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Pacheco Valencia, Marino, Noutsos, Poon

Institutions: State University of New York at Old Westbury

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Concentration-dependent change in hypothalamic neuronal transcriptome by the dietary fatty acids: oleic and palmitic acids

Fabiola Pacheco Valencia[1]^, Amanda F. Marino[1]^, Christos Noutsos[1], Kinning Poon[1]*

[1]Department of Biological Sciences, SUNY Old Westbury, Old Westbury NY, United States

^Authors contributed equally to this work

*Corresponding Author:

Kinning Poon

223 Store Hill Rd

Old Westbury, NY 11568, USA

1-516-876-2735

poonk@oldwestbury.edu

Abstract

Prenatal high-fat diet exposure increases hypothalamic neurogenesis events in embryos and programs offspring to be obesity-prone. The molecular mechanism involved in these dietary effects of neurogenesis are unknown. This study investigated the effects of oleic and palmitic acids, which are abundant in a high-fat diet, on the hypothalamic neuronal transcriptome and how these changes impact neurogenesis events. The results show differential effects of low and high concentrations of oleic or palmitic acid treatment on differential gene transcription. Gene ontology analysis uncovered significant gene enrichment in several cellular pathways involved in gene regulation and protein production, particularly with proliferation, migration, and cell survival. The enriched signaling pathways include Wnt, integrin, PDGF, and apoptosis, in addition endocrine function signaling pathways CCKR and GnRH. Further examination of proliferation and migration show low concentrations of oleic acid to stimulate proliferation and high concentrations of both oleic and palmitic acid to stimulate apoptosis. Oleic acid also reduced hypothalamic neuronal migration, with little effects by palmitic acid. The results show direct impact of the two most abundant fatty acids in a high fat diet to directly impact hypothalamic neuronal proliferation and migration. The results also uncovered signaling pathways affected by oleic and palmitic acid and suggest a mechanism of prenatal high-fat diet induced neurogenesis events is through these two abundant fatty acids.

Keywords: hypothalamic neurons, oleic acid, palmitic acid, proliferation, apoptosis, migration

1. Introduction

The hypothalamus is a heterogeneous brain region that regulates homeostatic processes of the body, including energy sensing in relation to hunger and satiety [1]. There are many types of neurons in the hypothalamus that contain a variation of neurotransmitters, neuropeptides, and receptors that control these homeostatic processes [2]. While the ingestion of a high-fat diet in adult animal models change the expression patterns of these neuronal regulators in the hypothalamus [3, 4], exposure to these diets during pregnancy has been widely accepted to program offspring to be more prone to ingesting these diets and becoming obese [5, 6]. Neuronal prenatal programming by a high-fat diet includes epigenetic changes [7], increased neurogenesis of orexigenic neuropeptides [6, 8], increased inflammation [9, 10], and altered neuronal patterning and connections to other brain regions [11] and to the gut [12]. The molecular mechanisms leading to these developmental changes while widely studied are still under speculation.

A large component of high-fat diets used in animal model studies is the saturated fatty acid, palmitic acid, and the monounsaturated fatty acid, oleic acid, which respectively constitutes 29% and 49% of these diets [13, 14]. These two fatty acids can promote cell proliferation and differentiation both within and outside of the central nervous system [15, 16]. The ingestion of a high-fat diet during pregnancy also increases availability of fatty acids in the placenta and to the embryo itself [17, 18]. Thus, it is possible that HFD ingestion during pregnancy increases the availability of fatty acids to the developing embryonic brain. The highly concentrated neural progenitor cell population may be disturbed by the overabundance of fatty acids and lead to changes in neurogenesis events, consequently producing hyperphagic offspring. To better examine the direct effect of fatty acids on neurodevelopmental processes, this study utilizes an immortalized rat embryonic hypothalamic neuronal cell line to examine differential gene expression changes

caused by oleic and palmitic acid. The findings reveal several changes in pathways relating to the cell cycle, proliferation, apoptosis, and migration, in addition to other endocrine related processes. Further examination of hypothalamic neurons shows low concentrations of oleic acid to stimulate proliferation and high concentrations of oleic and palmitic acid to stimulate cell death while inhibiting migration. These results provide potential cellular pathways that may be involved in the effects of a high-fat diet through oleic and palmitic acids on distinct aspects of hypothalamic neurodevelopment.

