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The Effects of Palmitate on the Expression of G-protein Coupled Receptors and GLUT Transporters in Neuro2A Cells

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The effects of palmitate on the expression of G-protein coupled receptors and GLUT transporters in Neuro2A cells

By

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Submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology from the Department of Biological Sciences of Seton Hall University
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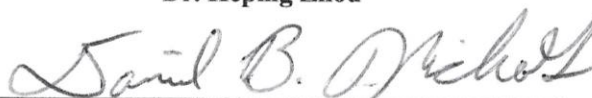
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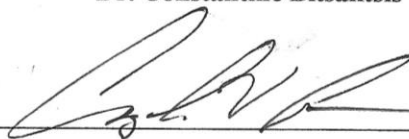_____

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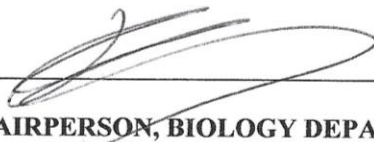
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iii

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My Friends and Family

Table of Contents

Figures/Images

Abstract: Palmitate (PA) is a saturated fatty acid that has been reported to be elevated in individuals with metabolic syndromes. The effects of fatty acids on the expression of various fatty acid receptors on neuronal cells is unknown, as is the effects of fatty acids on GLUT transporters. In this study, Neuro-2a (N2a) cells were treated with either 300μM of bovine serum albumin (BSA) or 300μM of palmitate conjugated to BSA (PA) at various time points to determine the effect of palmitate on the expression of various fatty acid receptors and GLUT transporters. RNA isolation followed by RT-PCR with primers specific to various G- protein coupled receptors (GPRs) and GLUT transporters was performed on these treated cells and further analyzed with statistical tests. 24 hour treatment with 300μM PA yielded increased mRNA expression of GPR41, GPR84, GPR119, GPR120 and GLUT5 when compared to vehicle control. In order to determine how GLUT5 may be regulated, N2a cells were treated with 300μM BSA along with PKA inhibitor KT5720 or GPR120 agonist GW9508. RNA isolation followed by RT-PCR showed that GPR41, GPR119 and GLUT5 expression increased with PKA inhibition, and GPR120 expression increased with GPR120 stimulation. These results suggest that PKA may play a role in the regulation of GPR41, GPR119 and GLUT5 in neuronal cells stimulated with palmitate.

Introduction

Obesity affects over one-third (36.5%) of the adult population in the United States (CDC 2017). In 2008, the estimated annual medical cost of obesity in the U.S alone was $147 billion dollars (CDC 2017). Many factors facilitate the progression of obesity, such as genetic, metabolic, and environmental influences, including the consumption of fat-rich diets (Stein 2004 and Milanski 2009). People who are obese are at higher risk for serious diseases and health conditions such as hypertension, dyslipidemia, coronary heart disease and type 2 diabetes mellitus (DM) (CDC 2017). Dyslipidemia is characterized by an abnormal amount of lipids in the blood while type 2 DM is characterized by insulin insensitivity with decreased glucose transport into muscle, liver and fat cells (Olokoba 2012). Conditions such as metabolic disorders and obesity have also been reported to be associated with higher plasma levels of free fatty acids (Boden 008 and Dresner 1999). These free fatty acids (FFAs) play multiple roles within the body, such as providing energy, acting as cell signaling molecules, and stabilizing membranes via palmitoylation and myristoylation (Zbigniew 2013).

A family of membrane-transversing proteins called G-protein coupled receptors (GPCRs) have been found to bind to various FFAs (Coleman 2017). These GPRs consist of a 7-transmembrane helix bundle (7TM) connected by 3 intracellular loops and 3 extracellular loops with the N-terminus on the outside and C-terminus on the inside (Wu 2017). Upon binding of the ligand to the N terminus of the GPCR, the heterotrimeric G-proteins associated with GPCRs are activated (Wu 2017) (Rosenbaum 2014). Free fatty acids have been found to bind to GPCRs such as GPR41, GPR84 and GPR120 (Nagasaki 2012). GPR41 is a member of the GPR40 family (also including GPR40, GPR42 and GPR43) shown to be activated by short chain fatty acids (Wu 2012). GPR84 has been found to be expressed in bone marrow, spleen, lymph nodes,

1

leukocytes, and thymus of mice and activated by medium chain fatty acids (Nagasaki 2012). GPR119 is highly expressed in the pancreatic islets and gastrointestinal tract of humans and has been suggested to be involved in glucose homeostasis (Kang 2013). GPR120 has been shown to be activated by long chain fatty acids and is attracting attention as a potential target for type 2 DM treatment (Milligan 2017). GPR120 knockout- mice subjected to high fat diets were found to develop obesity, glucose intolerance and exacerbated insulin resistance compared to wild type animals, suggesting its importance in metabolic functions (Alvarez 2016).

As a trimer consisting of α, β, and γ subunits, G-proteins play a critical role in transducing signals from ligand-GPCR interactions and modulate downstream effector proteins. Upon ligand binding to the GPCR, the conformation of G-proteins changes to facilitate the binding of GTP by Gα, giving rise to a Gα-GTP monomer and a Gβγ dimer (Miller 2017). There are several different types of Gα such as Gαs, Gαi/o, Gαq/11, and Gα12/13 (Miller 2017). The activation of Gαs leads to increased levels of the second messenger cAMP (Miller 2017). cAMP modulates the activities of protein kinase A (PKA), a kinase which consists of two regulatory subunits and two catalytic subunits (Sassone-Corsi 2013). Upon binding of cAMP to the regulatory subunits, these subunits dissociate from the catalytic subunits, thereby allowing the translocation and activation of PKA (Sassone-Corsi 2013). Activation of PKA leads to downstream activation of various transcription factors, including cyclic AMP responsive element- binding (CREB) protein (Alberts 2015) (Figure 1). Conversely, activation of Gαi signaling leads to the inhibition of adenylyl cyclase (Alberts 2015) (Figure 1). Finally, the activation of Gαq/11 leads to the activation of phospholipase C β, which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Alberts 2015). IP_3 is required for the release of intracellular calcium, which is the

second messenger that activates PKC with DAG (Alberts 2015). Activation of PKC leads to

downstream activation of various transcription factors, Such as NF-κB (Kang 2014).
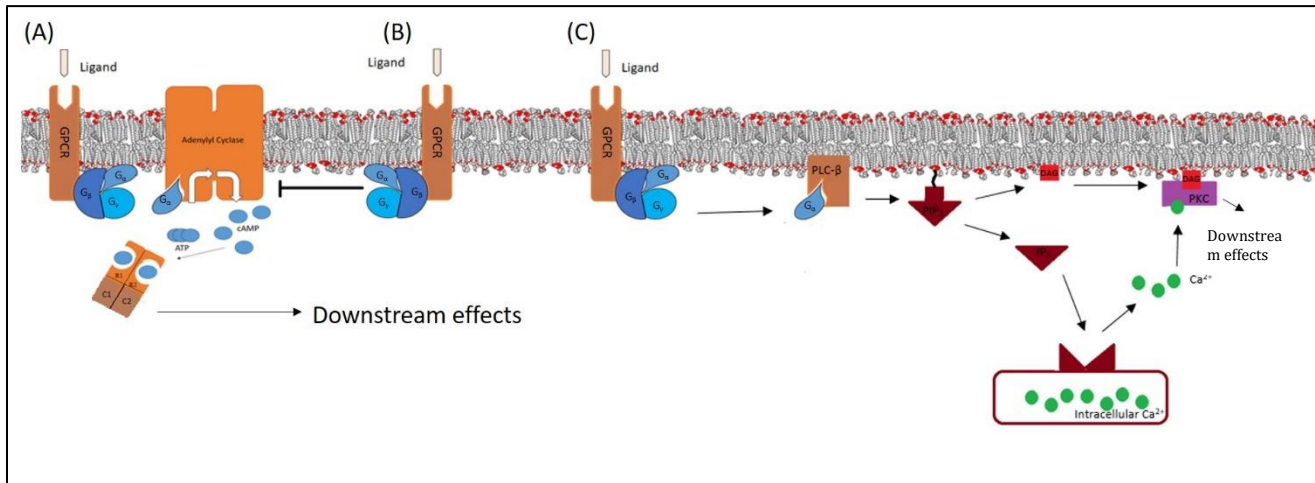
Figure 1: Downstream signaling of various heterotrimeric G proteins. (A) An example of Gαs signaling induced by binding of a ligand to the GPCR. (B) An example of Gαi signaling which leads to the inhibition of adenylyl cyclase. (C) An example of Gαq/11 signaling which leads to the release of intracellular calcium and the activation of PKC. (Alberts, 2015)

