronal cell death is induced by excitatory amino acids such as glutamate. A study using immunohistochemistry and kainic acid injections—a glutamate analog that directly stimulates the GluR6 glutamate receptors and is responsible for seizures—found that cFos in normal neurons creates AP-1 complexes that regulate GluR6 (glutamate receptor) and brain-derived neurotrophic factor (BDNF) expression (39). So although it has been found that caffeine can release glutamate, one of the major culprits in migraine excitation, it may be
possible that caffeine is regulating the excitation by transcribing cFos; the very same factor that indicates neural activation.

**CGRP and Cyclic Amino Monophosphate**

Migraine is a disabling neurological disorder affecting 14% of the general U.S. population (13). With every 1 in 7 Americans suffering from this chronic disorder, it is a crucial topic for extensive research in prevention and symptom mitigation. Even with the multitude of drugs such as NSAIDs, anti-epileptics, ergotamines etc., (18) there is still much room for improvement and there does not seem to be one absolute cure all. Recently, there has been a break through in the study of migraines that holds the most promise in migraine prevention drugs (24). Calcitonin gene related peptides (CGRP) have been under scrutiny in the last few years for its role in migraines. Now undergoing clinical research, antibodies specific for CGRP or the CGRP receptor have demonstrated at least a 15% increase in 100% reduction in migraine when compared to placebo with no significant adverse effects. CGRP is like nitric oxide in that it is a molecule that causes vasodilation by acting on the endothelial cells (24). CGRP is thought to have a direct involvement with the pathophysiology of migraines such as inflammation, dural and cerebral vasodilation. This hypothesis is proposed under the observations of the following migraine episode consequences: the increase in CGRP in plasma during migraine, the release of these molecules from the trigeminal nerve, and the ability for CGRP antagonists to reduce these symptoms (25). Further evidence is provided by CGRP sustainability for 4-72 hours; the approximate time of a migraine episode. CGRP is able to continue self regulation by the
inflammatory response it initially causes. Inflammatory mediators—mainly TNF-alpha---provide CGRP a continuous feedback mechanism (25).

One of the most potent and current treatments available for migraine prevention is triptan medication. Triptan reduces CGRP concentration by agonizing 5HT\textsubscript{1B/1D}. It is proposed that Triptans, such as sumatriptans work by increasing intracellular calcium concentration, increasing phosphatases, and thus inhibiting the CGRP feedback loop (25). However, for some people triptans are contraindicated due to its blood vessel constricting tendency (25). This makes CGRP antagonists and antibodies an ideal clinical solution; there is minimal adverse effects to where vasoconstriction is not a concern.

CGRP has also been shown to increase cyclic Adenosine Mono Phosphate (cAMP) accumulation. Adenylate Cyclase is part of the signaling process that breaks ATP down into cAMP. This cAMP is then used to activate Protein Kinase A that is involved in a vast majority of consequent signaling in the body. By increasing cAMP, PKA activation is increased as well. Vasodilation is often a consequence of this signal. This accumulation due to adenylate cyclase activity may be a downstream effect that potentiates migraines. A study attempted inhibition of phosphodiesterase type 3 (PDE3) by the PDE3 inhibitor drug cilozol in a clinical study (26). This inhibition created an increase of cAMP due to phosphodiesterase’s inability to break down the cycle. It was hypothesized that the clients in this trial would succumb to the downstream signaling and by succession migraine caused by this accumulation. The patients involved in this study were 13 women and 1 man. The patients were sufferers of migraine with migraine frequency of 1-6 attacks per month. Approximately 6 hours (with the range being 6-11 hours) after ingestion, 86%---that is 12 out of 14 patients on the medication—developed migraine episodes. This was far greater than the placebo group which experienced 14%—2 out of 14—
patients who had an onset of migraine. The treatments to relieve the attacks were also varied. In this study, the only medication that worked were triptans and these worked the majority of the time. This study strengthened the hypothesis that increased cAMP has a direct correlation to the onset of migraine headaches (26). Not only does CGRP increase cAMP accumulation, caffeine is theorized to also have this effect.

By blocking adenosine receptors, caffeine inhibits phosphodiesterases much like the drug cilotazol. This forces a cAMP build-up and a decrease of cAMP breakdown to AMP (27). Caffeine’s favorable effects such as vasoconstriction and energy enhancement begin 30 min after consumption and peak around two hours. It could be hypothesized that since an occurrence of headache only appeared 3-11 hours after the phosphodiesterase inhibitor cilotrazol was given, that a direct relationship with a later onset of headache induced by caffeine has been overlooked. It is interesting to note, however that caffeine is a very contradictory drug. Although it too does raise cAMP in the cytosol as well as Ca2+ levels, it decreases the TNF-alpha activity by MAPK inhibition. This decrease in TNF-alpha should reduce the amount of CGRP feedback available. However, in another study studying TNF-alpha’s apoptotic efficiency, apoptosis was greatly enhanced when paired with caffeine. So although the TNF-alpha transcription is reduced, if its cytotoxic effects are enhanced, would it attenuate or exemplify CGRP activity? Research points to both as an answer; CGRP is increased by TNF-alpha which is lessened in the presence of caffeine but CGRP too is increased when caffeine is present. A1 receptor inhibition; caffeine’s major mechanism; also has been shown to increase CGRP activity (28).

In an experiment testing caffeine’s effect on CGRP release in mice found that the caffeine causes direct release of CGRP in the spinal cord of rats. It does this by causing a release
of intracellular calcium from the ryanodine-sensitive channels in the spinal cord. The release from caffeine was inhibited by ryanodine and ruthenium red (29).

