VALIDATION OF REFERENCE GENES FOR RT-PCR STUDIES OF MIGRAINE TRIGGERS IN CA77 CELLS

by

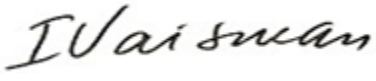
Katherine Mireles
A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

Committee:


_____ _____ Dr. Ancha Baranova, Thesis Chair


_ ____ Dr. Margaret Slavin, Committee Member


_____Dr. Aybike Birerdinc, Committee Member


_____ Dr. Iosif Vaisman, Department Chairperson


_ _____ Dr. Donna M. Fox, Associate Dean, Office of Student Affairs & Special Programs, College of Science


_____Dr. Ali Andalibi, Dean, College of Science

Date: _____5/22/2020_____ Spring Semester 2020
George Mason University, Fairfax,Va

Validation of Reference Genes for RT-PCR Studies of Migraine Triggers in CA77 Cells
A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Katherine Mireles
Bachelor of Science
Ferrum College, 2014

Director: Ancha Baranova, Professor
School of Systems Biology

Spring Semester 2020
George Mason University
Fairfax, VA

DEDICATION

I would like to dedicate this to my parents and my brother Rob. Thank you for always supporting me.

ACKNOWLEDGEMENTS

I would like to thank all of my committee members Dr. Baranova, Dr. Birerdinc, and Dr. Slavin for all of their help and support. I would also like to thank Dr. Lipsky for allowing me to use the equipment in his lab. Lastly, I would like to thank Sasha Stoddard for all of her assistance with troubleshooting and data analysis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS AND SYMBOLS

Cortical Spreading Depression..... CSD
Calcitonin Gene Related Peptide.....CGRP
Real Time Polymerase Chain Reaction ..... RT-PCR
Nerve Growth Factor.....NGF
Messenger Ribonucleic Acid .....mRNA
Complementary Deoxyribonucleic acid .....cDNA
Nanograms .....ηg
Microliter ..... µl
Degrees Celsius .....^{o}C
Cyclic Adenosine Monophosphate..... …….cAMP
N-methyl-D-aspartate…….….. NMDA
Receptor Activity Modifying Protein…….…. .....RAMP
Calcitonin Like Receptor…… ..... CLR
Receptor Component Protein……..….... ..... RCP
Prostaglandin E2……..….PGE_2
Magnetic Resonance Imaging ……..….MRI
Serotonin …….5-HT
Central Nervous System……...…..CNS
5-hydroxyindoleacetic acid…….. 5-HIAA
Adenosine Diphosphate…….ADP
Adenosine Triphosphate……ATP
Ribonucleic Acid……..RNA
Deoxyribonucleic Acid……DNA
Cycle Threshold……...CT
Potassium Chloride……KCl
Milliliter……ml
Microgram……µg
Fetal Bovine Serum……FBS
Delta Delta CT…….ΔΔCT

ABSTRACT

VALIDATION OF REFERENCE GENES FOR RT-PCR STUDIES OF MIGRAINE TRIGGERS IN CA77 CELLS

Katherine Mireles, M.S.

George Mason University, 2020

Thesis Director: Dr. Ancha Baranova

Migraine disorder is a debilitating neurological condition affecting 16-22% of the population. Migraines are severe unilateral headaches often accompanied by phonophobia and photophobia, and triggered by stress and some food compounds. Calcitonin Gene Related Peptide, which is encoded by the gene *CALCA*, is a neuropeptide involved in the propagation of the migraine. The purpose of this study was to identify reference genes in CA77 cells that could be used to normalize qRT-PCR data for the study of CGRP expression after exposure to various food-derived compounds. CA77 cells were exposed to treatments expected to either upregulate or down regulate CGRP expression. RNA samples were extracted, quantified, and converted to cDNA. qPCR data were analyzed using NormFinder to determine the most suitable reference gene(s) or combinations. The combination of *ACTB* and *GAPDH* was found most suitable for use as the references genes

in CA77 cells utilized for qRT-PCR studies of CGRP expression after exposure to various food-derived compounds.

CHAPTER ONE: INTRODUCTION

Migraine disorder is a debilitating neurological condition affecting between 16-22% of the United States population (Malone, Bhowmick, & Wachholtz, 2015). In the U.S. the cost of migraines is roughly 14 billion dollars annually between direct costs of health care and reduced productivity at work (Jackson, Shimeall, Sessums, DeZee, & Becher, 2010). Those with migraine are at higher risk of other medical conditions including depression, heart disease, and stroke (Katsarava et al., 2012).

A migraine is neurological condition appearing as a severe unilateral headache that may be accompanied by photophobia, phonophobia, or nausea. The pain is often described as throbbing and can be exacerbated by normal movement. A migraine may last from 4-72 hours (ICHD-3). Events that precipitate a migraine vary from individual to individual, but the most common ones are emotional stress, changes in sleep patterns, and diet. Women tend to have migraines more than men. Onset of migraine tends to occur around onset of puberty (Malone, 2015).

Migraine have different levels of classification. A migraine is a type of primary headache (ICHD-3). Primary types of migraine include migraine with aura, migraine without aura, and chronic migraine (Eikermann-Haerter & Ayata, 2010).

Around one third of those with migraine have an aura that precedes the attack (DeLange & Cutrer, 2014). Auras tend to last between 5-60 minutes (ICHD-3). An aura is a reversible sensory disturbance like changes in vision or hearing that is linked to cortical spreading depression, CSD. Individuals with the migraine with aura may not always have

a sensory disturbance preceding a migraine. Those with an aura are more likely to have a stroke. Smoking increases the risk for developing migraine with aura (Delange, 2014).

Habituation is a feature of learning which may be measured by a decrease in response to stimulus after repeated exposure. There are differences in habituation between migraineurs and non-migraineurs. During the interictal period, there is a habituation deficit that normalizes during the migraine attack (Coppola et al., 2013). One study showed that those with stress as a migraine trigger had significantly deficient habituation in comparison to either controls or the migraineurs who do not have stress as a trigger (Lisicki et al., 2018). There is a probable genetic link for habituation deficit in migraineurs (Lisicki et al., 2017).

Chronic migraine is diagnosed when an individual experiences 15 or more headache days a month with 8 of those days being migraines that last at least 4 hours (ICHD-3). Those with chronic migraine are more at risk for developing medication overuse headaches (Eikermann-Haerter & Ayata, 2010). Chronic migraine is not uncommon; it affects 1-2% of the general population, and develops from more typical, episodic migraines at an annual conversion rate of about 3% (May & Schulte, 2016)

Soluble Mediators of Migraine

The trigeminal vascular system is comprised of the trigeminal ganglion, trigeminal nucleus caudalis, and the cerebral blood vessels (Myren, 2012). It is believed to be the main mediator of nociception during migraine (Cipolla, 2009). Activation of the trigeminal vascular system leads to the release of both Calcitonin Gene Related Peptide (CGRP) and prostaglandin E_2 (PGE_2) (Jenkins, 2001). Both CGRP and PGE_2 are found in the saliva of

individuals experiencing a migraine. These compounds are capable of inducing a migraine in individuals with a migraine disorder. CGRP is expressed in nociceptive unmyelinated C-fibers along with substance P with its calcitonin-like expressed in Aδ fibers (Iyengar et al., 2019).