There is evidence that ligand binding to GPR120 may recruit TAB-1 through the receptor-β-arrestin-2 complex, inhibiting the downstream activation of IKKs and JNKs, causing an anti-inflammatory state in the cell (Alvarez 2016). CAAT/ enhancer binding protein β (C/EBPβ) has been shown to play a vital role in the transcriptional regulation of GPR120 (Chen et al 2016). GPR120 can also activate pathways through coupled G- heterotrimeric proteins such as $\alpha_{q/11}$, leading to the activation of phospholipase C (PLC) and downstream effectors, such as PKC (Chen 2012). Increased GLP-1 production due to GPR120 activation through this pathway is of interest for the treatment of DM (Liu 2015). Previous studies have also shown short chain fatty acids bind to GPR41 and activate mitogen activated protein kinase (MAPK) via Gβγ-mediated signaling (Inoue 2014).

The GLUT family of integral membrane proteins facilitate the transport of glucose and other sugars across the plasma membrane utilizing the diffusion gradients of these sugars (Wood 2003). This family of facilitative transporters are divided into three classes based on their sequence similarity, with GLUT1-4 belonging to class I; GLUT5, GLUT7 and GLUT 9 belonging to class II; and GLUT6, GLUT8, GLUT10, and GLUT12 belonging to class III (Wood 2003). A key feature of class II facilitative transporters lies in their ability to transport fructose (Augustin 2010).

The expression of certain GLUT transporters has been shown to be regulated by various fatty acids. GLUT4 mRNA in 3T3-L1 adipocytes has been shown to be downregulated due to increased levels of the phospholipid ceramide (Long 1996). Similarly, exposure of H9C2 cardiomyotubes to free fatty acids (Arachidonic, Stearic, Oleic and linoleic acids) has been reported to repress the promotor activity of GLUT4 (Armoni 2005). GLUT 5 has been suggested to be the primary fructose transporter (Augustin 2017), and is shown to be expressed in the

5

jejunum region of the small intestines at a higher level, while in the kidney, brain, skeletal muscles and adipose tissue at a lower level (Corpe 2002). The primary function of GLUT5 has been described as mediating the uptake of fructose across the membrane of the small intestines, as it has a high affinity to fructose (Mueckler 2013). In the gut, GLUT5 levels are regulated by the amount of fructose in the tissue (Jiang et al, 2001). Levels of fructose in individuals consuming diets high in fructose (as would be a risk factor for DM) can reach between 0.2-0.5 mM (Douard 2008). Like the gut, GLUT5 mRNA levels in the brain are found to increase with increased fructose levels (Douard 2008). The physiological importance of GLUT5 in the brain is currently unknown.

The aim of this study is to understand how high levels of fatty acids effect the expression of various GPRs and GLUT transporters in neuronal cells. Specifically, GPR41, GPR84, GPR119, GPR120, GLUT3, GLUT5, GLUT6 and GLUT8 expression levels were investigated in Nuero2A (N2A) cells treated with palmitate. First, a dose response assay was conducted to determine the proper dose of palmitate for treatment purposes. A time course MTT assay was then performed to determine the optimal duration of treatment. N2A cells were then treated with palmitate or vehicle for 24 hours, and their RNA was isolated to determine the mRNA expression of these genes. N2A cells were also treated with GW9508, an agonist for GPR120 and GPR40, and KT5720, a PKA inhibitor, to examine whether GPR120, GPR40, or PKA may potentially affect the expression of these genes.

Materials and methods

Cell culture

Neuro-2A (N2A) cells were cultured in Eagles Modified essential Media (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% pennicellin/streptomyicin antibiotics. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell viability/dose response assay

N2A cells were seeded in 96 well plates in EMEM supplemented with 10% FBS and 1% penn/strep. After a day, the media was changed to serum free EMEM and treated with either bovine serum albumin (BSA) or various concentrations of palmitate conjugated to BSA for specified periods of time. Ten µL of 5 mg/ml MTT reagent was added to the cells and incubated for three hours. At the end of incubation, 100 µl of MTT stop solution was added to solubilize formanzan crystals. The plate was then read at 560nm using the Spectramax M5 plate reader. The absorbance values for each treatment group were normalized to the BSA control group and relative cell viability was calculated and graphed.

RNA isolation

Total RNA was extracted from N2A cells using TRIzol (Sigma, St. Louis, MO) per manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis and quantified by spectrophotometry.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was synthesized utilizing the following: 1 µg of total mRNA, 1 µL of 20µM OligodT, 5 µl of 5x Moloney murine leukemia virus (MMLV)- reverse transcriptase buffer, 1.25 µl of 10 mM dNTP, 1 µL of reverse transcriptase, and nuclease-free water to bring the final volume to 25 µl. Following reverse trancription, PCR was performed utilizing appropriate

primers for *GPR41, GPR84, GPR119, GPR120, GLUT5, β-Actin* and *cyclophilin*. Each PCR

reaction consisted of the following: 0.5 µl of forward primer (20 µM), 0.5 µl of reverse primer

(20 µM), 18µl of Taq supermix and 1 µl of cDNA. Five µl of each PCR reactions were then run

on a 2% agarose gel, which was then imaged on the FlourChem E system (Protein Simple, San

Jose, California). The bands of interest were quantitated using alphaview software, and the data

were normalized with house-keeping genes such as *β-actin* or *cyclophilin*.

KT5720 and GW9508 treatment

N2A cells were seeded in 12 well plates in EMEM supplemented with 10% FBS and 1%

pen/strep. Next day the complete media was replaced with EMEM and treated with 100 µM

KT5720, an inhibitor for protein kinase A, 100µM GW9508, an agonist for GPR120 and GPR40,

or vehicle for 24 hours. Total RNA was then isolated from these cells as described previously.

Statistical analysis

All band intensities of RT-PCR were quantified using the alphaview software. After

normalization against actin or cyclophilin, the RT-PCR data from samples treated with vehicle or

PA were analyzed by student's t-test using the PRISM 6 software. One-way ANOVA was used

to analyze data from the PKA inhibitor and GPR120 agonist studies, and data from MTT assays.

Results

Viability assay of cells treated with different concentrations of palmitate for 24 hours

To determine the effects of palmitate on the viability of N2A cells, N2A cells were treated with 0, 25, 50, 100, 200, or 300 μM PA in unsupplemented EMEM for 24 hours or 48 hours, and cell viability was analyzed by MTT assay. Analysis of the data revealed that treatment with palmitate decreased cell viability in a concentration-dependent manner such that when palmitate concentration increased, cell viability decreased (Figure 2). Specifically, 300 μM PA treatment decreased cell viability by well over 50% while 200 μM PA treatment decreased cell viability by about 50%. 100 μM PA treatment decreased cell viability relatively less than 200μM PA treatment, while 25 μM and 50 μM PA treatment did not have noticeable impact on the viability of N2A cells.