As far as cAMP accumulation goes, the theory of blocking phosphodiesterase would assume that cAMP accumulation would build and increase PKA downstream signaling that would in turn increase CGRP and cause many of the same migraine pathology that clondizal did (25). However, some studies explain that cAMP may increase initially due to phosphodiesterase inhibition by caffeine, but cAMP concentration will quickly regulate and return back to homeostatic levels. These studies say that once caffeine begins to accumulate the cytosolic cAMP, the adenylate cyclase is inhibited and cAMP levels will normalize (29). This was discussed in an experiment that test cAMP levels in the ameba Dictyostelium discoideum. The research on Dicytostelium Discoideum explains that caffeine’s mechanism of action is not by inhibiting cAMP phosphodiesterase or inhibiting cAMP binding to the receptor but works instead by blocking cAMP dependendent activation of adenylate cyclase. Much like ionophore A23187 that employs the same effect as caffeine, the mechanism lies in the alteration of calcium concentration and distributions (29). While most studies would agree with the change in calcium distribution caused by caffeine, there is disagreement that this would cause cyclic AMP accumulation and directly contradicts current research that states that caffeine’s mechanism of action is through binding or at least inhibiting phosphodiesterases, binding adenosine receptors thus inhibiting adenosine, and that this causes a build up of cAMP.
Introduction

Migraine comes in all types of varieties. They are thought to have the same general mechanism of action yet different patients that present with migraine can have different triggers and symptoms. Although migraine pathology is the root of great mystery in research, more insight on the disease can be obtained by analyzing known and thoroughly studied genetic mutations that can cause a genetic predisposition to migraine. Migraine with aura is believed to be due to channelopathies (Samsam, 2012).

Familial Hemiplegic Migraine is a genetic migraine disease that is caused by mutated calcium channels. The neuronal P/Q-type voltage gated channel in neurons is responsible for familial hemiplegic migraines, episodic ataxia, and seizures (41). One type of calcium channel is calcium entry—such as voltage gated calcium channels-- which can be opened by depolarization and are used frequently at the presynaptic terminal for neurotransmission. There are also calcium release channels—such as ryanodine receptors—that are involved in the release of calcium into the cytoplasm to control excitation (41).

Calciumopathies are an important and recognized subset of the broader condition of channelopathies. Calciumopathies may also include dysfunction of the mitochondria which acts as powerhouse involving constant movement of calcium and interaction with the endoplasmic reticulum to control cellular metabolism. Such a dysfunction can cause monogenic mitochondrial migraine syndrome. Genes recognized for calcium channel mutation have been identified as FHM1/CACNA1A, FHM2/ATP1A2, and CACNA1C (42). Mutations of these genes have been found in familial hemiplegic migraines, seizures, and ataxia. But why does mutation of these genes and consequent channels cause these phenotypes? Since the calcium channels control
excitation, dysfunction of these channels cause hyper excitable tissues (42). Can caffeine cause hyper excitability to these susceptible tissues to cause an induced attack?

It is known that adenosine operates on the Cav2 channels to control calcium influx. When caffeine inhibits adenosine, calcium floods the synaptic terminals creating excitation of neurotransmitters (9). It is possible then, to hypothesize that caffeine could promote episodes of seizure, migraine, or whichever phenotype is present due to the specific calciumopathy. In mice with mutated Cav2.1 calcium channels specific to perkinje fibers only, the results support the hypothesis to be true (43). The effect of the Channelopathy being in the perkinje fibers created Cav2.1 mutant tottering mice with motor dysfunction during attacks. The mice Channelopathy was caused by a haplo insuffiency of the gene Cacna1a—a gene that is also found mutated in familial hemiplegic migraines. The experiment tested stress, caffeine, and ethanol as triggers and found all three to cause a substantial attack increase (43). When the mice were injected with caffeine, attack frequency rose from 0% to approximately 85%. When pretreated with ryanodine, the attack frequency was reduced from 85% to 20%. The significant decrease with ryanodine treatment supports the model of caffeine attenuating attacks by calcium influx at the excitatory synapses. Ryanodine works on the ryanodine receptors—a calcium release gate—to remove calcium from the synapses and into the cytoplasm (43).

Case Studies

Caffeine has long been contraindicated for those who experience seizures due to its inductive nature of epileptic consequences. Two case studies that followed two different patients, both with occasional seizures found that after reducing only their caffeine, the frequency of the seizures decreased greatly. Consider the first case of a 49-year-old male with a 36 years history of mixed seizure disorder. The patient was taking the anticonvulsants phenytoin and primidone
once daily. The patient drank four pints--approximately 1.9 liters--of diet caffeinated tea (diet to avoid increased caloric intake) daily. The patient experienced increased seizure frequency for the duration of the two months of this experiment. After the two months, the patient switched to only 1 pint—500 mL--of the diet tea drink and found his epileptic episodes returned to baseline. The patient repeated this experiment later on only to find the same results (44).

In another case study, a forty-year-old male patient with a history of seizures since eleven years old was experiencing five partial seizures daily and one complex seizure weekly. He was also consuming up to five pints of coffee daily. The patient spontaneously discontinued caffeine consumption and within one week of cessation, found his seizure frequency reduced to only one partial seizure daily and was without complex seizure for the six months reported after the dietary change. The patient was on the antiepileptic drug carbamazepine before during and after this experiment (45).