Brain Morphology in Migraineurs

Differences between the brains of migraineurs and non-migraineurs have been investigated using a combination of electrophysiological techniques and neuroanatomy mapping. As frequency of migraines increases, an increase in grey matter in the caudate nucleus is noted, with a concomitant decrease in the grey matter in the anterior cingulate cortex (de Tommaso et al., 2014). Any structural changes to the trigeminal somatosensory system increase the chance of developing migraines. Magnetic resonance imaging (MRI) studies have shown the changes in white matter along the orbitofrontal cortex in migraineurs (F Hu & Qian, 2016).

Serotonergic System in Migraineurs

Serotonin (5-HT) is a monoamine neurotransmitter derived from tryptophan. It can be found in multiple tissues including blood platelets and the central nervous system (CNS). 5-HT can act as a vasoconstrictor. 5-HT receptors are targets for abortive migraine drugs, like triptans. Triptans increase serotonin signaling by binding to serotonin receptors in cranial blood vessels which cause the cranial blood vessels to constrict. (Gasparini et al.,

2017). 5-HT is stored in the synaptic vesicles until depolarization occurs. Once released, 5-HT is either recaptured or metabolized into 5-hydroxyindoleacetic acid (5-HIAA).

In between migraines, individuals have lower levels plasma of 5-HT and higher levels of 5-HIAA. This indicates that low serotonin levels could be one cause of migraines. Depletion of serotonin can also induce other symptoms that are associated with migraine such as nausea and dizziness (Aggarwal et al., 2012).

Genetic Susceptibility

Several gene studies have been done to attempt to link various DNA polymorphisms to migraine. So far, analysis of the variants residing within the genes related to serotonin transport has led to conflicting results. A meta-analysis by Gormley et al examined 22 genome wide association studies and found 38 genomic loci related to migraine, many of which are associated the vascular health (Gormley et al., 2016). Later, analysis of gene expression patters in total cellular component of the blood samples collected from migraineurs and controls implicated immune-inflammatory pathways, and participation of microglial components (Gerring et al., 2018).

Stages of a Migraine

A migraine attack can be broken into several stages including the prodromal phase, aura, headache, and postdrome phase (Goadsby, 2017, ICHD3). Many of the stages have overlapping symptoms. **Figure 1** summarizes the four main phases of a migraine.

The prodromal phase occurs up to 72 hours before the headache phase of a migraine. During this phase there is an increase in hypothalamic blood flow. Events that are often considered a migraine triggers may be part of the premonitory phase. Habituation decreases during this phase, which allows sensitization to increase.

The aura phase is not distinctly recognized in all individuals with migraine. An aura is a sensory disturbance that precedes a migraine. In severe cases the aura phase and the headache stage overlap.

The headache phase is the 4-72-hour period of pain that is often unilateral, pulsing, and considered moderate to severe. (ICHD3). At this point habituation levels begin to normalize.

The postdrome phase of a migraine is not well understood, but it appears to mimic the premonitory phase. The most common reported symptoms include tiredness and neck stiffness.

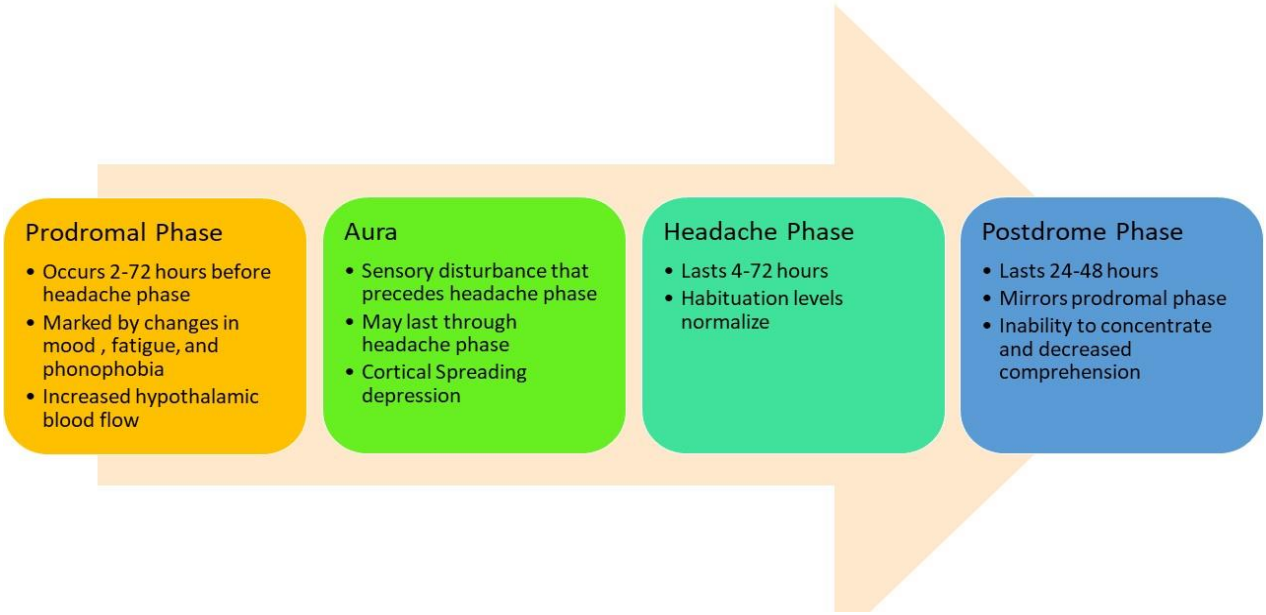


Figure 1 Summary of Migraine Phases

Cortical Spreading Depression

Cortical Spreading Depression, or CSD, causes the aura that precedes a migraine. CSD is a slow-moving wave of depolarization through cerebral grey matter. The wave moves on average of 3 mm/minute (Eikermann-Haerter & Ayata, 2010). Depolarization is believed to be brought on by activation of the N-methyl-D- aspartate (NMDA) receptor and elevated levels of extracellular K+ ions. The wave is then propagated by the intracellular transfer of ATP, H+ ions, and glutamate (Rodrigo, 2013). The release of ATP is believed to lead to the activation of nociceptors was observed during CSD (Gursoy-Ozdemir, 2004). CSD has been reported to cause a depletion of brain glucose levels which can trigger migraine symptoms (Charles & Brennan, 2009).

The conscious perception of the aura occurs once CSD has reached the primary sensory cortex. It is hypothesized that migraineurs who do not experience auras before a migraine may still experience CSD; in such a case the aura would occur in part of the brain that cannot be consciously perceived (Eikermann-Haeter, 2010).

CSD is linked to the release of CGRP and leads to the release of glutamate, K+ ions, and ATP. In turn, this leads to the release of CGRP from perivascular trigeminal fibers, but the mechanism behind this remains unknown. The trigeminal vascular system is activated when the peripheral terminals of nociceptive neurons are depolarized. The depolarization spreads to the medulla and cervical spinal cord.

Cerebral blood flow is affected by CSD. At the start, there is a brief period of vasoconstriction. This is followed by oligemia, a reduction in total blood volume. The aura starts during the oligemic phase, which then transitions into migraine. The migraine continues to through the hyperemic phase. Cerebral blood flow returns to normal at around 12 hours post migraine (Pusic, Grinberg, Mitchell, & Kraig, 2011).

Calcitonin Gene Related Peptide

Calcitonin Gene Related Peptide (CGRR) is one of the main neuropeptides involved in inflammation. CGRP consists of 37 amino acids, and is found in two different forms, α- CGRP and β- CGRP. These two forms are transcribed from two separate genes on chromosome 11. α-CGRP is an alternative mRNA splice product of the calcitonin precursor from the *CALCA* gene, as depicted in **Figure 2**. The α-CGRP form is found in the central nervous system and plays a role in migraine while the β- CGRP form can be found in the enteric nervous system (Russell 2014).