Following treatment for 48 hours, both 200 μM and 300 μM PA treatment significantly reduced N2A cell viability by over 50%. 100 μM PA treatment reduced cell viability by nearly 50%, while 25 μM and 50 μM PA treatment did not have a noticeable effect on the viability of the cells relative to vehicle (Figure 3).
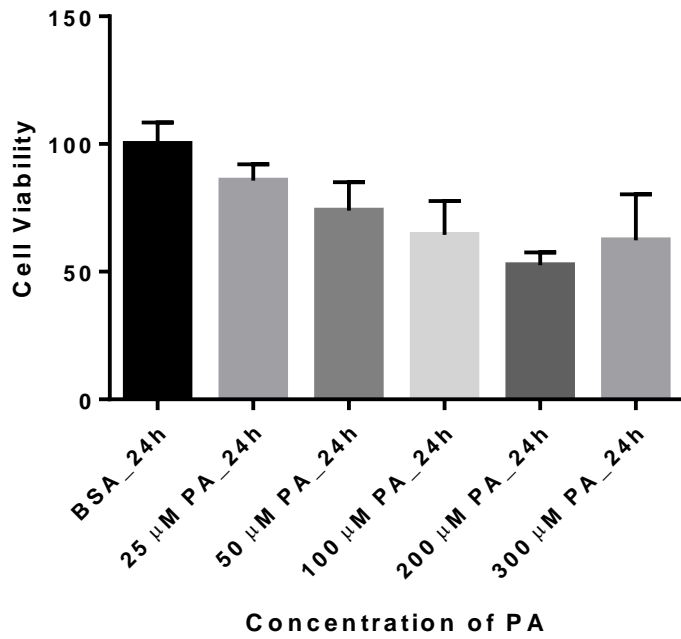
Figure 2: Viability of N2A cells treated with various concentrations of palmitate or BSA for 24 hours as measured with MTT assay. Data shown as mean ± SD. N=3.
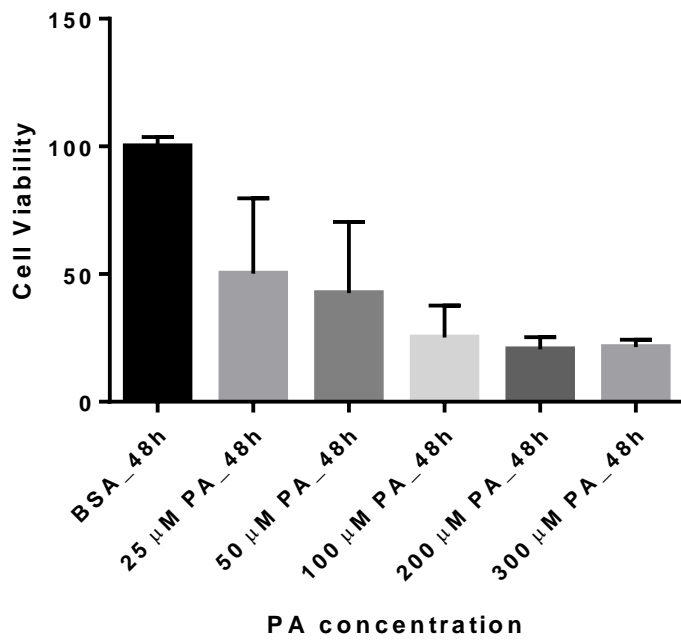
Figure 3: Viability of N2A cells treated with varying concentrations of palmitate or BSA for 48 hours as measured with MTT assay. Data shown as mean ± SD. N=3.

Viability of N2A cells treated with 300μM palmitate for different periods of time

To determine the optimal time for treatment, N2A cells were treated with 300 μM PA for 6, 24 and 48 hour, and cell viability was analyzed with MTT assay. Analysis of the data reveals that treatment with palmitate decreased cell viability in a time-dependent manner (Figure 4). Cell viability after 48 hour treatment with PA decreased over 50%, while 24 hour treatment showed about 50% decrease in viability, while treatment with PA for 6 hours did not significantly change cell viability compared to BSA.
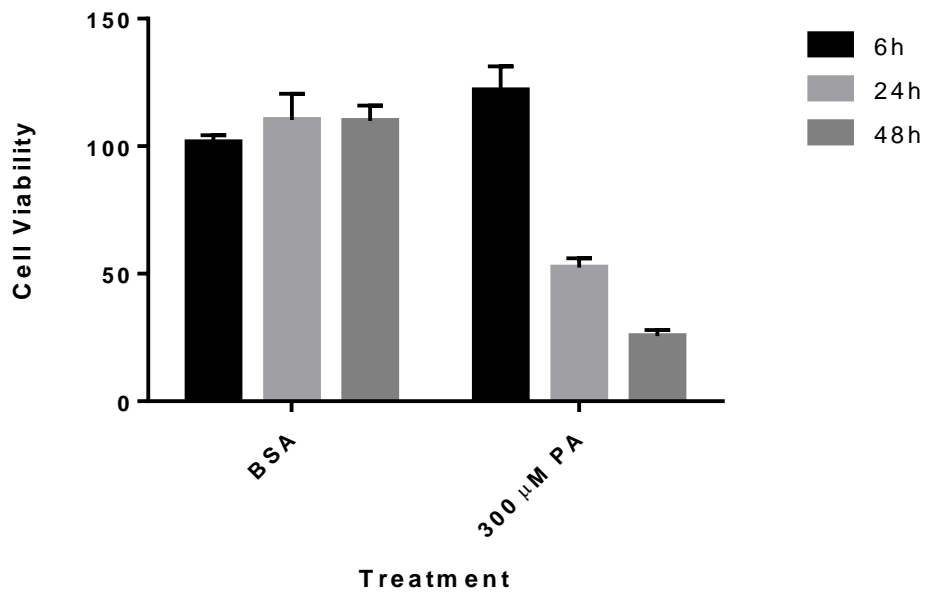
Figure 4: Viability of N2A cells treated with 300µM palmitate for 6, 24 and 48 hours as measured with MTT assay. Data shown as mean ± SD. N=3.

Effect of PA treatment on the mRNA expression of GPR41 and GPR84

GPR41 is a G-protein coupled receptor (GPCR) for short chain fatty acids, and may be involved in energy metabolism. GPR84 is a receptor for medium chain fatty acids, and may be involved in energy metabolism and immune response. To determine how palmitate affected the mRNA expression of GPR41 and GPR84, N2A cells seeded in 12 well plates were treated with 300 µM palmitate or vehicle for 24 hours. RNA was isolated from these cells using TRIzol. RT-PCR reactions using primers specific for GPR41 and GPR84 were performed and run on 2% agarose gel. The gel was imaged and gene-specific bands were quantified with alphaview software, and normalized against actin. As shown in Figure 5, treatment with 300 µM palmitate significantly increased the mRNA expression of GPR41 and GPR84 (Figure 5).

Figure 5: mRNA expression of GPR41 and GPR84 in N2A cells at 24 hours following treatment with BSA or 300 µM PA as measured by semi-quantitative RT-PCR. (A) RT-PCR result of GPR41 expression when treated with 300µM of palmitate or vehicle. (B) Relative mRNA expression of GPR41 as normalized against β-actin. Data shown as mean ± SD. N=3. (C) RT-PCR result of GPR84 expression when treated with 300µM of palmitate or vehicle. (D) Relative mRNA expression of GPR84 as normalized against β-actin. Data shown as mean ± SD. N=3.