In a case study discussing a five-year-old girl diagnosed with occipital benign epilepsy, the patient found her seizure-like paroxysmal kinesigenic dyskinesia (PKD) episodes were brought on by exhaustion. Her father had familial hemiplegic migraines and found his episodes brought on by exercise. They found a genetic mutation in this particular family of 649dpC in the PRRT2 gene, 2nd exon. She too was partially controlled by the antiepileptic drug carbamazepine. This studies conducted found that PRRT2 mutations can cause PKD as well as familial hemiplegic migraines (46).

Another study with mice used Cav2.1 knockin migraine mice to study spreading depression, the signature of migraine aura and possibly migraine activation mechanism. The Knockin mice carried a familial hemiplegic mutation (FHM1) that demonstrated cortical spreading depression due to calcium influx at the presynapses. The experiment found support for
the model that the activation of the P/Q-type presynaptic Ca2+ voltage gated channel released glutamate from the cortical pyramidal cell synapses, activate NMDA receptors, creating a positive feedback cycle with increasing extracellular K+ buildup. This excitation and potassium build up is enough to cause a cortical spreading depression. In the mutation tested, less K+ build is needed to overwhelm the cells’ mutated Ca2+ channel into the positive feedback cycle causing CSD (47).

What causes the mutation in these genes to express a phenotype of either migraines or seizures when induced? And what other proof is there that these two diseases are so closely linked? It has been proposed that the hyperactivity and overexcitation would produce cortical spreading depression instead of a seizure in migraines is that CSD is just a poorly controlled epileptic episode with disrupted potassium regulation. It has been shown that the three genes that when mutated can cause familial hemiplegic migraine can also cause seizures. The triggers for FHM can also trigger seizures. Anticonvulsants have also been used successfully as prophylactic medication for migraines (47).

**Medications**

Because anticonvulsants are used for migraines, it is important in this analysis to note the relationship that caffeine has with these drugs and thus on migraine prophylaxis. AEDs (antiepileptic drugs) are known to inhibit the enzyme cytochrome P450. This enzyme metabolizes caffeine in the body and if inhibited would increase the the toxicity of caffeine (44). However, when looking at the reverse relationship, caffeine does not appear to alter the concentration of AED concentration or presence in the blood when taken (44).

As well as the use of anticonvulsants or AEDs, there are several medications used to prevent migraines that alter the channel responses in neurons. Opiates are used to decrease the
calcium influx into the neuron, decreasing the excitatory neurotransmission at the synapses. Opiates also activate K+ efflux from the cell to reduce the positive charge of the excited cell (48). Calcium channel blockers are also used, often successfully, to reduce the calcium influx and decrease the excitation of neurons to prevent over activity from triggering a migraine episode (18).

Glutamate, another protein activated indirectly by caffeine consumption, can be inhibited to prevent migraines. Glutamate inhibitors can decrease neuronal activity in the brainstem’s trigeminal nucleus which has been proposed to be the activation site of migraines (18).

The protein cFos that has been mentioned throughout this analysis has been used to indicate cellular activation due to the fact that it is one of the very first transcription factors expressed to transcribe DNA to become protein. In an experiment studying trigeminal activation, cFos was used as a marker to indicate activation of the neurons (49). For these reasons cFos protein was the major marker tested for in the in vitro experiments analyzed in this report.
LABORATORY METHODS

To say there is more research needed on caffeine, migraines, and the relationship between the two is an understatement. There are an abundance of theories and heaps of experiments and research that all seem to contradict one another. Because so many different components are involved, it is difficult to determine if the scientific community will ever really understand the source and the mechanisms of migraine, and what causes the susceptibility of having a migraine episode greater in one person than another. With the ever rising popularity of caffeine consumption, it is imperative to understand what is being put in our bodies and how it can affect us. To see the effect of caffeine on neurons can shed light to its possible effect on our nervous system and may increase our understanding of its effects in pathological conditions where increased neuronal activity might contribute to the disease pathophysiology.

The goal of the following experiments is to help do just that. The goals of this experiment were to learn neuronal cell culture growth maintenance and morphology. Investigate neuronal cell response by introducing caffeine into cell culture. Detect the concentration that caffeine causes neuronal cell toxicity and death. Finally, to look for CGRP activity following caffeine induction of the neuronal cell culture. The experiments performed were to see neuronal morphology and response evaluation at different physiologically relevant caffeine concentrations tested by Trypan Blue, crystal violet, and Fluorojade. Excitation due to caffeine exposure was then tested by Immunocytochemistry analyzing cFos expression.

Aim of the Study:

The aim of our study was to look for the neuronal changes following application of various concentrations of caffeine for various amounts of time in neuronal cell culture. For future additions, we want to look for such changes in endothelial cells and coculture with neurons.

Neuronal Cell Culture
**Methods**

Human Neural Stem cells were taken from cryo conditions and thawed quickly at room temperature and placed in T-75 suspension flask and suspended in 20 mL of HNSC media.

Cultures were incubated at 37 degrees Celsius and fed every 3-5 days. Feeding was performed by 50/50 media exchange of removing old media/debris and adding new HNSC media. Once cells reached approximately 90-100% confluency, the cells were passaged or split into two flasks.

After three to four weeks the cells were cultured enough to allow for differentiation. This was performed by removing all HNSC media and placing cells into T-25 adherent flask with 10 mL of NT-2 media. This media was exchanged 50/50 every 3-5 days for two weeks until differentiation was achieved.

*Culture with Caffeine*

Caffeine powder (sigma) was diluted with deionized water to a working stock of 200microgram/mL (1.04 mM). Physiologically relevant caffeine concentrations were applied to the neuronal cell cultures that the tests in this experiment were performed on.