The mechanisms behind regulation of CGRP levels are not entirely understood. CGRP has been found to be upregulated in response to nerve damage (Iyengar, 2017). After translation, α-CGRP is stored in vesicles at the terminals of afferent nerves. Neurons containing CGRP can be found in the hypothalamus, cerebellum, trigeminal vascular system, and the inferior colliculi (Edvinsson 2010).

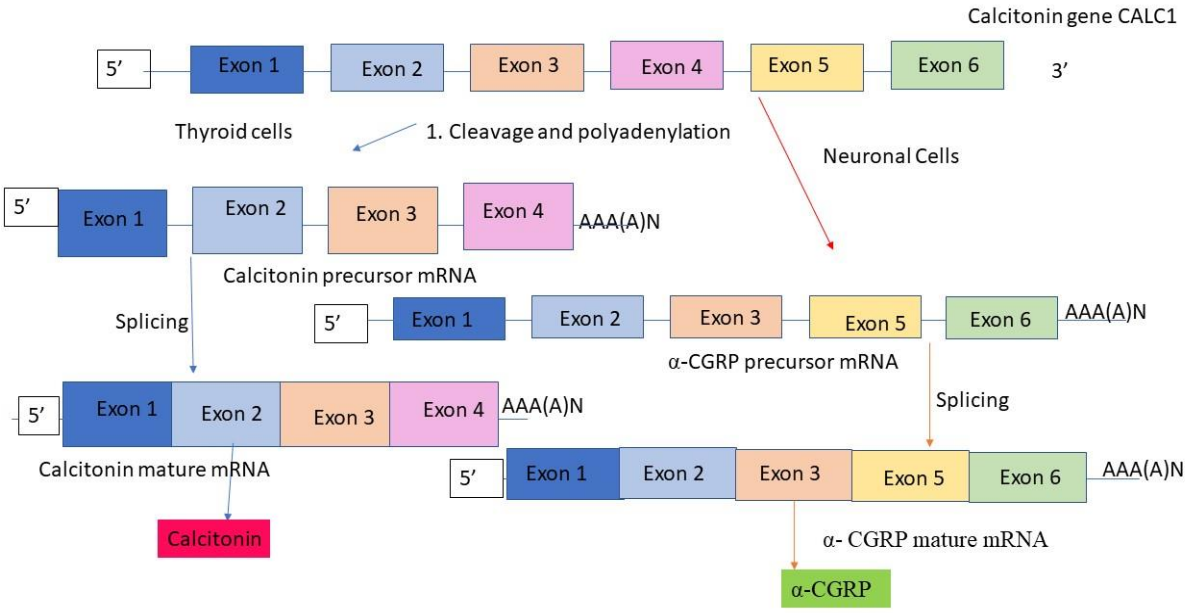


Figure 2. Alternative Splicing of the *CALCA* gene mRNAs into ones encoding calcitonin and CGRP

CGRP is thought to be involved when the trigeminal vascular system is activated. During a migraine, this occurs along the accumulation of vasodilators including adenine diphosphate, ADP, when blood flow is reduced in response to the migraine trigger (Aggarwal 2012). Reactive oxygen species can lead to the release of CGRP from trigeminal

terminals by exciting trigeminal nociceptors. In the central nervous system, CGRP acts as a vasodilator. It is one of the most potent vasodilators in the body.

The receptor for CGRP is made by the heterodimerization of the calcitonin like receptor (CLR) and one of the receptor activity modifying proteins (RAMP). CLR protein has 7 transmembrane domains; it is a G- protein-coupled receptor. As the name implies, the CLR does resemble the calcitonin receptor (Edvinisson, 2010). RAMP proteins are expressed separately from the CLR and stay together as homodimers until needed. Once a RAMP1 forms a dimer with CLR, the receptor is transported to the plasma membrane. Upon binding with its receptor, CGRP starts multiple signaling pathways that lead to the increase in CGRP expression itself (thus forming a positive feedback loop), an increase in substance P, and an increase in cyclic adenosine monophosphate, cAMP (**Figure3**).CGRP may also bind to the Amylin 1 receptor (Iyengar, 2017). The receptor component protein (RCP) is a small hydrophilic protein that while not necessary for CGRP binding , does increase the efficacy of CGRP receptor(Russell et al., 2014).

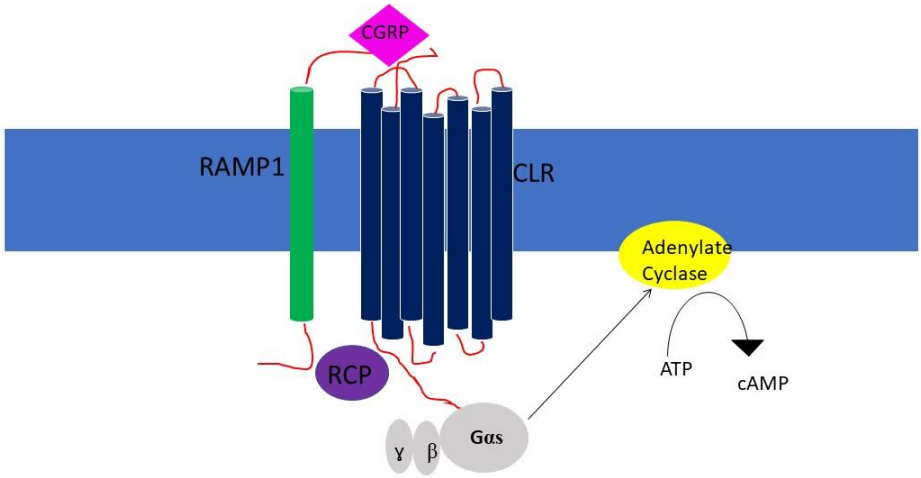


Figure 3 CGRP binding to the calcitonin receptor like receptor made of the dimerization of the CLR and RAMP1

Patients with migraines have higher basal levels of CGRP in plasma. Levels are higher in patients with chronic migraine in comparison to patients with episodic migraine. Administration of CGRP in migraineurs can induce a migraine (Park & Russo, 2008). Even though the exact mechanisms of CGRP release have not been worked out, there is still a clear link to CGRP levels and migraine. The location of neurons containing CGRP can be linked to various aspects of a migraine (Iyengar, 2017). Administration of CGRP to transgenic rats that overexpress human RAMP-1 induces a photophobic response to bright light, which did not occur in wild type rats (Iyengar et al., 2019). Nitroglycerin, a compound that can be used to induce migraines in humans, when administered peripherally to rats causes an increase in CGRP levels in the brainstem and trigeminal ganglia (Wattiez et al., 2019).

While CGRP can act as a vasodilator, it does not control systemic blood pressure; the treatments with its receptor antagonists have not shown a change in blood pressure, thus, making CGRP a viable drug target for migraine treatment. In 2018, three different monoclonal antibodies targeting CGRP signaling were approved in the U.S. for use for migraines. Triptans work to reduce CGRP levels by constricting cranial blood vessels which, in turn, this effect inhibits the release of CGRP (Gasparini et al., 2017).