Effect of PA treatment on medium chain fatty acid receptor GPR119 and GPR120 expression

GPR119 has been suggested as a receptor for fatty acid amides and monoacylglycerol and a potential target for the treatment of type 2 DM. GPR120 is a receptor for medium and long chain fatty acids. It has also been shown to bind to ω-3 unsaturated fatty acids and a potential target for the treatment of type 2 DM. The effects of palimitate on the mRNA expression of these two receptors were thereby determined. Gel electrophoresis depicted GPR119 band intensity higher for the cells treated with 300μM palmitate than those treated with 300μM vehicle (Figure 6). The mRNA expression of GPR119 and GPR120 was significantly increased in cells following 24 hour-treatment with 300 µM PA as compared to the cells treated with BSA (Figure 6).
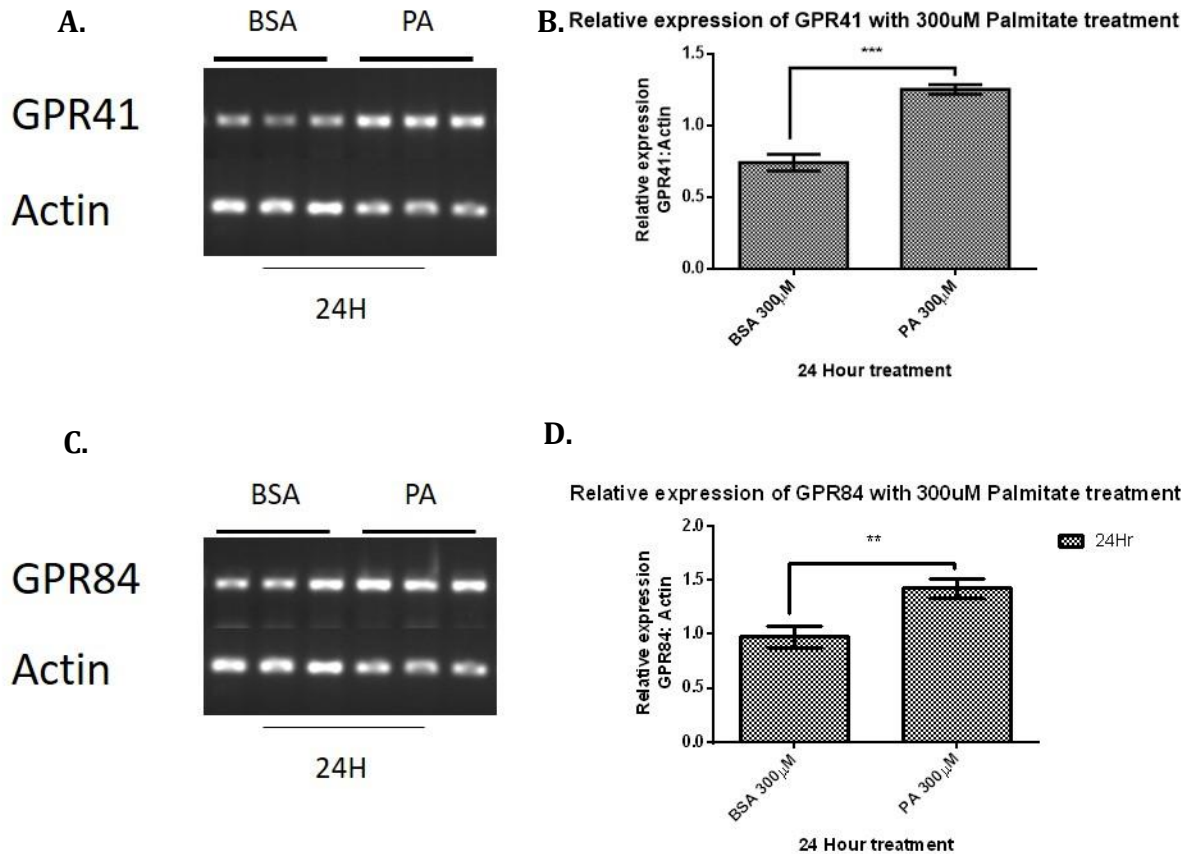
Figure 6: mRNA expression of GPR119 and GPR120 in N2A cells at 24 hours following treatment with BSA or 300 µM PA as measured by semi-quantitative RT-PCR (A) RT-PCR result of GPR119 gene expression when treated with 300µM of palmitate or vehicle. (B) Relative mRNA expression of GPR119 as normalized against β-actin. Data shown as mean ± SD. N=3. (C) RT-PCR result of GPR120 expression when treated with 300µM of palmitate or vehicle. (D) Relative mRNA expression of GPR120 as normalized against β-actin. Data shown as mean ± SD. N=3.

Effect of PA treatment on the mRNA expression of class I and class III GLUT transporters

GLUT3 is a class I transporters that has been found to transport glucose. GLUT6 and GLUT8 are class III transporters that have been reported to transport glucose. To examine whether treatment with palmitate affected the expression of these glucose transporters, N2A cells were treated with 300 µM palmtate or BSA for 24 hours, and RT-PCR was conducted to examine the mRNA expression level of these genes. The mRNA expression of GLUT3, GLUT6 or GLUT8 genes was comparable in cells treated with palmitate and those cells treated with vehicle (Figure 7).
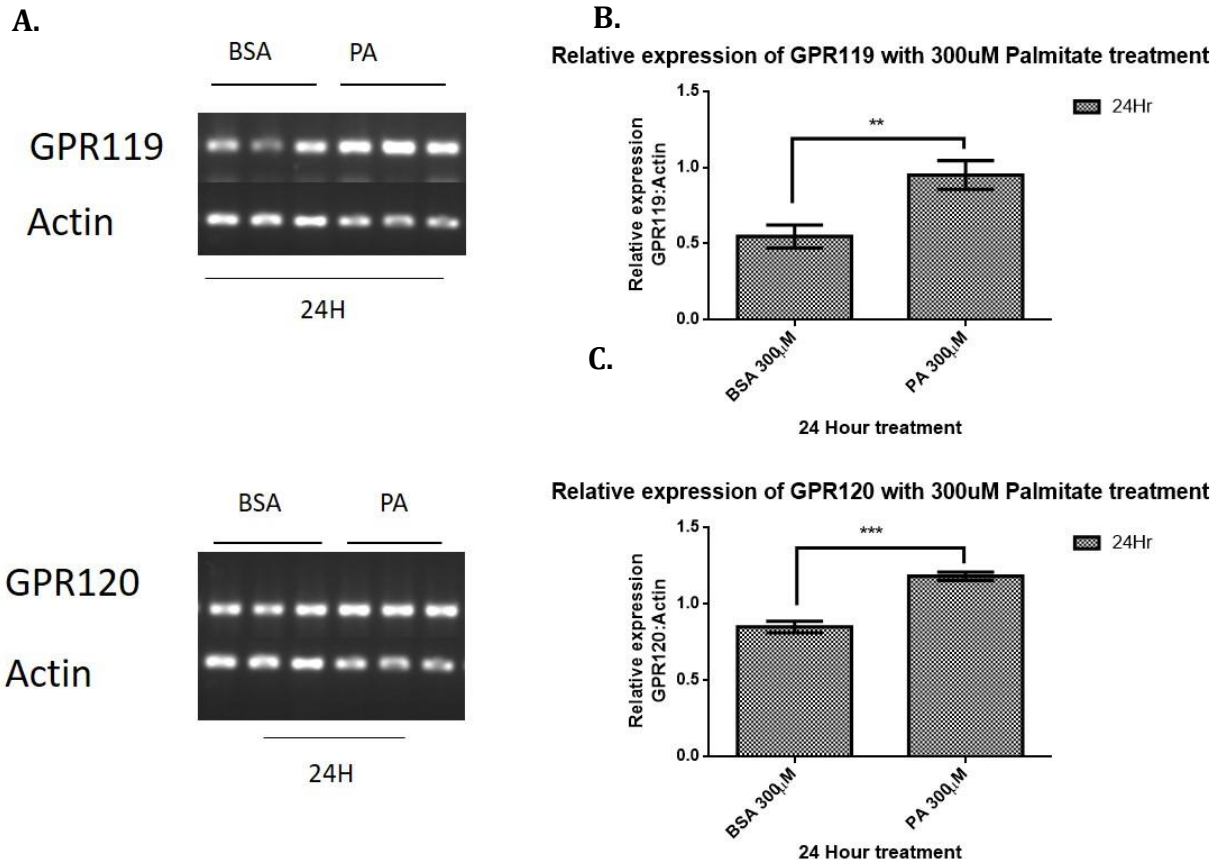
Figure 7: mRNA expression of GLUT3, GLUT6 and GLUT8 genes in N2A cells at 24 hours following treatment with BSA or 300 µM PA as measured by semi-quantitative RT-PCR. (A) RT-PCR result of GLUT3, GLUT6 and GLUT8 gene expression when treated with 300µM of palmitate or vehicle. (B) Quantification of GLUT3 PCR bands as normalized against β-actin. Data shown as mean ± SD. (C) Quantification of GLUT6 PCR bands as normalized against β-actin. Data shown as mean ± SD. (D) Quantification of GLUT8 PCR bands as normalized against β-actin. Data shown as mean ± SD.