**Protocol**

**Materials**

- Human neural stem cells (HNSCs) and media provided by Dr. Kiminobu Sugaya
- HNSC culture media
  - 500 mL DMEM/F12
  - 0.04% (2mL) 500 U/mL heparin
  - 0.2% (1mL) 10ug/mL EGF
  - 0.2% (1mL) 10ug/mL bFGF
  - 2% (10 mL) B27 stock
- Neuron differentiation media (NT2 Media)
Culture protocol

• Once HNSCs are thawed from the cryo tube, they are quickly added to a T75 suspension flask with 20mL HNSC media pre warmed to 37 degrees Celsius.
• Cells are able to grow and proliferate when incubated in this media at 37 degrees Celsius.
• Allow cells to grow and proliferate for about 4-6 weeks.
• During this time, every 3-5 days, replace 10 mL of culture HNSC media with fresh HNSC media while avoiding floating cell colonies. This allows for removal of waste product and introduction of more nutrients to continue proliferating.
• After about 2 weeks, the cells may need to be passaged and/or split into two flasks. This happens when the cell colonies near 100% confluence. If the colonies look large enough to be cut mechanically, passage the cells under the hood with a razor.
• Cut the colonies finely and place back into flask and continue proliferation.
• Passaging may need to be performed once weekly as the cells continue to grow and colonize.
• After about 2-3 passages, the cells will be ready to differentiate with NT-2 media.

Differentiation protocol

• Remove the 20mL of media and any suspended cells from flask to conical tube.
• Allow cells to settle to the bottom of the tube.
• Using 5 mL of NT-2 media, dislodge any adherent cells by gently rinsing.
• Transfer the cells and NT2 media to an adherent T-25 flask.
• Remove the HNSC supernatant from the conical tube and resuspend the cells with 5mL of NT-2 media. Transfer this 5 mL to the T-25 flask to have a total of 10mL.
• Continue to do 50% media exchange with NT-2 media every 5 days for a week or two of differentiation.

Seeding

• Remove NT2 media. Differentiated neurons will be adhered to the flask.
• Wash gently with PBS to remove any media as the media will deactivate the following enzyme:
• Add 1 mL of collagenase to the flask.
• Incubate at 37 degrees celsius for 30-45 minutes.
• Neutralize the collagenase with 2mL of NT2 media
• Rinse the bottom of the flask with the media dislodge any stubborn neurons
• Centrifuge the cells and the added 3mL of media at 0.2 rcf for 3 minutes
• Remove supernatant
• Replace with 5mL of NT2 media
• Aspirate and seed into either chamber slides or well plates.
Caffeine exposure

Caffeine powder (sigma) was diluted to 200 microgram/mL.

The four concentrations were used:

1. Control: 0 microgram/mL, 0mL
2. 1-2 cups of coffee in plasma equivalent: 10 microgram/mL, 52 micromolar
3. Therapeutic concentration thought to protect against alzheimers: 20 microgram/mL, 104 micromolar
4. Neurotoxic concentration: 50 microgram/mL, 260 micromolar

In 200 microliter chambers

1. 0UL caffeine in 200UL media
2. 10 UL caffeine in 190 UL media
3. 20 UL caffeine in 180 UL media
4. 50 UL caffeine in 150 UL media

In 400 microliter wells

1. 0UL caffeine in 400 UL media
2. 20 UL caffeine in 380UL media
3. 40 UL caffeine in 360UL media
4. 100 UL caffeine in 300 UL media

Incubate at 37 degrees Celsius in incubator for 24 hours
RESULTS

Morphology of the neuronal cell culture following exposure to different concentrations of caffeine with and without Crystal Violet Staining.

Caffeine is one of the few molecules that can cross the blood brain barrier. In less than an hour after consumption, enough has crossed to begin causing noticeable and often desirable effects. Although caffeine can directly have an effect in the brain and body, up to 80% of caffeine may be metabolized to paraxanthine. Paraxanthine has a stronger binding affinity to adenosine receptors. Therefore, it was important to test the levels used in the following experiments to represent physiological relevance. It has been discovered that the one to two cups of coffee equivalent of caffeine in the plasma is 10 microgram/mL or 50 micromolar. Therapeutic levels up to 4-5 cups of coffee is 20 microgram/mL or 100 micromolar. Finally, toxic levels of caffeine have been reported to be 50 microgram/mL or 250 micromolar (51).

Human neural stem cells (HNSCs) were differentiated for two weeks into neurons in individual wells. The cells with the four different concentrations—0 microgram/mL, 10 microgram/mL, 20 microgram/mL and 50 microgram/mL—were exposed to the caffeine for 24 hours. There were two sets of each to ensure reliability of the results.

Because the neurons were adhered to the bottom of the well, one set of the four different concentration wells were lifted with accutase and the other with trypsin. In order to include any dead floating cells, the media with debris was removed first and later added to the lifted cells, and all centrifuged down together to create a pellet. The pellets' supernatants were removed and replaced with 100 microliters of media and later 100 microliters of trypan blue creating a 1:1 dilution.
Figure 1
Neuronal Cell Culture Morphology without Crystal Violet Staining after three weeks of neuronal differentiation. These cultures display mature neurons in all three cultures. Some debris is visible at the 250 micromolar of caffeine level.
A: 0 micromolar of caffeine applied
B: 50 micromolar of caffeine applied
C: 100 micromolar of caffeine applied
D: 250 micromolar of caffeine applied

Figure 2
Neuronal Cell Culture morphology without Crystal Violet Staining. Cell cultures recorded during
the first week of stem cell differentiation into neurons. Caffeine concentrations applied may have matured the differentiation of the cells as seen from the axon projections and separation from the neurospheres seen between cultures A (0 micromolar of caffeine) and B (50 micromolar of caffeine) as well as C (100 micromolar of caffeine). After applying 250 micromolar of caffeine, there was visible debris possibly due to cell death or decreased viability as seen in D₁. In another reference frame from the same cell culture, the cells were viable with maturing neurons as seen in D₂.