Cell Model for Migraine

CA77 cells are derived from rat medullary thyroid carcinomas. Their neuronal-like properties make them practical model for studying migraines. These cells produce a higher ratio of CGRP to calcitonin than other parafollicular cell lines (Birnbaumsp, Mahoneyli, & Roossij, 1986). CA77 cells will put down neuronal like processes when treated with nerve growth factor (NGF) or when plated on laminin. The cells exhibit serotonergic properties, including the 5-HT_{1b}receptor and 5-HT transporter protein (Clark, Lanigan, Page, & Russo, 1995). In addition to this, CA77 cells express neuronal transcription factors like MASH-1 and express all three neurofilament proteins (Clark, Lanigan, Page, & Russo, 1995). The use of NGF can upregulate CGRP expression, while the addition of Sumatriptan, a medication used for the treatment of migraines and cluster headaches, downregulates CGRP expression (Johansen, 1995). CA77 cells provide a model of screening compounds that may modulate α-CGRP expression.

CA77 cells were successfully employed as a model for investigate the effects of ginger, grape pomace extract and S-petasin on two non-mRNA driven migraine-related

mechanisms in vitro: CGRP secretion and calcium uptake (Slavin et al., 2016) Because of that, CA77 cells are also suitable as a model for screening of the compounds capable of modulating α-CGRP expression up- or downward at mRNA levels.

Real Time Polymerase Chain Reaction

Real-time PCR, or qRT-PCR, is a technique for quantifying mRNA expression. It is preferred over other RNA quantitation techniques due to its sensitivity and accuracy (Gilsbach, 2006). The most common type of qRT-PCR employs non-specific fluorescent dyes, for example, SYBR Green or Eva Green, that intercalate with any double-stranded DNA proportionally to its amounts. Another group of techniques employs one or another type of fluorophore-labeled oligonucleotide (Navarro et al., 2015). There are several stages of RT-PCR. The first stage is the linear ground phase, where the background fluorescence is calculated (Wong, 2005). The next phase is the early exponential phase. At this phase the fluorescence is now higher than the baseline levels, and the cycle threshold is recorded. The exponential phase is when peak amplification is reached. The plateau stage is where fluorescence is no longer useful for calculations (Wong, 2005).

Quantification errors are common in qRT-PCR, with variations in amount of starting material, enzymatic efficiency and presence of inhibitors leading to uneven, biased, or not efficient amplification. When normalizing data for RT-PCR, reference genes are commonly used. A reference gene is one that does not significantly change expression, when cells exposed to various stimuli. It is ideal to have more than one reference gene to prevent bias. While *18S* RNA is commonly used, it is not optimal for every reaction due to

its high expression level in eukaryotic cells, which exceed expression levels of other genes by orders of magnitude (18S rRNA, the Best Internal Control).

Better reference gene candidates are found among housekeeping genes like *GAPDH*, due to lower levels of their expression as compared to 18S. Housekeeping genes are genes that are involved in maintenance of the cell and are often expressed at consistent levels (Eisenberg, 2013; Silver 2006). However, even housekeeping gene expression may change depending on the state of the cells. It is important to test various experimental conditions to find the reference genes ideally fitting to these conditions. Examples of previously validated reference genes include *ACTB* and *RPII* for human visceral adipose tissue (Mehta et al., 2010), *ACTB, C1orf43* and *PSMB4* for neural stem cells (Artyukhov et al., 2017) and *YWHAZ/14-3-3-zeta* for differentiating keratinocytes (Lanzafame et al., 2015).

CHAPTER TWO: MATERIALS AND METHODS

Cell Culture Technique for CA77 Cell Line

CA77 cells were received from Dr. Andrew Russo, Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA, USA. Privately procured stock was used because at the start of cell treatment, CA77 cells were not available from the ATCC repository. Now, CA77 cells are available from the ATCC repository (Manassas, VA) under the item number CRL-3234.

Cells were plated on 10 centimeter dishes coated with laminin. The cells were grown in DMEM-12 low glucose media supplemented with 1% Penicillin- Streptomycin and 10% fetal bovine serum. The cells were grown in a humid 5% CO_2 incubator at 37.1 degrees Celsius (^oC). Cells were treated with 1X trypsin EDTA in HBSS and passaged once they were between 75-80% confluent.

Primer Design

Primers for housekeeping genes and CGRP variants were designed using the NCBI Primer BLAST tool. The primers were design to cross exon-exon junctions and to minimize GC content when possible. In rats there are three CGRP mRNA variants that can be found in nature. The primers for the three CGRP variants were computationally verified through alignment of their sequences using ClustalW, (**Table 1 and Table 2**).

Primer Reconstitution

Primers were reconstituted from their lyophilized form. In order to reconstitute the primers, they were first centrifuged to ensure that none of the primers were stuck to the lid. RNase free water was added to every primer, and they were then incubated at 60^{o}C for five minutes. The primers were centrifuged again before a working stock was made.

Table 1. Housekeeping Genes Evaluated as Possible Reference Genes Suitable for CA77 Cells

Gene	Nucleotide Sequence		Product Size
Mapk-1	FWD	5'TGCTTTCTCTCCCGCACAAA3'	185 bp
	REV	3'AGCAATGGGCTCATCACTTG5'	
Mapk-6	FWD	5'TAAAGCCATTGACATGTGGG3'	129 bp
	REV	3'TCGTGCACAACAGGGATAGA5'	
Gapdh	FWD	5'AGACAGCCGCATCTTCTTGT3'	85 bp
	REV	3'CGATACGGCCAAATCCGTTC5'	
B2m	FWD	5'CCGTGATCTTTCTGGTGCTT3'	148 bp
	REV	3'ATTTGAGGTGGGTGGAACTG5'	
Hprt-1	FWD	5'AAGCTTGCTGGTGAAAAGGA3'	185 bp
	REV	3'CCGCTGTCTTTTAGGCTTTG5'	
Actb	FWD	5'CCCGCGAGTACAACCTTCTT3'	70 bp
	REV	3'TCATCCATGGCGAACTGGTG5'	
Rplp-1	FWD	5'AAAGCAGCTGGTGTCAATGTT3'	87 bp
	REV	3'GCAGATGAGGCTTCCAATGT5'	
Ppia	FWD	5'CTGTCTCTTTTCGCCGCTTG3'	114 bp
	REV	3'ACTTTGTCTGCAAACAGCTCG5'	

Table 2. Nucleotide Sequences for *CGRP* Variants in CA77 Cells

Gene	Nucleotide Sequence		Product Size
Cgrp V1	FWD	GAGGGCTCTAGCTTGGACAG	278 bp

	REV	GCCCAGAGAACAGCCAGAGA	
Cgrp V2	FWD	ACCAGGTGAGCCCTGAGGTT	76 bp
	REV	CTGACAACCAGGAAAGGGGAG	
Cgrp V3	FWD	GTCATCGCTCACCAGGGAGGCAT	669 bp
	REV	GCTACCAGATAAGCCAGAACCAT	
Cgrp (all)	FWD	AGGTAGGTCCTGTTGATGTTCC	993 bp
	REV	ATGGCCAGTCTACCCAAACC	

Treatment of Cells with Compounds Expected to Change CGRP Expression

The treatments were designed to either upregulate or down regulate CGRP expression. Serum-free media was used as a control. A list of treatments with specific concentrations can be found in **Table 3**. Cells were treated for either 30 minutes, or 4 hours, or 16 hours. The 16-hour treatment time was chosen because it was the longest the cells could be treated and RNA was able to be successfully extracted.

To begin with, cells were plated at approximately 500,000 cells per well in a treated 6-well dish in whole media. After 24 hours of being plated, the cells were serum starved in DMEM F12 low glucose media. On the day of treatment, cells were washed with serum free media. All treatments were diluted in serum free media and then put into the wells. At the end of treatment, the treatment was aspirated, and the cells were washed with serum free media.