Effect of PA treatment on the mRNA expression of GLUT5

GLUT5 is a class II glucose transporter and has been found to transport fructose. To determine how palmitate treatment affected the mRNA expression of GLUT5, N2A cells were treated with palmitate or BSA for 24 hours, and the mRNA expression level of GLUT5 gene was examined using RT-PCR. As shown in Figure 8, the mRNA expression of GLUT5 gene was significantly higher cells treated with 300μM palmitate than those treated with BSA vehicle.
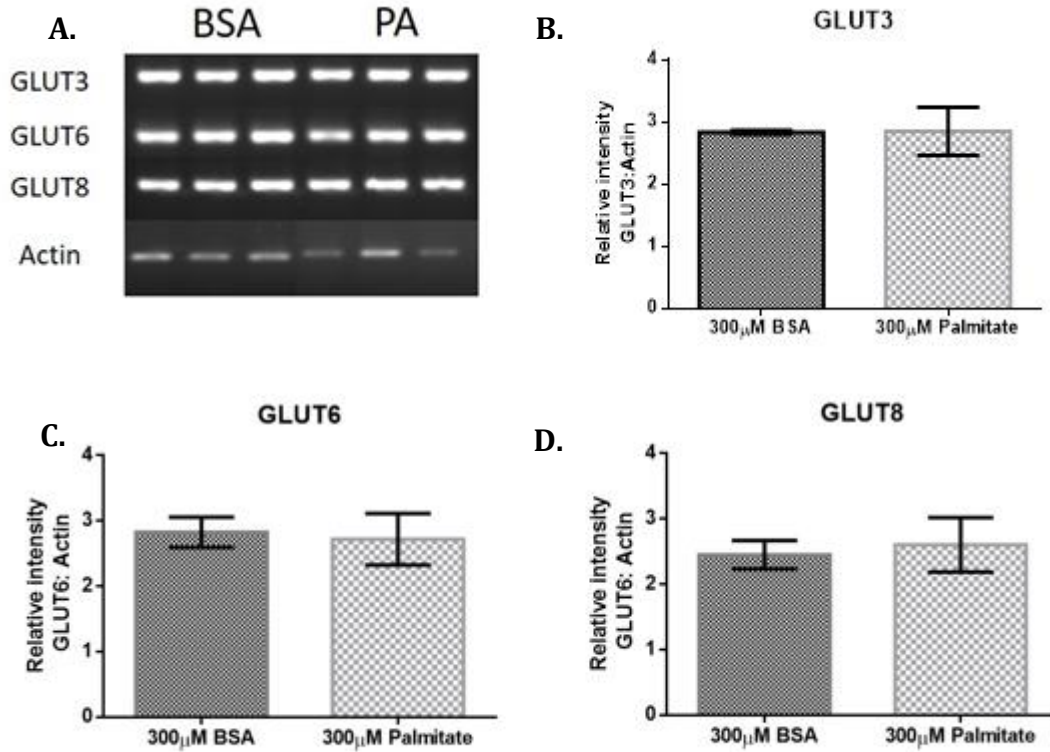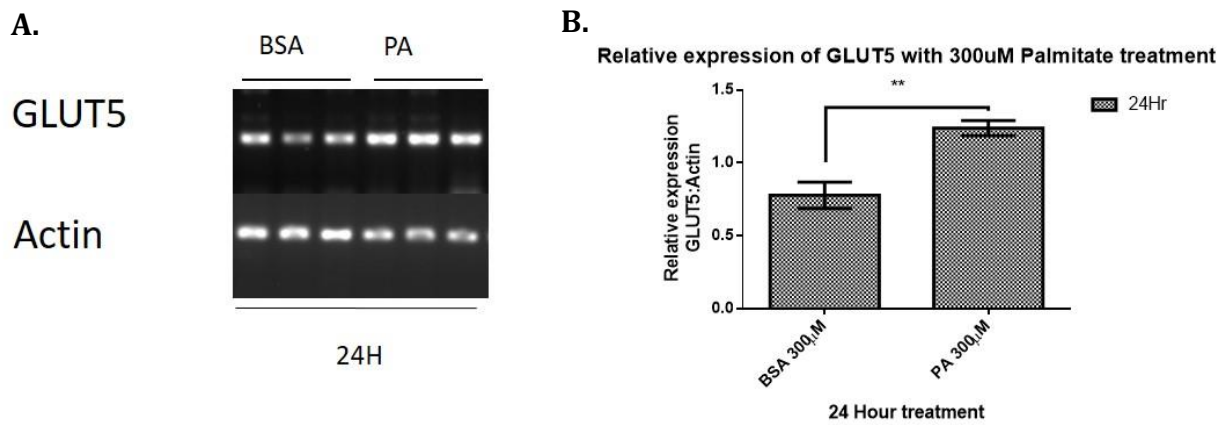
Figure 8: mRNA expression of GLUT5 in N2A cells at 24 hours following treatment with BSA or 300 µM PA as measured by semi-quantitative RT-PCR. (A) RT-PCR result of GLUT5 expression when treated with 300µM of palmitate or vehicle. (B) Relative mRNA expression of GLUT5 as normalized against β-actin. Data shown as mean ± SD. N=3.

Effects of KT5720 and GW9508 on the mRNA expression of GPRs

KT5720 is an inhibitor of PKA. GW9508 is an agonist for GPR40 at lower concentration and GPR120 at higher concentration. In order to examine whether GPR signaling pathway was involved in palmitate induced changes in the mRNA expression of GPRs, N2A cells were treated 100 µM KT5720, 100 µM GW9508, 300 µM PA, and BSA for 24 hours, RNA was isolated, and RT-PCR was conducted with primers specific for GPR41, GPR119, and GPR120 respectively. Treatment with 300µM palmitate increased the mRNA expression levels of GPR41, GPR119 and GPR120 mRNA expression relative to the control ($\alpha < 0.05$, Figure 9). The mRNA expression levels of GPR41 and GPR119 were higher in the cells treated with 100 µM KT5720 as compared to those treated with BSA alone while the mRNA level of GPR120 was not significantly altered by the treatment with 100µM KT5720 (Figure 9). Treatment with 100 µM GW9508 did not significantly change the mRNA expression for GPR41 or GPR119 as compared to the BSA control. However, treatment with 100 µM GW9508 did increase the mRNA expression level of GPR120 gene as compared to the control (Figure 9C and 9F).