2. Methods

2.1 Cell culture. Immortalized embryonic day 18 (E18) hypothalamic rat neurons (rHypoE-9) were acquired from Cedarlane (Burlington, NC). Neurons were maintained in culture using 10% FBS in 1X DMEM supplemented with 1X penicillin/streptomycin (Thermofisher Scientific, Waltham, MA). Cells were placed in a humidified 5% CO_2 incubator at 37°C. Cells were passaged every 3-4 days when the confluency reached ~95%. Fatty acids (Sigma-Aldrich, St. Louis, MO) were dissolved in 100% ethanol at 1000x's the usable concentration and diluted immediately prior to use, as previously described [14, 19, 20].

2.2 RNA-seq. The hypothalamic neurons were treated with 1, 10, and 100 μM oleic acid or palmitic acid for 24 hours prior to experimentation (n = 3), as previously described [14]. Cell culture samples were collected with RNAprotect Cell Reagent (Qiagen, Germantown, MD) and the mRNA was extracted using a Qiagen RNeasy kit (Qiagen, Germantown, MD), as previously described [21]. The yield was initially quantified with a Nanophotometer (Implen, Germany), with resulting ratios of absorbance at 260 to 280 nm of total RNA from all samples ranging between

1.90 and 2.10, indicating high purity. The mRNA samples were sent to Genewiz (South Plainfield, NJ) to determine RNA integrity (RIN > 9.9 for all samples), library preparation, quantification and quality control analysis. Genewiz also performed RNA sequencing with polyA selection using Illumina HiSeq 2x150 bp single index sequencing. Sequencing yielded libraries with an average of 34 million reads. Data was returned in FastQ formats on an external hard drive. The data analysis was performed utilizing Seawulf HPC at Stony Brook University.

2.3 Transcriptome Data Analysis. The RNAseq analysis was first aligned and annotated to the Rattus Norvegicus Rnor 6.0 genome using STAR v2.7.6 [22] with default settings. The output ".bam" files were then quantified using Stringtie v2.1.4 [23] to estimate transcript abundances as FPKM (Fragments Per Kilobase of exon per Million fragments mapped) which normalizes transcript expression for transcript length and the total number of sequence reads per sample. The reads were first quantified to the Rnor 6.0 reference annotation sequences, merged and requantified to the global merged transcripts. Following this, the reads were analyzed in R using DEseq2 v3.12 [24] to determine differential expression across all treatment groups. Over 90,000 genes were aligned and quantified across all experimental condition.

Gene expression analysis was performed across all concentrations of palmitic and oleic acids using Genesis [25] to select genes that were differentially expressed. The data was transformed to Log2 fold change and using 1 – Pearson correlation metric and were hierarchically clustered (HCL) for differences in expression as a function of the fatty acid or genes. To understand the biological meaning of the differentially expressed genes, the resulting gene IDs was loaded into Panther (http://www.pantherdb.org) or ENRICHR (https://maayanlab.cloud/Enrichr/) for Gene Ontology (GO) enrichment analysis. To further classify the pathways affected by each individual fatty acid

treatment, manual selection of only downregulated or upregulated genes for each treatment was examined to determine enrichment across each condition. For all analyses, a threshold of $p < 0.05$ was set. Ranking of GO was computed by the combined *p*-value multiplied by the z-rank (see ENRICHR). Verification of GO analysis descriptions in addition to examining function of each individual gene was manually searched using The Rat Genome Database (https://rgd.mcw.edu/GO/). Additional function of genes was cross referenced at The Human Gene Database (https://www.genecards.org).