**Figure 3**
Crystal Violet stain to show the morphology of caffeine 3 weeks after stem cell differentiation into non specific neurons
A1/A2=control B1/B2= 50 micromolar caffeine, C1/C2= 100 micromolar caffeine, D1/D2=200 micromolar caffeine

**Neurotoxicity to determine physiological significance using Trypan Blue, Crystal Violet staining, and Fluorojade Immunochemistry**

The experiment was optimized to test physiologically relevant caffeine concentrations to in vivo pathology. Many in vitro experiments use extremely toxic caffeine concentrations approximately 1mM. The use the in vivo plasma value of toxic caffeine concentration is 250 micromolar and non toxic average caffeine consumption concentrations ranging between 50 and 100 micromolar. These concentrations were tested in the neuronal cell cultures in vitro. If toxicity results were present at 250 micromolar and not at 50-100 micromolar of caffeine in vitro, caffeine concentrations in vitro—for both neurotoxicity and theoretically excitation--results mimic in vivo pathology.

**Trypan Blue**

By using the four corner squares of the hemocytometer, each sample gave between 30-50 cells total. Based on these results, the average consumption (1-2 cups of coffee) kept the viability around the control's viability percentage within 4%. At the reported therapeutic levels, viability was decreased up to 8.4%. Finally, as predicted, Toxic levels decreased viability much lower than 10-20 microliters as previously discussed. Toxic levels decreased viability up to 31%.
### Percent Viability of Neuron Cultures after Caffeine Exposure at different Concentrations

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Accutase</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 microliters (control)</td>
<td>58.3%</td>
<td>60%</td>
</tr>
<tr>
<td>10 microgram/mL 50 μmol (Avg. consumption)</td>
<td>57.6%</td>
<td>63.9%</td>
</tr>
<tr>
<td>20 microgram/mL 100 μmol (therapeutic)</td>
<td>52.2%</td>
<td>51.6%</td>
</tr>
<tr>
<td>50 microgram/mL 250 μmol (Toxic)</td>
<td>26.5%</td>
<td>38.2%</td>
</tr>
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</table>

### ADJUSTED

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Accutase</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 microliters (control)</td>
<td>100.000%</td>
<td>100%</td>
</tr>
<tr>
<td>50 μmol (Avg. consumption)</td>
<td>99.300%</td>
<td>104%</td>
</tr>
<tr>
<td>100 μmol (therapeutic)</td>
<td>93.900%</td>
<td>92%</td>
</tr>
<tr>
<td>250 μmol (Toxic)</td>
<td>68.200%</td>
<td>78%</td>
</tr>
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</table>
Figure 4:
Percent viability of neuronal cell cultures after caffeine exposure at different concentrations show in a table and chart. Neuronal cell cultures were lifted from flask using either accutase or trypsin enzymes. While accutase is known to be more gentle than trypsin, both applications are known to cause possible death as seen in the control culture as a baseline for viability. Adjusted table and chart show the same data but set the control groups with nuclease induced cell death as 100% viability.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Viability (%)</th>
<th>Enzyme</th>
</tr>
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<tbody>
<tr>
<td>0 microliters (control)</td>
<td>100%</td>
<td>Accutase</td>
</tr>
<tr>
<td>50 μmol (Avg. consumption)</td>
<td>99.30%</td>
<td>Trypsin</td>
</tr>
<tr>
<td>100 μmol (therapeutic)</td>
<td>93.90%</td>
<td></td>
</tr>
<tr>
<td>250 μmol (Toxic)</td>
<td>68.20%</td>
<td></td>
</tr>
</tbody>
</table>

0μM

50μM

100μM

250μM
Figure 5:
Neurotoxicity of neuronal cell cultures after Crystal Violet Staining. Crystal Violet staining is able to infiltrate and thus dye cells with ease as permeation increases indicating cellular death. The control cells as seen at 0uM do indicate some darker cell clusters. These darker cell clusters are seen as indicated in the culture with 50uM of caffeine. As the concentrations increase, the darker clusters become more frequent and prominent only slightly at 100uM caffeine but undeniably at 250 uM of caffeine.

Figure 6
Fluoro Jade (green) and DAPI (stain nuclei blue) Fluorojade’s target of immunochemistry is currently an unknown neurodegenerative factor seen in green (seen at wavelength identical for viewing FITC) Neurotoxicity signal is greatest at 250 micromolar of caffeine. It is nearly non
existant for control and 100 micromolar of caffeine. At 500 micromolar of caffeine, neurodegeneration is still visible but begins to fade; this may be due to total death of the neurons.

**Excitation: cFos immunocytochemistry**

**Methods**

After differentiation, cells were lifted from T-25 adherent flask and seeded onto a chamber slide coverslip (Protek) containing 8 individual 1 mL chambers. These working chambers were best utilized with 200-500 microliters of media. The cultures were left to adhere in 37 degree incubation in NT-2 media for 48 hours before any other experimentation. Cells were exposed to 0, 50, 100, and 250 µmol of caffeine for 24 hours. After 24 hours, the caffeine was removed, the samples washed and fixed with Paraformaldehyde (PFA) in 4 degrees Celsius overnight. Immunocytochemistry was performed with mouse anti-cFos polyclonal antibody (Abcam) and rabbit monoclonal anti-CGRP antibody (Biocompare).