Figure 9: mRNA expression of GPR41, GPR119 and GPR120 in N2A cells at 24 hours following treatment with BSA, 100μM of KT5720, 100μM of GW9508, or 300 μM PA as measured by semi-quantitative RT-PCR. (A, B and C) RT-PCR result of GPR41, GPR119 and GPR120 expression when treated with 300μM of vehicle, 100μM of KT5720, 100μM of GW9508, or 300μM of palmitate. (D, E and F) Quantification of PCR bands as normalized to β-actin. Data shown as mean ± SD. N=3.

Effects of treatment with KT5720 and GW9508 on the mRNA expression of GLUT5

In order to examine whether GPR signaling pathway was involved in palmitate induced changes in the mRNA expression of GLUT5, N2A cells were treated with 100 μM KT5720, 100 μM GW9508, 300 μM PA, or BSA for 24 hours. RT-PCR with primers specific for GLUT5 was performed. The mRNA level of GLUT5 gene was higher in cells treated with 300μM palmitate and cells treated with 100uM KT5720 as compared to those treated with BSA alone (Figure 10). Treatment with 100 μM GW9508 did not induce significant changes in mRNA expression for GLUT5 gene (Figure 10).

A.

B.

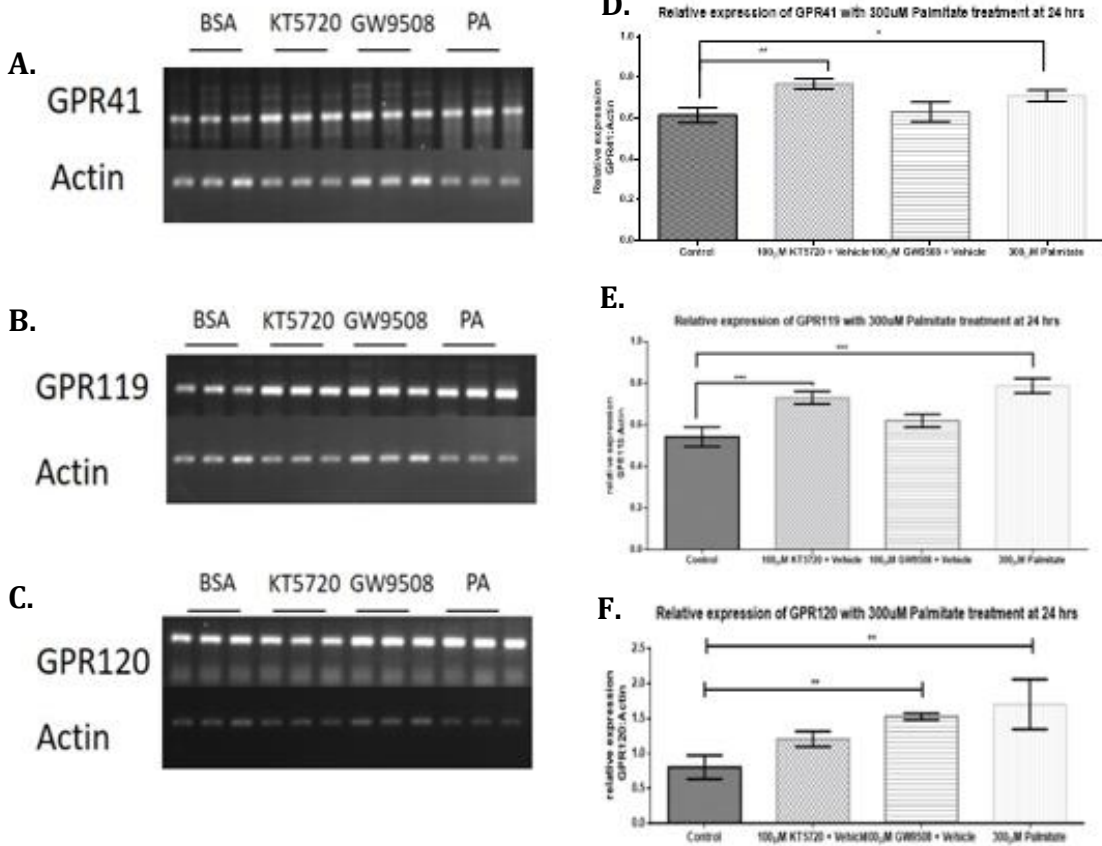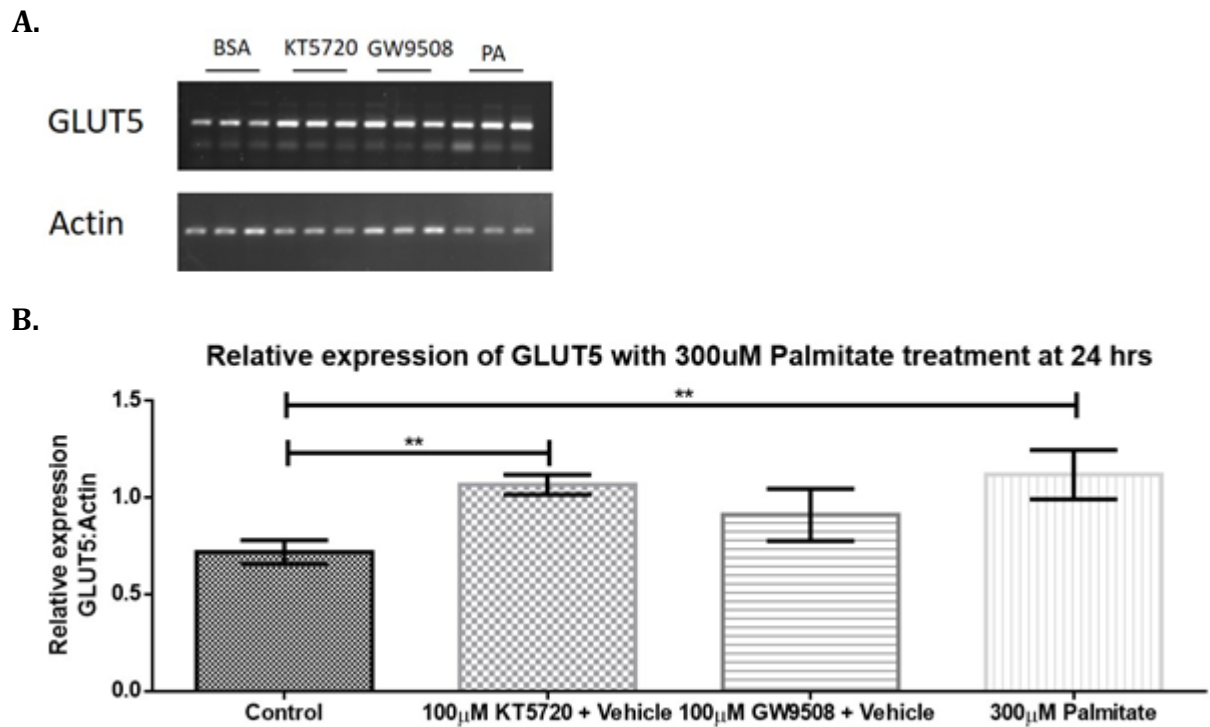**Figure 10:** mRNA expression of GLUT5 in N2A cells at 24 hours following treatment with BSA, 100µM of KT5720, 100µM of GW9508, or 300 µM PA as measured by semi-quantitative RT-PCR. (A) RT-PCR result of GLUT5 expression when treated with BSA, 100µM of KT5720, 100µM of GW9508, or 300µM of palmitate. (B) Quantification of PCR bands normalized to β-actin. Data shown as mean ± SD. N=3.