This analysis was repeated under only oleic acid or palmitic acid conditions to examine clustering of differentially expressed genes as a function of concentration. Lastly, the data was manually sorted, filtered using Genesis and only differentially expressed genes under all three oleic or palmitic acid conditions were selected for further GO analysis. The Volcano plots and bar graphs representing GO analysis were created using ENRICHR, and HCL plots and heatmap using Genesis.

2.4 Proliferation Assay. The hypothalamic neurons were seeded in a 96-well plate with 10,000 cells per well. Neurons were treated for 24 hr with 1, 10, 50, 100, 200 and 400 μM oleic acid or palmitic acid ($n = 8$ per condition). A standard curve was generated from additional neurons that were seeded at a density ranging from 50 – 50,000 cells 4 hr prior to performing the cell proliferation assay to allow for attachment. The cell proliferation assay was performed as per manufacturer's instruction using a CyQuant kit (Thermofisher Scientific), which measures the levels of DNA via fluorophore labeling. The labeled DNA for the standards, control and treated neurons were measured at 480nm. The number of neurons were calculated based on the standard curve.

2.5 Cell migration scratch assay. The hypothalamic neurons were seeded in a 6-well plate with 1 x 10^6 cells per well. Once the cells reached ~75% confluency, a scratch was drawn across the middle of the plate with a 10 μL micropipette tip. Neurons were treated for 48 hours with 1, 10, 50, 100, and 200 μM oleic acid or palmitic acid (n = 8 per condition). Images were taken at timepoint 0 and again at 24 hr. The area within the scratch space was blind analyzed using ImageJ. The difference in area between 0 and 24 hr for control and treatment conditions were calculated and compared.

2.6 Statistical Analysis. Blind-data analysis was performed for all experiments. Statistical significance for RNA-seq data analysis was built into the programs and set at a threshold of $p < 0.05$. Statistical significance for the cell proliferation and migration assays were examined using a one-way ANOVA followed by Bonferroni *post hoc* test.

3. Results

3.1 Differential gene expression changes as a function of fatty acid treatment

Examination of 1, 10 and 100 μM oleic or palmitic acid treatment on hypothalamic neurons reveal hundreds of genes to be significantly differentially expressed in comparison to control (1 μM OA = 222, 10 μM OA = 493, 100 μM OA = 450, 1μM PA = 236, 100 μM PA = 439, 10 μM PA = 465; $p < 0.05$). Further examination of differential expression reveal overlap in many genes across 2 or more conditions (**Table 1**). To examine gene expression patterns, hierarchical clustering analysis (HCL) was performed to reveal groups of genes that show similar responses to fatty acid treatment across different conditions. The HCL analysis for all treatment groups did not show a clear

clustering pattern in differential gene expression. This may be attributed to the lack of overlap in gene expression among the low to high concentrations of fatty acid in addition to the heterogenous mixture of neurons in the hypothalamus. The data was next separated into either only oleic acid condition or palmitic acid condition and reanalyzed. The resulting HCL analyses reveal two distinct clusters between 100 μM oleic and palmitic acid and 1 and 10 μM oleic and palmitic acid. (**Figure 1**). Many genes that are not differentially expressed at 100 μM concentrations are either down or upregulated at 1 and 10 μM oleic and palmitic acid and vice versa. The lack of clear patterning of differentially expressed genes across 1 and 10 μM oleic and palmitic acid indicates that each fatty acid concentration itself produces distinct effects. Gene enrichment via gene ontology (GO) analysis was performed on the 3 oleic acid or palmitic acid treatment groups. The most gene enrichment occurred under cellular processes, followed by biological process and molecular function. The top 10 processes in each of these categories show significant enrichment in pathways related to chromosomal regulation, cell cycle, and mRNA and protein processing (**FIGURE 2**). These results suggest that differences in gene expression can be attributed to a concentration effect and that fatty acids impact cellular processes involving cellular processes.