**Results**

Caffeine concentrations were optimized as discussed using in vivo plasma levels of neurotoxicity due to caffeine consumption and tested them in vitro. Once optimized, Immunocytochemistry was performed to detect excitation with anti-cFos antibody and the neurotransmitter CGRP was selected against using anti-CGRP antibody. The nuclei were stained with DAPI mounting medium. It was found that, possibly due to natural deterioration of the anti-CGRP antibody, that it was less effective in targeting or binding correctly to CGRP receptors. However, the results show that cFos is expressed. The staining with DAPI did not select specifically for neurons, as glial cells were also created during differentiation. In order to identify specifically neuronal cells, Beta Tubulin III marker was used in later experiments.
Figure 7

ICC performed with mounting media with Dapi, dilution of polyclonal anti-cFos at dilution of 1:50 cFos (red), 1:25 CGRP (green) Neurons are visualized by blue--DAPI, CGRP--green, cFos--red, and the final picture is overlapped of all three. There is no CGRP expression in the cell cultures but may be expressing some signal after 100 micromolar caffeine exposure. In all pictures except the 250 micromolar cFos expression was seen.
Immunocytochemistry was performed again but this time without anti-CGRP antibody.

**Figure 8**
Mounting media contained Dapi (blue), and anti-cFos polyclonal antibody at a dilution of 1:100 (TRITC/red). The DAPI binds to the nucleus of all cells and is shown as blue. The anti-cFos antibody was visualized by TRITC fluorescence as indicated in red. The images displayed revealed varying levels of cFos expression after caffeine exposure. However, since the cells differentiated nonspecifically in the neuronal cell culture, it was impossible to know if the cells expressing activity were glial or neurons.

**Immunocytochemistry using anti-Beta Tubulin III (FITC) and anti-cFos antibodies (TRITC) after 24 hours of caffeine exposure**
Immunocytochemistry was performed again using anti-beta tubulin III to visualize neurons. This was because human neural stem cells do not specifically differentiate into neurons. They differentiate non specifically to glial/astrocytes and neurons. This is important to allow for proper functioning and cellular maintenance in vitro.
Figure 9
Immunocytochemistry indication cFos expression (TRITC) and neurons with Beta III Tubulin (FITC). Areas where the signals overlay are seen in a yellow color. The increase of yellow upon increasing caffeine exposure indicates

cFos expression in Neuronal Cell Cultures after 24 hour exposure to different caffeine concentrations

<table>
<thead>
<tr>
<th>Caffeine concentration</th>
<th>cFos Expression</th>
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<tbody>
<tr>
<td>Control</td>
<td>22.92%</td>
</tr>
<tr>
<td>50 µmol</td>
<td>47.22%</td>
</tr>
<tr>
<td>100 µmol</td>
<td>76.74%</td>
</tr>
<tr>
<td>250 µmol</td>
<td>54.55%</td>
</tr>
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</table>
Figure 10

cFos expression in Neuronal Cultures after 24 hour exposure to different caffeine concentrations displayed in a table and chart. Increase of cFos expression is seen through 100 micromolar of caffeine exposure. This cFos expression begins to decrease at 250 micromolar caffeine exposure. This may be due to the toxicity at this level causing a combination of death as well as excitation of the neurons.

15 min

<table>
<thead>
<tr>
<th>Caffeine Concentrations</th>
<th>0 Micromolar Caffeine</th>
<th>100 micromolar caffeine</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 μmol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μmol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 μmol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 11**
cFos expression after caffeine application after 15 min, 24 hours and repeated exposure. The repeated exposure was conducted by adding just NT-2 media to the control neuronal cell cultures. The experimental cell culture had 100 micromolar of caffeine added. The cultures were permitted to incubate for 24 hours. After 24 hours the control cell culture media was removed, rinsed with PBS, and new NT-2 media was added. The caffeine exposed culture had its media removed, rinsed with PBS, new NT-2 media was added with new 100 micromolar of caffeine. The cultures were then permitted to incubate for 2 more hours. The control sample after 15 min shows significant increase in cFos expression possibly due to the manual manipulation of added NT-2 media. 15 minutes is approximately the amount of time that cFos transcription factor is expressed after stimulation.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>100 μmol</th>
<th>0 μmol</th>
<th>Percent Changed</th>
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<tbody>
<tr>
<td>15 min</td>
<td>94.4%</td>
<td>75%</td>
<td>20%</td>
</tr>
<tr>
<td>24 hours</td>
<td>79.2%</td>
<td>30%</td>
<td>49.20%</td>
</tr>
<tr>
<td>24 hours +2 hours</td>
<td>75.3%</td>
<td>0%</td>
<td>75.3%</td>
</tr>
</tbody>
</table>

Table 2

**Figure 12**
cFos expression after Caffeine Application at different time intervals as represented in a chart and a table. cFos expression is highest at after 15 minutes of 100 micromolar of caffeine. The control group is also elevated to 75% excitation. This may be due to the stimulus of changing the media. As the cultures recover from the manual shock, the control groups begin a decline of cFos expression, while the 100 micromolar caffeine exposed samples show a plateau of 75% excitation. This elevated expression is seen after 15 minutes, 24 hours, and after two exposures.
DISCUSSION

The experiments performed in the lab were conducted to better determine caffeine’s effect in the brain, particularly its role in migraine. Human neural stem cells were differentiated non specifically to neuronal and glial cells in vitro. Caffeine has been the base of many experiments in vitro and in vivo. However, in most of the in vitro experiments, caffeine is tested at molarities not reflective of true human physiological serum concentrations. Many experiments test caffeine of at least 2mM, when in human serum the toxic level is 250 µmol approximately 50mg/L (50). Average consumption of caffeine plasma levels is approximately 50 µmol, 10mg/L, amounting to approximately 1-2 cups of coffee. The therapeutic 100 µmol or 24mg/L is approximately up to 5-6 cups of coffee. These concentrations, 0 µmol, 50 µmol, 100 µmol, and 250 µmol were utilized our experiments to construct a physiologically relevant in vitro experiment.