Discussion

This study examined the effects of palmitate on the mRNA expression of select GPCRs and GLUT transporters in neuronal cells. Palmitate is the most abundant saturated free fatty acid in plasma, constituting up to 30% of total FFAs in the serum (Weigert 2004). Furthermore, the concentration of FFAs can rise to over 1 mM following a fatty meal, as would be expected with individuals with metabolic disorders (Weigert 2004). The results of the viability assay pertaining to the varying palmitate concentrations at 24 or 48 hours revealed that the highest dose of palmitate (300 µM) produced the greatest difference in viability when compared to vehicle (Figures 2 and 3). Therefore, the 300 µM dosage of palmitate was used for treatment. To determine the time-point to use for palmitate treatment, an MTT assay was performed using the 6, 24 and 48 time points with the 300 µM palmitate. The results of this assay showed that 48 hours of treatment with 300 µM palmitate caused a severe reduction in cell viability compared to BSA control. Treatment with 300 µM palmitate for 6 hours did not significantly influence cell viability, while 24 hour treatment decreased cell viability by about 50% (Figure 4). Therefore, for the purpose of the gene expression studies, the 24 hour time point treatment with 300 µM PA was used for physiological relevance.

The effects of palmitate treatment on the mRNA expression of GPRs were examined in this study. GPRs have been of interest for the study of fatty acids due to their roles in regulating metabolic and immune processes (Alvarez 2016). Increased GPR120 mRNA may be attributed to C/EBPβ activation (Chen 2012). C/EBPβ has been shown to play a vital role in the transcriptional regulation of GPR120 and its role in N2A cells treated with palmitate is currently unknown (Chen et al 2016). GPR119 is of interest in the study of fatty acids due to the release of glucagon like peptide-1 (GLP-1) upon stimulation of the receptor (Hansen 2012). The regulation

behind GPR119 expression, however, is not yet so clear. GPR41 (FFAR3) is unique in that it's transcribed as a bicistronic mRNA, with GPR40 upstream of it (Halpbern et al 2012). The HR2 enhancer is important for the expression of this bicistronic mRNA (Halpbern et al 2012). Within the regulatory sequences of HR2 lie consensus sites for the signal transducer and activator of repression (STAT) family of transcription factors, pancreatic duodenal homeobox-1 (PDX-1) and BETA2 transcription factors (Ridner 2008). This receptor is known to be activated by short chain fatty acids and upon activation, it is known that this receptor plays a role in mediating anti-inflammatory effects (Alvarez 2016). GPR84 is a medium chain fatty acid receptor that has been suggested to be regulated by the transcription factor NF-κB (Nagasaki 2012).

Treatment with palmitate for 24 hours upregulated the mRNA levels of GPR41, GPR84, GPR120, and GPR119 (Figures 5 and 6). The responsive increase in short, medium and long chain fatty acid receptor mRNA expression in response to palmitate treatment may be explained through the activation of various signaling pathways, include protein kinase C (PKC) and glycogen synthase kinase 3β (GSK3). Downstream signaling from PKC has been shown to lead to the activation of NF-κB (Kang 2014). Glycogen synthase kinase 3β directly activates C/EBPβ through phosphorylation (Chen 2012). Thus, it seems as if palmitate- induced activation of these two pathways may help to explain the observed increase in the mRNA expression of GPR 84 and GPR120. This palmitate-induced activation may occur through GPR120 or GPR40 stimulation. Further promotor studies are required to understand the cause of both GPR41 and GPR119 increased mRNA expression.

GLUT1, GLUT4 and GLUT5 have been found to be important for the transport of sugars in white adipose tissue and the overall function of WAT (Wood 2003). The enlargement of adipocytes and increased fasting level of FFAs has been reported in obese individuals

(Greenberg 2006). In Caco-2 cells, the glucocorticoid receptor is thought to play a role in regulating the expression of GLUT5 (Takabe 2008). Whether this receptor is involved in the observed effects by palmitate in N2a cells remains unknown. However, fructose concentrations have also been shown to regulate GLUT5 expression (Douard 2008). Many studies have shown a correlation between increased fructose consumption and increased incidence of type 2 diabetes and obesity (Douard 2008). Typically the brain is dependent on glucose as its main energy source (Maher 1991). However, recent studies suggest that the polyol pathway contributes to the production of fructose in the CNS (Hwang 2017). While the mRNA expression of GLUT3, GLUT6, and GLUT8 genes was not significantly altered by the treatment with 300 µM PA, the mRNA expression of GLUT5 was significantly upregulated by treatment with 300 µM PA for 24 hours (Figures 7 and 8). Other studies of different GLUT genes have shown that fatty acid treatment decreases the mRNA levels of GLUTs (Long 1996). The observed increase in mRNA level of GLUT5 in response to palmitate treatment warrants investigating the role of fructose in neuronal cell metabolism. Further testing at lower PA concentrations is also needed to attenuate any variability resulting from apoptotic events.

The use of KT5720 to inhibit PKA and elucidate its role in response to a stimuli is well documented (Batty 2017). In this study, N2A cells were treated with vehicle or 100 µM of KT5720 to determine whether it is likely for PKA to be involved in palmitate treatment induced changes. Treatment with 100 µM KT5720 increased the mRNA expression of GLUT5 as did the treatment with palmitate (Figure 10). Similarly, the mRNA expression of GPR41 and GPR119 was upregulated by the treatment with KT5720 as well as the palmitate (Figure 9). These data suggest that PKA-mediated signaling may negatively regulate the mRNA expression of GLUT5, GPR41 and GPR119. Interestingly, other studies have found that inhibiting PKA in neonatal rats

in a fructose-induced state with PKA inhibitor or H89 did not affect GLUT5 mRNA level in the small intestine (Cui 2004). Whether PKA might play a role in palmitate induced changes in the mRNA expression of GLUT5, GPR41, and GPR119 in neuronal cells needs further investigation.

GW9508 acts as an agonist to both GPR40 and GPR120 with 100 fold lower binding affinity for GPR120 than for GPR40 (Briscoe 2003; Milligram 2017). Activation of GPR120 through G_α leads to the coupling of the $G_{\alpha q}$ to phospholipase C (PLC) (Liu 2015). Downstream signaling from PLC leads to increased diacylglycerol (DAG) and calcium concentrations, activating PKC and further downstream transcription factors (Liu 2015). Similarly, GPR40 activation from fatty acid binding leads to increased DAG and calcium concentrations through $G_{\alpha q/11}$ signaling (Burant 2013). However, activation of GPR120 via phosphorylation of its C-terminus promotes a recruitment of TAB1, a protein which disrupts the TAK1/TAB2/3 complex and consequently inhibits IKK and JNK signaling (Liu 2015). Activation of GPR40 has also been shown to increase phosphorylation of cyclic amp responsive binding element (CREB) and ERK 1/2 (Miyamoto 2016). Clarifying which pathway is activated in response to stimulation with GW9508 is still required. It also remains unclear whether GW9508 treatment activates both pathways in the neuronal cells and what the net effects are. Also, it remains to be determined whether unsaturated and saturated fatty acids induce an additive, synergistic, or antagonizing effects in neuronal cells.

Overall, this study examined the effects of palmitate treatment on the mRNA expression of GPRs and GLUTs in N2A cells. Further investigation may be conducted to better understand the mechanisms of palmitate-induced effects in N2A cells. These studies may shed light on how

increased levels of palmitate may contribute to the development of diabetes and diabetes-associated neuropathy.

References

Alberts, Bruce. Molecular biology of the cell 6th edition. 2014. Garland science: New York and Abingdon, UK.

Alvarez-Curto E, Milligan G. Metabolism meets immunity: the role of free fatty acid receptors in the immune system. Biochemical pharmacology. 2016. 114 pp. 3-13

Armoni, M et al. Free fatty acids repress the GLUT4 gene expression in cardiac muscle via novel response elements. J Biol Chem. 2005. 280 pp. 34786-34795.