3.2 Gene ontology analysis reveals trends in affected cellular pathways

The differentially expressed gene sets across varying concentrations of fatty acid suggest distinct effects on hypothalamic neurons. To examine each concentration of oleic and palmitic acid independently, GO analysis was performed for each individual treatment. The GO analysis of the upregulated and downregulated genes for each treatment revealed significant enrichment in several cellular pathways ($p < 0.05$). The top signaling pathways that were affected in at least two fatty acid conditions are: Wnt, cholecystokinin receptor (CCKR), apoptosis, platelet-derived growth

factor (PDGF), integrin, and gonadotropin-releasing hormone receptor pathway (**Table 2**). The genes involved in each of the pathways were distinct across the concentrations of oleic and palmitic acid and further evidence a concentration effect on differential gene expression. The top pathways were examined in greater detail.

3.3 Wnt signaling pathway

The activation of canonical Wnt signaling by Wnt receptors leads to an increase in β-catenin signaling and changes gene transcription, resulting in cell proliferation and cell survival [26, 27]. Some Wnt receptors include the G-protein coupled receptor *FRIZZLED* and *LRP5/6* [28], in addition to a few tyrosine receptor kinases (*RYR* and *ROR*; **FIGURE 3**) [29, 30]. Several g-protein subunits and kinases were downregulated at 1 or 10 μM oleic acid or palmitic acid treatment (*Csnk2b, Prkcd, Gng12, Gnb1*) or upregulated at 10 μM oleic acid (*Lrp6*) and at 100 μM palmitic acid (*Csnk2a1*). Genes associated with regulating Wnt signaling were also changed. Genes that inhibit Wnt signaling [31, 32] were found to be upregulated at 100 μM palmitic acid (*Smad5, apc, Tle4*) and downregulated at 1 and 10 μM oleic acid (*apc, tle2, and dvl2*). Other changes involve the SWI/SNF (switch/sucrose nonfermentable) family of protein complexes, which activates Wnt signaling [33]. The SWI/SNF family of proteins acts as a switch in changing chromatin structure and forms large complexes to regulate gene transcription [34]. This complex is required for stem cell proliferation [35], including neuronal development [36, 37]. The SWI/SNF family of genes were downregulated at 10 and 100 μM oleic acid (*Smarcd3, SRCAP, Ppp3c, Pcdhga2, Arid1b*) while *Smarcc2* was the only upregulated gene at 10 μM oleic acid. Interestingly, genes that are involved in Ca^{2+} signaling were increased with 10 μM oleic acid (*PPP3ca, Ppp3cc, Itpr3*) and may play a role in the activation of the noncanonical Wnt activated gene transcription pathway [38].

Two genes that were upregulated at 100 μM palmitic acid had unknown function (*AC109100, Celsr2*). These gene expression changes show activation of Wnt signaling at low oleic acid concentrations suggesting cell proliferation and survival and the opposite effect at high oleic and palmitic acid concentrations.

3.4 CCKR signaling pathway

Cholecystokinin (CCK) is a peptide released from the small intestine that signals satiety and senses lipids in the gut [39]. In the hypothalamus, neurons from the nucleus of solitary tract also contain CCK neurons that project into the hypothalamus to control hunger and satiety signaling [40]. Thus, it is not surprising that this pathway arose in GO analysis of fatty acid treatment on hypothalamic neurons. The CCK signaling pathway also overlaps with apoptosis and Wnt signaling pathways [41], and suggests that the overlap in hunger and satiety signaling with cell proliferation and apoptotic pathways may be a side effect of metabolic signaling. The pathways leading to CCK and CCKR transcription and the downstream pathways of CCKR signaling are complex (**FIGURE 4**). At 10 and 100 μM palmitic acid, several genes involved in pathways leading to gene transcriptional changes were downregulated (10: *Hbegf, Yes1, Cdc42, Gnb1, Orkcd*; 100: *Hbegf, Eif4e, Men1, Yes1*). At 10 μM palmitic acid (*Akap1, Camkk1, Eif4e, Mef2d*) and 100 μM oleic acid (*Camkk1, Egr1, Hbegf, Mef2d*), several genes involved in calcium signaling pathways were upregulated. G protein beta subunit 1 (*Gnb1*) is highly downregulated at 10 μM palmitic acid and may be coupled to CCKR receptors to activate downstream signaling. Knockdown of this G-protein subunit has been implicated in the development of obesity [42]. CCK can directly activate the eukaryotic initiation transcription factor 4 (*Eif4e*) to initiate translation [43] and is upregulated at 10 μM but down regulated at 100 μM palmitic acid. Protein kinase C delta (*Prkcd*) and YES proto-oncogene