RNA was then extracted using the RNeasy Mini kit (Qiagen). The RNA was quantified using a Nanodrop device. A 1% agarose gel was then run to confirm that the RNA was not degraded. The RNA samples were then converted to cDNA using the RT^2 First Strand kit (Qiagen) and then stored at -20^{o}C.

Table 3. Types of Migraine- Modifying Treatments Selected for Study in CA77 Cells

Treatment	Reason for Selection	Conditons	Time Points Tested
Whole Media	Cell culture conditions	Contains FBS and Pen-Strep	All
Serum Free Media	Control	Control	All
KCl	Upregulation	40mM in KCl in serum free media	4 hours only
Nerve Growth Factor	Upregulation	100ηg/ml in serum free media	All
Sumatriptan	Downregulation	50µM in serum free media	All

qRT-PCR

qRT-PCR was done using a CFX96 thermocycler (Bio-Rad). Reactions were run in quadruplicate or triplicate depending on the amount of RNA available. Eva Green SsoFast SuperMix was used as a DNA-binding dye. No template controls were used on every plate. Directions were followed per manufacturer guidelines. Every well contained 8µl of MasterMix and 2µl of cDNA. The components in the MasterMix are listed in **Table 4**. The thermocycler instructions are outlined in **Table 5**.

Table 4 Components of Mastermix for qPCR

Reaction Component	Volume
RNAse free water	1.5µl
Primer	2.5µl
EvaGreen Ssofast	5µl

Table 5 Settings for CFX96 Thermocycler

Temperature	Time	Cycles

95ºC	30 Seconds	1
95ºC	5 Seconds	1-40
Annealing Temperature	5 Seconds	1-40
Melt Curve 65-95ºC	5 seconds per step	1

Optimization of mRNA Primers of Housekeeping Genes in CA77 Cells

RNA was extracted from untreated CA77 cells using the Qiagen RNeasy Mini kit. After the RNA was quantified, it was converted to cDNA using the Qiagen RT^2 First Strand kit. This cDNA was used to optimize all of the primers.

Annealing temperature and cDNA concentration were the conditions optimized. cDNA concentrations tested ranged from 10-30 ηg. The range of temperatures included 55ºC to 65ºC. After RT-PCR, the samples were loaded into a 1% TAE agarose gel. SYBR Safe dye was the fluorescent dye used to visualize the PCR products. Optimal conditions were determined by the presence of only one distinct band on the agarose gel of the correct size. If multiple conditions produced one distinct band, the one with the lowest CT value was used. **Table 6** lists the optimal annealing temperatures as well as the optimal cDNA concentrations for each primer pair.

Table 6 Optimal RT-PCR Conditions for Primers

Gene	Annealing Temperature (ºC)	cDNA concentration (ηg/µL)

Mapk-6	59	30
Gapdh	59	30
Hprt-1	55.7	20
B2m	59	30
ActB	63.3	30
Rplp-1	63.3	30
Ppia	55	30
CGRP V1	65	30
CGRP VII	65	30

Data Analysis Using *GeNorm*

Stability of expression of candidate reference genes was assessed across all samples using the *GeNorm* statistical algorithm, according to developer's recommendations. The *GeNorm* algorithm was developed in 2002 by Vandesompele et al. The algorithm utilizes a pair-wise comparison of one candidate reference gene to all other candidate genes in a manner independent of its relative level of expression in a given sample or amount of starting material. In this way, expression stability (M) is determined. Ideally, same expression ratio of two reference genes should be observed in all profiled samples. Raw non-normalized expression values are used for calculation of a pairwise variation in terms of the standard deviation (Vjk) of each gene' logarithmically transformed expression ratios (aij). This is done for each tissue sample (m), for any combination of two internal control genes (j or k) (Vandesompele et al., 2002):

$$(\forall j, k\epsilon[1,n] and\ j \neq k):$$

$$Ajk = \left\{\log_2 \frac{a_{1j}}{a_{1k}}, \log_2 \frac{a_{2j}}{a_{2k}}, \ldots \log_2 \quad \frac{a_{mj}}{a_{mk}}\right\}$$
$$= \left\{\log_2 \frac{aij}{aik}\right\} i = 1 \rightarrow m$$

$$Vjk = St.dev(Ajk)$$

Accordingly, an expression stability measure (Mj) is calculated as the mean of pairwise variations of expression levels for a particular gene compared to that of all other genes, then the genes are ranked according to the stability of their expression.

$$Mj = \frac{\sum_{k=1}^{n} Vjk}{n-1}$$

Genes with lowest stability values (i.e. the highest Mj values) meaning genes that have the most variation in expression are excluded at iterative steps until only the genes with the lowest Mj values and most stable expression remain.

The optimal number of reference genes is defined as the minimum number of genes for which the pair-wise variation Vjk/Vjk+1, is smaller than 0.15. The normalization factor is calculated as the geometric mean of the final set of reference genes, highlighted as optimal.

CHAPTER 3: RESULTS AND THEIR DISCUSSION

Results of Reference Gene Selection

In this study, we attempted to find the pair of reference genes most stably expressed in CA77 model cell under the set of conditions, designed to either induce or suppress CGRP-encoding mRNA. Tested conditions included treatments with potassium chloride, KCl (40mM), nerve growth factor (NGF) (100 ηg/ml), and sumatriptan (50μM) as well as serum free media used as control. To each treatment except KCl, CA77 cells were exposed for 30 minutes, 4 hours or 16 hours. In the case of KCl, only 4 hour exposure time point was tested.

For each candidate reference gene, qPCR data were analyzed using NormFinder (http://moma.dk/normfinde-software). Normfinder uses a model-based approach that estimates gene expression variation. It determines intragroup and intergroup variation and combines them to determine the stability value, thus making the gene with the lowest stability value the best candidate reference gene (Andersen et al., 2004). A lower stability value indicates that there is less intergroup and intragroup variation.

For the purpose of this analysis, mRNA samples were grouped by either duration of treatment or type of treatment. The combination of *ACTB* and *GAPDH* provided the lowest combination stability value of 0.479 when grouped by time. Based off of the grouping by time, *ACTB* has the lowest stability value of 0.501 on its own. The combination of *ACTB* and *GAPDH* have a lower stability value than either gene on their own when grouped by time making the combination of the two better reference gene

candidates than either gene individually. When grouped by treatment, *ACTB* and *GAPDH* had the lowest combination stability value of 0.332. When not grouped by time or treatment, *ACTB* and *GAPDH* had very similar stability values of 1.045 and 1.047 respectively. Again, together *ACTB* and *GAPDH* show less variation when paired together than individually.

After 30 minutes of treatment *ACTB* had the lowest stability value of 1.793. *GAPDH* gene had a very low stability value of 0.126 after 4 hours of treatment. In addition, the expression levels for this gene also had the highest standard error of all genes tested at this time point, making it not suitable for a reference gene in CA77 model for this treatment length.

Similarly, neither *MAPK6* nor *HRPT1* genes were found suitable as the reference for CA77 cells, due to their high stability values, indicating high intra-group and inter-group variation in all qRT-PCR experiments analyzed. Final ranking of candidate reference genes according to their expression stability in CA77 cells was as follows: *ACTB* > *GAPDH* > *B2M* > *RPLP* > *PP1A* > *HPRT1* > *MAPK6* (**Table 7** and **Figure 4**).

When intra-group and inter-group variations of gene expression levels were analyzed, a value closer to zero indicates higher stability. Accordingly, measurements of the expression of *ACTB* displayed the least amount of intra-group (**Table 8**) and inter-group variation (**Table 9**). When intra-group and inter-group variations of gene expression levels were analyzed, a value closer to zero indicates higher stability.