Batty N, Fenrich K, Fouad K. The role of cAMP and its downstream targets in neurite growth in the adult nervous system. Nuerosci. Lett. 2017. 653 56-63.

Binienda Z, Sarkar S, Sivia-Ramirez S. Gonzalez C. Role of free fatty acids in physiological conditions and mitochondrial dysfunction. *Food and Nutrition Sciences*, Vol. 4 No. 9A, 2013, pp. 6-15. doi: 10.4236/fns.2013.49A1002.

Boden G. Obesity and free fatty acids. Endocrinol Metab Clin North Am 2008. 37(3)635-46.

Briscoe C.P, Tadayyon M, Andrews J, Benson W, Chambers J, Eilert M, Ellis C, Elshourbagy N.A, Goetz A, Minnick D, Murdock P, Sauls Jr. H, Shabon U, Spinage L.D, Strum J, Szekeres P.G, Tan K, Way J.M, Ignar D, Wilson S, Muir A.I. The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids J. Biol. Chem., 278 (2003), pp. 11303-11311

Burant C. Activation of GPR40 as a therapeutic target for the treatment of type 2 diabetes. Diabetes care. 2013. 36 (suppl 2) pp. S175-S179

Center for disease control and prevention (CDC). Adult Obesity Facts. 2017. Web https://www.cdc.gov/obesity/data/adult.html

Center for disease control and prevention (CDC). The health effects of overweight and obesity. 2017. Web https://www.cdc.gov/healthyweight/effects/

Chen K, Zhou J, Zhang F, Zhang F, Zhang R, Zhan M, Tang X, Deng B, Lei M, Xiong Y. Transcription factor C/EBPβ promotes the transcription of the porcine GPR120 gene. J. Mol. Endocrinol. 2016. 56 pp. 91-100.

Cui X, Ananian C, Perez E, Strenger A, Beuve A, Ferraris R. Cyclic AMP stimulates Fructose transport in Neonatal Rat Small intestines. J. Nutr. 2004. 137 (7) 1697-1703.

Douard V, Ferraris R. regulation of fructose transporter GLUT5 in health and disease. Am J Physiol Endocrinol Metab. 2008 Aug; 295(2): E227–E237.

Dresner A, Laurent D, Marcucci M, et al. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. J Clin Inves 1999. 103(2) 253-259.

Greenberg A, Obin M. Obesity and the role of adipose tissue in inflammation and metabolism. Am J Clin Nutr. 2006. 83(2) pp. 461-465

Halpern K, Veprik A, Rubins N, Naaman O, Walker M. D. GPR41 Gene expression is Mediated by Internal Ribosome Entry Site (IRES)- dependent Translation of Bicistronic mRNA Encoding GPR40 and GPR41 proteins. J. Biol. Chem. 2012. 287 (24) pp. 20154-20163.

Hansen H, Rosenkilde M, Holst J, Schwartz T. GPR119 as a fat sensor. Trends in pharmacological sciences. 2012. 33 (7) pp. 374-381

Hwang J, Jiang L, Hamza M, Dai F, Belfort-Deaguiar R, Cline G, Rothman D, Mason G, Sherwin R. The human brain produces fructose from glucose. JCI insight 2017. 2 (4) e90508.

Inoue D, Tsujimoto G, Kinura I. Regulation of energy homeostasis by GPR41. Front. Endocrinol. 2014. 5:81. doi: 10.3389/fendo.2014.00081

Jiang L, David E, Espina N, Ferraris R. GLUT5 expression in neonatal rats: crypt-villus location and age-dependent regulation. Am J Physiol Gastrointest Liver Physiol 2001. 281 (3) G666-G674.

Kang J. Protein Kinase C (PKC) Isozymes and Cancer. New J Sci. 2014. 14 pp. 36

Kang S. GPR119 agonists: a promising approach for T2DM treatment? A SWOT analysis of GPR119. Drug discov today 2013. 18, (23-24) 1309-1315.

Liu H, Wang W, Xu Z, Liu C, He D, Du L, Li M, Yi X, Sun J. FFA4 receptor (GPR120): A hot target for the development of anti-diabetic therapies. Eur. J. Pharmacol. 2015. 763B pp.160-168.

Long S, Pekala P. Lipid mediators of insulin resistance: ceramide signaling down-regulates GLUT4 gene transcription in 3T3-L1 adipocytes. Biochem K. 1996. 319, pp. 179-184.

Milanski M, Degasperi G, Coope A, et al. Saturated fatty acids produce an inflammatory response predominately through the activation of TLR4 Signaling in hypothalamus: Implications for the pathogenesis of Obesity. J Neurosci 2009. 29(2):359-370.

Miller s et al. A GPR119 signaling system in murine eye regulates intraocular pressure in a sex-dependent manner. Invest Ophthalmol Vis Sci. 2017 Jul; 58(7): 2930–2938.

Milligan G, Alvarez-Curto E, Hudson B et al. FFA4/GPR120: pharmacology and therapeutic opportunities. Trends Pharmacol Sci. Web http://ac.els-cdn.com.ezproxy.shu.edu/S016561471730127X/1-s2.0-S016561471730127X-main.pdf?_tid=5ac66d74-74ef-11e7-8f08-00000aab0f26&acdnat=1501395883_2de96b84f87d642e8bcc342aa3cfa97f

Miyamoto J, Hasegawa S, Kasubuchi M, Ichimura A, Nakajima A, Kimura I. Nutritional Signaling via Free fatty acid receptors. International journal of molecular sciences. 2016. 17, 450; doi:10.3390/ijms17040450

Mueckler M, Thorens B. The SLC2 (GLUT) family of membrane transporters. Mol Aspects Med. 2013. 34(0) 121-138.

Nagasaki H, Kondo T, Fuchigami M, Hashimoto H, Sugimura Y, Ozaki N, Arima H, Ota A, Oiso Y, Hamada Y. Inflammatory changes in adipose tissue enhance expression of GPR84, a medium-chain fatty acid receptor: TNFα enhances GPR84 expression in adipocytes. FEBS Letters. 2012. 586 (4) pp. 368-372.

Ridner G, Bartoov- Shifman R, Zalogin T. Avnit-Sagi T, Bahar K, Sharivkin R, Kantorovich L, Weiss S, Walker M. Regulation of the GPR40 locus: towards a molecular understanding. Biochem, Soc, Trans. 2008. 36 (3) pp. 360-362.

Sassone-Corsi. The Cyclic Amp Pathway. Cold Spring harb Perspect Biol 2012;4:a011148

Stein C, Colditz G. The Epidemic of Obesity. J Clin Endocrinol Metab. 2004. 89 (6): 2522-2525

Takabe S, Mochizuki K, Goda T. De-phosphorylation of GR at Ser203 in nuclei associates with GR nuclear translocation and GLUT5 gene expression in Caco-2 cells. Arch Biochem Biophys 2008. 475 (1) pp. 1-6

Weigert C, Brodbeck K, Staiger H, Kaisch C, Machinao F, Haring H, Schleincher E. Palmitate, but not unsasturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-κB. Journal of biological chemistry. 2004. 279 (23) pp. 23942-233952.

Wood I, Hunter L, Trayhurn P. Expression of Class III facilitative glucose transpoter genes (GLUT10 and GLUT12) in mouse and human adipose tissues. Biochemical and biophysical research communications. 2003. 308 (1) pp.43-49

Wood S, Trayhurn P. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. Br J Nutr 2003. 89, 3-9.

Wu J, Zhou Z, Hu Y, Dong S. Butyrate-induced GPR41 activation inhibits histone acetylation and cell growth. J Genet Genomics 2012. 39(8):375-84.