1 (*YES1*) are downstream of both CCKR and EGFR signaling and are downregulated with 10 μM palmitic acid treatment. This pathway may be involved in the transcription of *Hbegf* [44]. Other transcription factors that were upregulated at 10 μM palmitic acid and 100 μM oleic acid include myocyte enhancer factor 2D (*Mef2d*), A-kinase anchoring protein 1 (*Akap1*), and early growth response 1 (*Egr1*). These proteins are involved in cell survival, proliferation, and migration processes [45-47]. One gene that was upregulated has not been previously identified (*AABR07045405*). These results suggest that at high concentrations of fatty acids, cellular pathways leading to cell survival are inhibited while at 10 μM oleic acid and palmitic acid there is both up and downregulation of genes involved in proliferation and migration.

3.5 Apoptosis signaling pathway

The classical apoptosis pathway results in the activation of caspase cascades that leads to irreversible apoptosis [48]. A method of activation of apoptosis is through the mitochondrial *Bax/Bak* receptors (**Figure 5**). Activation *Bax/Bak* stimulated by cellular damage or stress results in the release of cytochrome c, which initiates the caspase pathway [49]. The *Bcl2* and *TMBim6* family of proteins under normal conditions inhibits the activation of *Bax/Bak*. At 100 μM palmitic acid, *Bcl2l2* (Bcl-w) is highly downregulated, suggesting increased activation of the apoptotic pathway. Upstream transcriptional activation of *Bcl2l2* expression include β-catenin [50], which is also downregulated at 100 μM palmitic acid and suggests overlap with Wnt signaling. *Pik3ca* is also downregulated at 100 μM palmitic acid, with downstream activation of *Akt* signaling to also activate *β-catenin* driven *Bcl2* gene expression [51, 52]. Downregulation of *Map4k2* at 100 μM palmitic acid can inhibit *Bcl2l2* through *JNK* signaling [53]. Jun dimerization protein 2 (*Jdp2*) is

a transcription factor involved in cell survival [54] and was also downregulated at 100 μM palmitic acid.

Similar gene expression changes occurred at 100 μM oleic acid, with downregulation of *Tmbim6, Bak1* and *pik3ca*. *Prkcd* was also downregulated at 10 μM oleic acid and is downstream of pik3ca anti-apoptotic cell signaling [55]. Heat shock proteins have also been implicated in anti-apoptotic pathways, including activation of *Akt* and inhibition of *Bax/Bak* [56, 57], and one isoform (*Hspa2*) was greatly downregulated at 10 μM oleic acid. The downregulation of genes involved in anti-apoptotic pathways and upregulation of apoptosis signaling at 100 μM oleic and palmitic acid concentrations show high levels of fatty acids to be toxic to hypothalamic neurons.

3.6 Integrin signaling pathway

Integrins are a group of receptor proteins that interact with the extracellular matrix to induce signaling cascades involved in cell proliferation and migration processes [58]. Integrin receptor signaling can activate MAP kinase cascades and gene expression changes [59] of proteins that regulate the cell cycle (**FIGURE 6**) [58]. Integrin receptors can also be activated through an inside-out reverse signaling mechanism [59, 60]. One pathway of inside-out activation is through rap gtpases (*Rap2c*), which recruits talin (*tln1*) to *PIP3*, resulting in changes in intracellular Ca2+ levels [61]. Vasodilator stimulated phosphoprotein (*vasp*) similarly induces inside out activation of integrin receptors [62]. Extracellular matrix proteins such as collagen type IV α2 chain (*Col4a2*) can bind to integrin receptors to initiate gene transcription of *PI3K-Akt* and promote cell proliferation in tumors [46]. Several genes in the integrin signaling pathway were downregulated at both 1 μM oleic acid (*Col4a2, Rap2c*) and palmitic acid (*Cal4a2, Mapk1, Itga3, Tln1, Vasp*) treatment. The downregulation of integrin signaling pathway proteins suggest a decrease in

proliferation [63, 64] and changes in migration. However, integrin signaling is complex and thus migratory processes cannot be inferred from the differentially expressed genes alone [65].