Table 7 CA77 specific Stability Values for Candidate Reference genes

Gene name	Stability value	Standard Error
PPIA	1.86	0.27
RPLP	1.67	0.26
ACTB	1.05	0.24
GAPDH	1.05	0.24
B2M	1.15	0.24
HRPT1	2.70	0.33
MAPK6	5.78	0.62

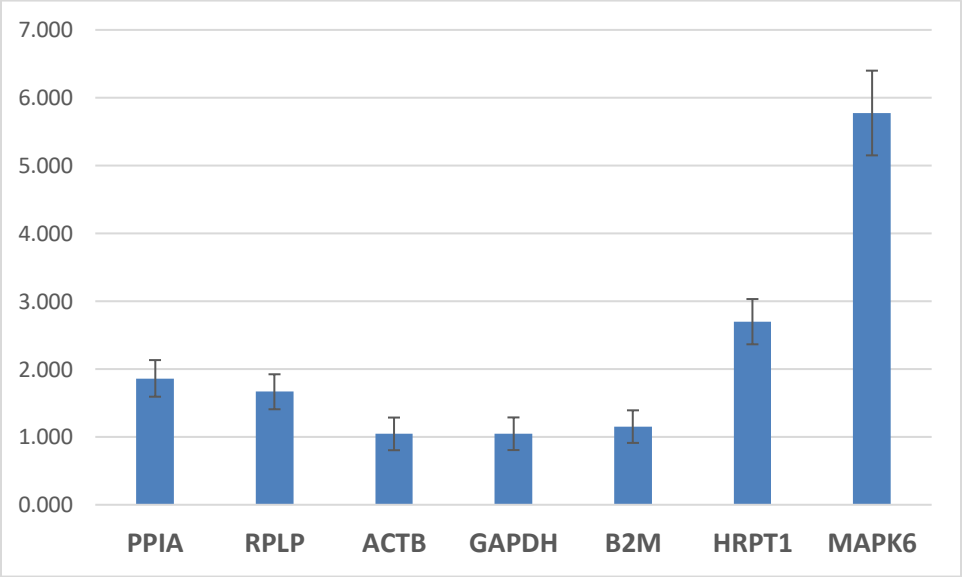


Figure 4 Stability Values for all Housekeeping Genes

In subsequent experiments, the combination of *ACTB* and *GAPDH* were used to normalize the data quantifying expression of a target mRNAs encoding CGRP variants.

While *ACTB* had the least amount of intra-group variation and inter-group variation on its own, when paired with *GAPDH* the stability value was lower than the stability value of *ACTB* on its own, meaning there is a smaller standard deviation.

Table 8 Intragroup Variation of Expression Values for Each Candidate Reference Gene

Gene	Serum Free	Whole Media	Sumatriptan	NGF
PPIA	11.17	0.31	0.12	0.81
RPLP	10.46	0.12	0.002	0.66
ACTB	3.70	0.07	0.68	0.83
GAPDH	4.10	0.05	0.01	0.02
B2M	2.95	0.23	0.002	0.53
HRPT1	6.63	9.79	0.37	10.63
MAPK6	49.36	1.60	34.34	24.36

Table 9 Intergroup Variation of Expression Values for Each Candidate Reference Gene

Gene	Serum Free	Whole Media	Sumatriptan	NGF
PPIA	-1.05	-0.01	0.67	0.40
RPLP	-0.49	-0.26	0.58	0.17
ACTB	0.15	-0.30	0.07	0.08
GAPDH	0.82	-0.69	0.02	-0.150
B2M	1.04	-0.88	0.17	-0.34
HRPT1	0.60	-1.06	1.28	-0.82
MAPK6	-1.07	3.20	-2.80	0.67

Analysis of CGRP mRNA Variants

CGRP mRNA variant expression was calculated using the delta delta CT methods using *ACTB* and *GAPDH* as the reference genes. The average CT value for the reference gene in control conditions is subtracted from the average CT value for the gene of interest (CGRP) to get the ΔCT for the control. Then the ΔCT for each experimental condition is calculated by subtracting the average CT value of the reference gene when exposed to the experimental treatment from the average CT value of the gene of interest when exposed to experimental treatment. The ΔΔCT value is calculated by calculating the difference between the ΔCT experimental and the ΔCT control. The fold change, R, is determined using the following equation:

$$R = 2^{-\Delta\Delta CT}$$

The fold change data for both *CGRP* mRNA variants can be found in **Tables 10 and 11.** The greatest fold change occurred with cells were exposed to known migraine curtailing medicine, sumatriptan, for 16 hours. While sumatriptan was performing as expected and have led to a decrease in the levels of *CGRP* expression after short-term exposure, the long term exposure to this compound led to an increase in respective levels of *CGRP* encoding mRNA. It is well known that prolonged exposure to sumatriptan in humans can lead to medication overuse headaches (Meng et al., 2011). Observed rebound upregulation of mRNA variant 1 of *CGRP* following a 16-hour exposure to sumatriptan may indicate the reversal of a protective effect after increased exposure and is in line with clinical observations.

The down regulation of both *CGRP* mRNA variants after exposure to NGF was not expected. Typically, CGRP protein levels are upregulated in response to an exposure to NGF. It is possible that observed increase in CGRP protein levels in response to NGF may be due to another non-transcription related mechanism, such as decreased degradation of CGRP or its receptor. It is also possible that longer treatment times may be necessary in order to achieve an upregulation in expression of *CGRP* mRNAs, especially in stressed cells. Twenty-four-hour exposure to NGF was attempted, but the cells died, which precluded extraction of RNA of suitable purity and integrity.

Downregulation of *CGRP* in response to whole FBS-containing media treatment was expected in comparison to the control treatment with serum free media, where the cells were starved. In this experiment, serum starved cells were more stressed than FBS treated ones. As CGRP is a neuropathic peptide which is produced in response to stress, downregulation of CGRP encoding gene in FBS-treated cells is reflecting physiological regulation.

The overall trend for upregulation of the *CGRP* mRNA variants observed after 16 hours may indicate that the decrease in viability of the CA77 cells after long-term treatments. At 16 hours post-treatment, all cell cultures experiences a substantial increase in a cell death, which may reflect some stress and lead to higher levels of expression of CGRP-encoding mRNAs.

Table 10 Trends in the Relative Fold Changes of *CGRP* V1 expression between shortest and longest exposure

CGRP V1	ΔΔ CT	Fold Change
Sumatriptan 30 minutes	5.07	0.03
Sumatriptan 16 hours	-2.35	5.11
NGF 30 minutes	5.30	0.03
NGF 16 hours	0.47	0.72
Whole media 30 minutes	6.84	0.01
Whole media 16 hours	-0.14	1.11

Table 11 Trends in the Relative Fold Changes of *CGRP* V2 expression with exposure time

CGRP V2	ΔΔ CT	Fold Change
Sumatriptan 30 minutes	12.14	0.0002
Sumatriptan 4 hours	-0.17	1.12
Sumatriptan 16 hours	-0.94	1.91
NGF 30 minutes	7.56	0.01
NGF 4 hours	-1.72	3.31
NGF 16 hours	1.37	0.39
Whole media 30 minutes	4.79	0.04
Whole media 4 hours	0.01	0.99
Whole media 16 hours	-0.71	1.64

CA77 as a Model for the studies of CGRP regulation

According to our experiments, CA77 cells might be useful as a model for the studies of CGRP gene regulation. Even though the mRNA regulation patterns observed in our experiment do not match the trends showing in proteomic assays, the changes in CGRP mRNA expression may still be used for evaluation of possible migraine triggers, and for a search of potential anti-CGRP therapies that work on the mRNA rather than the protein

level. For the future studies performed in CA77 cell, collecting both proteomic and genomic data is paramount, as their comparison may provide novel insights into the pathogenesis of the migraine.