The neurotoxic levels were first tested to ensure that the known toxic level in vivo would indeed cause a neurotoxic episode in vitro. The neurons were permitted to be exposed to these concentrations for 24 hours. The cultures were then tested with Trypan Blue—a dye specific for dyeing dead cells a deep blue—by hemocytometer counting to calculate percent viability after being exposed to caffeine. This was performed after using two different lifting agents: Trypsin and Accutase. Acutase is slightly more gentle for lifting than Trypsin and this is seen in the results displayed in table 1. There was a significant increase in dead cells when counted with the hemocytometer as caffeine concentration increased. Percent viability decreased from 60% from the control, therefore, an adjusted chart and table were created to exclude the baseline death caused by the nucleases. In the unadjusted information, Percent viability decreased from 60% to 50% in 100 µmol and finally down to nearly 30% at 250 µmol. These results reflect the
neurotoxicity in vitro to be the same as in vivo at 250 µmol. Taking the baseline death into consideration, meaning the control 60% is set as 100% viability. This shows that the cell cultures stay within 90-100% viability until they reach the toxic level of 250 micromolar of caffeine where they drop to as low as 68.2% viability. However, the results also show that the proclaimed therapeutic concentration may also be a cause for slight toxicity in some neurons since they do not remain at 100% viability at 100 micromolar of caffeine.

To visualize the toxicity further to determine the relevance of these averages in vitro when compared to in vivo, a crystal violet staining was performed. The four concentrations were tested again on the neurons and were exposed for 24 hours before being fixed with paraformaldehyde. Dark purple stains are indicative of significant stain infiltration of the crystal violet into the malleable and dying cells. Indeed, the results show the darkest purple stains in the 250 µmol well alluding to the predicted neurotoxicity. Finally, neurotoxicity was visualized with a fluorojade immunochemistry. Fluorojade is a relatively new immunochemistry reagent that binds to an unknown factor indicating neurodegeneration (52). The images reveal little to no signal when not exposed to caffeine and at the therapeutic level of 100 µmol. The fluorescence signals of fluorojade were hypothesized to be strongest at 250 µmol and 500 µmol. The results of all three of these neurotoxic tests indicate that the serum levels of toxicity in vivo are applicable in vitro. The average and therapeutic levels reported in vivo are physiologically relevant and non toxic in vitro. This gave clearance to analyze further caffeine’s effect on neurons in vitro.

Mentioned in the prior analysis is the new research on calcitonin gene related peptide (CGRP), its correlation with migraine, and new drugs in clinical trials for migraine relief that inhibit this protein. There was mouse anti-CGRP antibody conjugated with FITC fluorescence already present in the lab. However, due to improper storage for over 365 days in a 4 degrees
Celsius refrigerator, there was no saying if the antibody maintained its binding properties to CGRP. The antibody was tested on the neurons regardless and this data proved to be inconclusive as shown in the results section. It may be that the antibody used had lost its binding ability over time or it may be that the specific neurons that differentiated do not express CGRP.

The cFos protein is commonly used to show excitation as it is one of the first transcription factors present when neurons are activated. This anti-cFos antibody was first targeted with a DAPI counter stain. The results show that the cFos was indeed visible but further experimentation needed to be performed. DAPI binds non specifically to the nucleus of cells, and since the neurons specifically were of focus in the experiments, neurons needed to be more accurately identified. For this reason, the experiment was repeated with anti-beta III tubulin antibody. Beta III tubulin is a well known neuronal cell marker and when bound by anti-beta III tubulin antibody can be used to visualize the entirety of the cell. This counter stains against the anti-cFos antibody at the different concentrations mentioned were performed. The cells were counted and a dose dependent correlation between excitation and caffeine exposure was discovered.

The experiment was performed again, this time knowing that the greatest cFos expression was seen at the physiological therapeutic level of 100 µmol. Because tolerance to caffeine builds quickly the cells were exposed to caffeine at different time intervals. The first set of cells were exposed to either 0 µmol or 100 µmol of caffeine for 15 minutes before being fixed in paraformaldehyde. The cFos expression is thought to occur within the first 15 minutes of cellular activation which is why it is deemed as one of the first transcription factors to be translated. Indeed, the expression increased 20% from 75% to 95% cFos expression in the neurons. The expression may have been already higher than predicted due to the recent movement of the
media being added to both wells. This physical stimulation may have caused an expression of cFos alone. The second set of neurons were exposed to caffeine overnight for 24 hours, much like the first experiment and found the cFos expression increase from 30% to 79.2%. Finally, the last set of neurons were exposed to caffeine for 24 hours, cultures rinsed with PBS, and media replaced with 0 μmol and 100 μmol of caffeine and exposed for 2 hours before fixing with PFA. The cells in the control well expressed no cFos protein while the neurons that were exposed to the caffeine twice showed a 75% expression of cFos.