3.7 PDGF signaling pathway

Platelet-Derived Growth Factor (PDGF) signaling pathway regulates a diverse set of functions, including proliferation, cell survival, differentiation, modulation of receptors, and the development of specific neuronal subtypes (**FIGURE 7**) [66]. The activation of tyrosine receptor kinases is important for neurogenesis processes in the brain [66]. At all three concentrations of palmitic acid and at 1 μM oleic acid, several genes involved in cell migration processes were upregulated. The vav guanine nucleotide exchange factor 2 (*Vav2*) found to be upregulated at 1 μM oleic acid and 1 μM palmitic acid is downstream of PDGF signaling and targets *RhoA*, *CDC42*, and *RAC* to change gene transcription [67]. MAPK interacting serine/threonine kinase 1 (*Mknk1*) is upregulated in all but 100 μM palmitic acid and activates *Eif4* mediated mRNA translation of proteins involved in migration [68, 69]. Other genes downstream from PDGF activation that were upregulated at 1 μM oleic acid are phosphoinositide-3-kinase regulatory subunit 3 (*Pik3r3 or PIP3*) and inositol 1,4,5-trisphosphate receptor type 3 (*Itpr3*). Downstream *Pik3r3* is AKT serine/threonine kinase 2 (*Akt2*) and E74 like ETS transcription factor 2 (*Elf2*), which are upregulated at 10 μM palmitic acid. Other genes downstream from PDGF activation that were upregulated at 1 μM palmitic acid include StAR related lipid transfer domain containing 13 (*Stard13*), which is downstream from β-catenin signaling, and *Srgap2* that is involved in stimulating cell migration [70]. At 100 μM palmitic acid, *Mapk6, Srgap2*, and *Nin* are upregulated. *Mapk6* is involved in neuronal morphogenesis and spine formation while negatively regulating cell proliferation [71, 72]. *Nin* is involved in microtubule formation during migration and in

stimulating axogenesis [73]. These selective pathways suggest that palmitic and oleic acids play a role in migration.

3.8 Gonadotropin-releasing hormone signaling pathway

Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of follicle stimulating hormone and luteinizing hormone from the pituitary [74]. Several neuropeptides that regulate food intake either co-express with or are connected to GnRH neurons [75-78]. At 10 and 100 μM oleic and palmitic acid, several genes involved in gonadotropin signaling or gonadotropin gene expression were downregulated (*Prkab1, Prkab2, Prkc, Sdf4, Irs2, Ksr1, Map4k2, Smad2, Smad4, Per1, Bmpr2, Cdc42, Gnb1*). Only three genes were upregulated at 100 μM palmitic acid (*Bmpr2, Map4k5, Smad5*). The protein kinase AMP-activated non-catalytic subunit beta 1 (*Ampk*) and insulin receptor substrate 2 (*Irs2*) are expressed in hypothalamus and acts as nutrient sensors through control of orexigenic neuropeptides [76, 78, 79] and as stimulants of gonadotropin release [80, 81]. The SMAD family, period circadian regulator 1 (*per1*), *Cdc42*, and *Mapks* are downstream from GnRH signaling [82, 83]. *Smads* and bone morphogenetic protein receptor 2 (*Bmpr2*) are also involved in the transcriptional activation of GnRH [84]. These results are consistent with fatty acids modulating the activity of hypothalamic neurons involved in controlling ingestive behavior in addition to sex hormone production.