In course of this study we found out that the long term viability of CA77 cells exposed to anti-CGRP treatment overnight or longer is low. This may be either due to intrinsic sensitivity of these cells to the stress, or to the necessity of the decrease in concentration of compounds used for CA77 treatments. Nevertheless, even with current study design, CA77 cell may be used for a study of fast acting migraine abortive medications, or mechanistic studies of CGRP regulation.

Co-profiling of CGRP along with other inflammatory molecules, such as substance P, may provide a better understanding of the mechanisms behind the induced changes in CGRP expression. Compounds that interact with the serotonin receptor can be studied since the CA77 cell line has the 5-HT1_b receptor. This would possibly allow for a clearer understanding of the mechanisms between serotonin and CGRP.

Additionally, it should be noted that both variants of CGRP encoding mRNAs have a high GC content, which makes the process of identifying primers that will not dimerize challenging. In particular, we encountered problems with designing the primers specific for CGRP mRNA V3 as varying the location and length of the primer, primer concentration, and annealing temperature did not allow us to obtain amplification either due to either forming a product of primer dimerization, or no expression of CGRP mRNA V3 in selected CA77 cell model.

Current experiments were started under the hypothesis that the CA77 cell line could be useful for testing the effects of common dietary triggers on CGRP expression. Dietary triggers vary between individuals, with the most common being alcohol, dairy, and citrus (Rist et al., 2015). Finding out which dietary compounds and in what amounts alter CGRP gene regulation may provide better dietary guidelines for migraineurs. Examples of dietary compounds that could be tested include ginger derivatives and S-petasin.

Migraines occur more frequently in females than males, and the age of onset coinciding with the start of puberty. Menstrual period related migraines tend to be more severe, and more difficult to treat than intra-period migraines (Delaruelle et al., 2018). Approximately 53% of adolescent girls have headaches at the onset of menses (Delaruelle et al., 2018). During puberty, the resetting of the hypothalamic hormones can alter the trigeminovascular system leading to an increased susceptibility to migraine. Throughout the menstrual cycle, there are the cyclic changes in the concentrations of the gonadal hormones within the grey and white matter, with estrogen providing excitatory stimulus, while progesterone inhibiting the neuronal systems (Borsook et al., 2014). Because of that, the role of estrogen and progesterone in migraines is of particular interest; an influence of these hormones on CGRP mRNA levels may also be studied in CA77 cell line.

In conclusion, thus study provides a rationale for the study of CGRP expression in CA77 cell line after exposure to various food-derived compounds, and describes a set of primers useful for evaluating expression of reference genes in this cellular model.

References

Aggarwal, M., Puri, V., & Puri, S. (2012). Serotonin and CGRP in migraine. *Annals of neurosciences, 19*(2), 88-94.

Andersen, C. L., Jensen, J. L., & Ørntoft, T. F. (2004). Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Research*, *64*(15), 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Artyukhov, A. S., Dashinimaev, E. B., Tsvetkov, V. O., Bolshakov, A. P., Konovalova, E. V., Kolbaev, S. N., Vorotelyak, E. A., & Vasiliev, A. V. (2017). New genes for accurate normalization of qRT-PCR results in study of iPS and iPS- derived cells. *Gene, 626*, 234–240. https://doi.org/10.1016/j.gene.2017.05.045.

Arulmani, U., MaassenVanDenBrink, A., Villalon, C., & Saxena, P. (2004). Calcitonin Gene-Related Peptide and Its Role in Migraine Pathophysiology. *European Journal of Pharmacology, 500*(1), 315-330.

Birnbaumsp, R., Mahoneyli, W., & Roossij, B. (1986). Biosynthesis of Calcitonin by a Rat Medullary Thyroid Carcinoma Cell Line. *The Journal of Biological Chemistry, 261*(2), 699-703.

Borsook, D., Erpelding, N., Lebel, A., Linnman, C., Veggeberg, R., Grant, P.E., Buettner, C., Becerra, L., Burstein, R. (2014) Sex and the migraine brain. *Neurobiol Dis*, 68:200-14.

Chan, V., McCabe, E., & MacGregor, D. (2009). Botox Treatment for Migraine and Chronic Daily Headache in Adolescents. *Journal of Neuroscience Nursing, 41*(5), 235-243.

Charles, A., & Brennan, K. (2009). Cortical spreading depression—New insights and persistent questions. *Cephalalgia : An International Journal of Headache*, *29*(10), 1115–1124.

Clark, M., Lanigan, T., Page, N., & Russo, A. (1995). Induction of a serotonergic and neuronal phenotype in thyroid C-cells. *Journal of Neuroscience, 15*(9).

Coppola, G., Di Lorenzo, C., Schoenen, J., & Pierelli, F. (2013). Habituation and sensitization in primary headaches. *The Journal of Headache and Pain*, *14*(1), 65.

DeLange, J., & Cutrer, F. (2014). Our Evolving Understanding of Migraine with Aura. *Current Pain and Headache Reports, 18*(10), 453.

Delaruelle, Z., Ivanova, T. A., Khan, S., Negro, A., Ornello, R., Raffaelli, B., Terrin, A., Mitsikostas, D. D., & Reuter, U. (2018). Male and female sex hormones in primary headaches. *The Journal of Headache and Pain*, *19*(1). https://doi.org/10.1186/s10194-018-0922-7

de Tommaso, M., Ambrosini, A., Brighina, F., Coppola, G., Perrotta, A., Pierelli, F., Sandrini, G., Valeriani, M., Marinazzo, D., Stramaglia, S., & Schoenen, J. (2014).

Altered processing of sensory stimuli in patients with migraine. *Nature Reviews Neurology, 10*(3), 144–155.

Durham, P. (2006). Calcitonin Gene-Related Peptide (CGRP) and Migraine. *Headache, 46*.

Durham, P., & Papapetropoulos, S. (2013). Biomarkers Associated With Migraine and Their Potential Role in Migraine Management. *Headache: The Journal of Head and Face Pain, 53*(8), 1262-1277.

Durham, P., & Russo, A. (1999). Regulation of Calcitonin Gene-Related Peptide Secretion by a Serotonergic Antimigraine Drug. *J. Neurosci., 19*(9), 3423-3429.

Edvinsson, L., & Ho, T. (2010). CGRP receptor antagonism and migraine. *Neurotherapeutics, 7*(2), 164-175.

Eikermann-Haerter, K., & Ayata, C. (2010). Cortical Spreading Depression and Migraine. *Current Neurology and Neuroscience Reports, 10*(3), 167-173.

Eising, E., A Datson, N., van den Maagdenberg, A., & Ferrari, M. (2013). Epigenetic mechanisms in migraine: a promising avenue? *BMC medicine, 11*, 26.

Estemalik, E., & Tepper, S. (2013). Preventive treatment in migraine and the new US guidelines. *Neuropsychiatric disease and treatment, 9*, 709-20.