Due to the slight decline of cFos expression in the toxic level of 250 μmol, it is hypothesized that the cFos expression was not present or impossible to bind in dead cells. The cFos protein has also been proposed to counteract neurotoxicity when over stimulated. This hypothesis can be used to explain why the cFos expression increases as caffeine also increases. It could be that the neurons are increasingly excited releasing more of the transcription factor cFos, or that they are working harder to combat the over stimulation by producing more cFos for protection. It also makes sense in the slight depression in cFos expression due to dying cells. The dying cells that could no longer fight off the toxicity would no longer be expressing cFos.

Migraine is believed to be a brain disorder, a sensory demodulation that is triggered by activation of specific parts of the brain stem and hypothalamus (53). In particular, the headache phase of migraine is associated with vasodilatation and release of vasoactive neurotransmitters, such as CGRP, and neuromodulators (54). For these reasons, an increase in excitation of neurons is the leading proposal of migraine causation. This over excitation causes a cascade effect that results in cortical spreading depression, dilation and neuroinflammation of peripheral vessels in the dura mater of the brain as well as activation of the nociceptor pathway creating pain.
In particular, patients with Channelopathy are more susceptible to neuronal excitation due to the lower threshold and their deficiency to control the positive feedback loop that is the calcium influx at calcium voltage gates at the synapses. Caffeine being a base analog of adenosine uses the inhibition of adenosine to create a multitude of excitation events. It has been proposed that caffeine inhibits phosphodiesterase, causing a build up of cAMP to increase biochemical signaling. Caffeine has also been shown to excite neurons by drawing calcium into calcium voltage gated channels at the synapses. Caffeine also disrupts adenosine’s natural tendency to depress the nervous system by reducing firing rates and inhibiting glutamate. Caffeine for this reason causes increased firing uninhibited in the brain.

Caffeine also indirectly causes vasoconstriction due to its adenosine receptor binding action. It also allows for more efficient metabolism of NSAIDs which is why it so often is used acutely to relieve headaches. With all of these events that happen in the brain, it could be hypothesized that caffeine has the potential to over excite the neurons in the brain which could leave those vulnerable to migraines at risk of episodes, particularly those who have been diagnosed with familial hemiplegic migraines. Caffeine also lowers the seizure threshold in those with genetic Channelopathy that leaves them susceptible to epileptic episodes (50).

Channelopathy heredity such as seizures have genetic ties to familial hemiplegic migraines. These two diseases are caused by mutations in the same three genes (42).
CONCLUSION

What is the effect of caffeine on migraine headaches? The leading theory on migraine causation is that excitation of neurons in the hypothalamus and brain stem are the cause (53). This creates activation of the nociceptive pathways and neurogenic inflammation of the vessels causing the headache phase of migraine. The experiments performed were to first optimize the caffeine concentrations utilized to correlate with in vivo physiology. This was found by testing known toxic and non toxic caffeine plasma concentrations in vitro. Next, was to test if caffeine could excite the cells to the point of activation. This was found by targeting cFos using immunochemistry. Finally, we tested the effect of multiple exposures of caffeine on neural cell cultures as related to excitation. We hypothesized that knowing caffeine’s stimulatory mechanisms there should be activation of the neurons.

The toxic levels of caffeine in vivo begin at 250 micromolar caffeine. This was found to be the case from the results of the in vitro studies. Trypan blue testing revealing percent viability found a 30-40% decrease in the toxic cell cultures than in both the control and physiologically acceptable caffeine concentrations. The Fluorojade immunochemistry revealed that only at concentrations tested at 250 micromolar of caffeine, was neuro degeneration detectable. Finally, the increase of crystal violet infiltration into the permeable cells of 250 micromolar caffeine suggest increased degeneracy.

We then tested for excitation by targeting the transcription factor cFos with immunochemistry. We found a 53.82% increase of cFos expression from the control (exposed to no caffeine) to the neuronal cell culture exposed for 24 hours with 100 micromolar of caffeine. Upon different time intervals of caffeine exposure, we found this similar pattern of cFos expression again. From 15 minutes we saw a 20% increase of excitation from the control to 100
micromolar caffeine exposed group. After 24 hours exposed to 100 micromoles of caffeine, we found a 49% increase in this particular neuron culture from the control group. Finally, after repeated exposure of 100 micromoles of caffeine, we saw the control group level out with no cFos expression and the caffeine exposed group beginning to plateau at 75% cFos expression.

The results obtained from the experiments performed and analyzed in this report demonstrate that physiological concentrations and pathologies seen in vivo are relevant in neuronal cells in vitro. They also reveal that caffeine causes an increased state of excitation and neuron activation at average consumption levels in the form of cFos expression in as little as 15 minutes and begins to plateau with repeated exposure. These results support the notion that caffeine at the physiological level could induce an over activated state of neurons that could lead to a migraine episode.

**Implications for future experiments**

Unfortunately, time and resources did not permit for differentiation of peripheral blood stem cells to endothelial cells or work on rat dura mater in this study; these could help clarify many unknowns about caffeine’s effect on the brain. It would be beneficial in the future to create an in vitro co-culture of endothelial cells and neurons to see the relationship of the two types of cells’ interactions and cross talk once exposed to caffeine, in particular by targeting the CGRP proteins. It may also be performed in the future the physical changes that the vasculature undergoes at real time when exposed to caffeine. Finally, future studies regarding familial hemiplegic migraines in knock in mice or case studies where caffeine is the independent variable could provide further insight if caffeine’s effect on the calcium channels negatively or positively affects Channelopathy in the form of migraines.
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