Gasparini, C. F., Smith, R. A., & Griffiths, L. R. (2017). Genetic and biochemical changes of the serotonergic system in migraine pathobiology. *The Journal of Headache and Pain, 18*(1). https://doi.org/10.1186/s10194-016-0711-0

Gerring, Z. F., Powell, J. E., Montgomery, G. W., & Nyholt, D. R. (2018). Genome-wide analysis of blood gene expression in migraine implicates immune-inflammatory pathways. *Cephalalgia, 38*(2), 292–303. https://doi.org/10.1177/0333102416686769

Goadsby, P. (2005). Migraine pathophysiology. *Headache, 45 Suppl 1*, S14-24.

Goadsby, P. (2016). Bench to bedside advances in the 21st century for primary headache disorders: migraine treatments for migraine patients: Table 1. *Brain, 139*(10), 2571-2577.

Gormley, P., Anttila, V., Winsvold, B. S., Palta, P., Esko, T., Pers, T. H., Farh, K.-H., Cuenca-Leon, E., Muona, M., Furlotte, N. A., Kurth, T., Ingason, A., McMahon, G., Ligthart, L., Terwindt, G. M., Kallela, M., Freilinger, T. M., Ran, C., Gordon, S. G., … Palotie, A. (2016). Meta-analysis of 375,000 individuals identifies 38 susceptibility loci for migraine. *Nature Genetics, 48*(8), 856–866. https://doi.org/10.1038/ng.3598

Gursoy-Ozdemir, Y., Qiu, J., Matsuoka, N., Bolay, H., Bermpohl, D., Jin, H., . . . Moskowitz, M. (2004). Cortical spreading depression activates and upregulates MMP-9. *The Journal of clinical investigation, 113*(10), 1447-55.

Hu F, Qian ZW. (2016). Characteristic analysis of white matter lesions in migraine patients with MRI. *Eur Rev Med Pharmacol Sci.* 20(6):1032-6.

Iyengar, S., Johnson, K. W., Ossipov, M. H., & Aurora, S. K. (2019). CGRP and the Trigeminal System in Migraine. *Headache*, *59*(5), 659–681.

Jackson, J., Shimeall, W., Sessums, L., DeZee, K., & Becher, D. (2010). Tricyclic antidepressants and headaches: systematic review and meta-analysis. *British Medical Journal*. 341:c5222.

Katsarava, Z., Buse, D. C., Manack, A. N., & Lipton, R. B. (2012). Defining the Differences Between Episodic Migraine and Chronic Migraine. *Current Pain and Headache Reports*, *16*(1), 86–92. https://doi.org/10.1007/s11916-011-0233-z

Kokavec, A. (2016). Migraine: A disorder of metabolism? *Medical Hypotheses*, *97*, 117-130.

Lanzafame, M., Botta, E., Teson, M., Fortugno, P., Zambruno, G., Stefanini, M., & Orioli, D. (2015). Reference genes for gene expression analysis in proliferating and differentiating human keratinocytes. *Experimental Dermatology*, *24*(4), 314–316.

Lisicki, M., Ruiz-Romagnoli, E., D'Ostilio, K., Piedrabuena, R., Giobellina, R., Schoenen, J., & Magis, D. (2017). Familial history of migraine influences habituation of visual evoked potentials. *Cephalalgia*, *37*(11), 1082–1087.

Lipton, R., Bigal, M., Diamond, M., Freitag, F., Reed, M., & Stewart, W. (2007). Migraine prevalence, disease burden, and the need for preventive therapy. *Neurology*, *68*(5), 343-9.

Lyseng-WIlliamson, K., & Yang, L. (2007). Topiramate: a review of its use in the treatment of epilepsy. *Drugs*.

Malone, C., Bhowmick, A., & Wachholtz, A. (2015). Migraine: treatments, comorbidities, and quality of life, in the USA. *Journal of pain research*, *8*, 537-47.

May, A., & Schulte, L. H. (2016). Chronic migraine: Risk factors, mechanisms and treatment. *Nature Reviews Neurology*, *12*(8), 455–464.

Mazzotta, G., Floridi, F., Alberti, A., & Sarchielli, P. (2004). Antiepileptic drugs in migraine prophylaxis. *Journal of Headache Pain*, 67-70.

Mehta, R., Birerdinc, A., Hossain, N., Afendy, A., Chandhoke, V., Younossi, Z., & Baranova, A. (2010). Validation of endogenous reference genes for qRT-PCR analysis of human visceral adipose samples. *BMC Molecular Biology*, *11*, 39.

Meng, I.D., Dodick, D., Ossipov, M.H., Porreca, F. (2011) Pathophysiology of medication overuse headache: insights and hypotheses from preclinical studies. *Cephalalgia*, 31, 851-60.

Millichap, J., & Yee, M. (2003). The diet factor in pediatric and adolescent migraine. *Pediatric Neurology*, *28*(1), 9-15.

Navarro, E., Serrano-Heras, G., Castaño, M. J., & Solera, J. (2015). Real-time PCR detection chemistry. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *439*, 231–25

Nguyen, T.-V., & Low, N. (2013). Comorbidity of migraine and mood episodes in a nationally representative population-based sample. *Headache, 53*(3), 498-506.

Park, K.-Y., & Russo, A. (2008). Control of the calcitonin gene-related peptide enhancer by upstream stimulatory factor in trigeminal ganglion neurons. *The Journal of biological chemistry, 283*(9), 5441-51.

Pietrobon, D., & Moskowitz, M. (2013). Pathophyiology of Migraine. *Annual Review of Physiology*, 365-391.

Pusic, A., Grinberg, Y., Mitchell, H., & Kraig, R. (2011). Modeling neural immune signaling of episodic and chronic migraine using spreading depression in vitro. *Journal of visualized experiments : JoVE*(52).

Raddant, A., & Russo, A. (2011). Calcitonin Gene Related Peptide in Migraine: intersection of peripheral inflammation ans central modulation. *Expert Review in Molecular Medicine, 23*.

Rist, P. M., Buring, J. E., & Kurth, T. (2015). Dietary patterns according to headache and migraine status: A cross-sectional study. *Cephalalgia : An International Journal of Headache*, *35*(9), 767–775.

Russell, F., King, R., Smillie, S.-J., Kodji, X., & Brain, S. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiological reviews, 94*(4), 1099-142.

Schifi-Er, S., Johansen ?, T., & Johnsens, A. (1995). Molecular Characterization of Calcitonin Gene- Related Peptide (CGRP) in a Rat Medullary Thyroid Carcinoma Cell Line. -*Petxides, 16*(8), 1489-1496.

Slavin, M., Bourguignon, J., Jackson, K., & Orciga, M.-A. (2016). Impact of Food Components on in vitro Calcitonin Gene-Related Peptide Secretion-A Potential Mechanism for Dietary Influence on Migraine. *Nutrients*, *8* (7). https://doi.org/10.3390/nu8070406

Stucky, N., Gregory, E., Winter, M., He, Y.-Y., Hamilton, E., McCarson, K., & Berman, N. (2011). Sex differences in behavior and expression of CGRP-related genes in a rodent model of chronic migraine. *Headache, 51*(5), 674-92.

Tolner, E., Houben, T., Terwindt, G., de Vries, B., Ferrari, M., & van den Maagdenberg, A. (2015). From migraine genes to mechanisms. *PAIN, 156*, S64-S74.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), research0034.1-research0034.11.

Wattiez, A.-S., Wang, M., & Russo, A. F. (2019). CGRP in Animal Models of Migraine. *Handbook of Experimental Pharmacology*, *255*, 85–107. https://doi.org/10.1007/164_2018_187

BIOGRAPHY

Katherine Mireles graduated from Ocean Lakes High School in 2011. She received her Bachelor of Science from Ferrum College in 2014.