3.9 Differentially expressed gene overlap in oleic and palmitic acid treatment

The individual GO analysis revealed overlap in cellular pathways affected by the individual fatty acids. Using Panther, only differentially expressed genes across all three oleic acid or palmitic acid concentrations were examined and 27 genes returned matches for oleic acid and 36 genes returned

matches for palmitic acid. The HCL analysis revealed 4 clusters with oleic acid treatment and 3 clusters of genes with palmitic acid treatment (**FIGURE 8**). The limited number of genes did not yield significant enrichment in any pathway during GO analysis but may be examined in future studies on modulation of neuronal function.

3.10 Oleic and palmitic acid stimulates proliferation and cell death and inhibits migration

Based on the transcriptome analysis, both oleic and palmitic acid is involved in the progression of cell cycle processes, migration and apoptosis. To examine whether these fatty acids affect hypothalamic neuronal proliferation and death, a cell proliferation and viability test was performed. Treatment with 1, 10, 50, 100, 200 and 400 μM oleic or palmitic acid for 24 hr had a significant effect on the number of hypothalamic neurons (F (12, 91) = 65.49, $p < 0.01$). Bonferroni *post hoc* tests reveals significant change in the number of neurons at all concentrations of oleic acid and at 1, 50, 200 and 400 μM palmitic acid (**Table 3; Figure 9B**). While both low concentrations of oleic (1, 10 μM) and palmitic acid (1 μM) stimulated cell proliferation, concentrations greater than 50 μM reduced cell viability. Visual examination showed that many cells died with 400 μM oleic and palmitic acid treatment.

The fatty acids were also examined for its ability to affect hypothalamic neuronal migration using a simple scratch assay. Treatment with 1, 10, 50, 100 and 200 μM oleic or palmitic acid for 24 hr reveal significant effects on migration (F (8, 63) = 8.37, $p < 0.05$). (**Figure 9A, C**). Bonferroni *post hoc* test reveals a significant decrease in migration at all concentrations of oleic acid and a significant decrease at 100 μM palmitic acid (**Table 3**). The measured decrease in migration at the high concentrations of fatty acid may be due to the increase in cell death. These

results suggest that oleic acid inhibits while palmitic acid does not impact hypothalamic neuronal migration.

4. Discussion

The molecular process governing maternal high-fat diet-induced neurogenesis events in the brain have been a focus on understanding prenatal programming of ingestive behavior. In these offspring, the hypothalamus has an increased number of orexigenic peptide neurons that induces a hyperphagic phenotype [6, 8]. A potential mechanism involved in high-fat diet outcomes on hypothalamic neurogenesis events directly involve the fatty acids, oleic and palmitic acids.

4.1 Distinct concentration-dependent effects of oleic and palmitic acid on differential gene expression

The initial analysis of the combined fatty acid groups displayed nonspecific gene clusters that could be attributed to both a concentration effect and differences in fatty acid pathway activation. Examining only oleic or palmitic acid alone revealed clustering differences between 100 μM to 1 and 10 μM, without any further emerging patterns. The individual GO pathway analysis revealed each fatty acid to change the expression of genes involved in specific aspects of each signaling cascade while maintaining the findings of the HCL analysis. For each concentration of fatty acid, only a particular subset of genes involved in each pathway was found to be differentially expressed. Low concentrations of oleic or palmitic acid stimulated anti-apoptotic targets and inhibited apoptotic targets in Wnt and apoptosis signaling pathways while the opposite effect was observed at primarily 100 μM concentrations. A single fatty acid can have a concentration effect to impact

different aspects of a signaling cascade and would account for the diverse programming effects observed during the neurodevelopmental period.

4.2 High fat diet-induced neurogenesis is partially mediated by fatty acids

Neurogenesis from stem cell populations involves proliferation, differentiation and migration steps for proper formation of the embryonic brain [85]. This delicate process can be disturbed and impact the modeling of brain tissue, particularly during exposure to excessive prenatal dietary fat [6]. The RNAseq analyses reveal enrichment in several pathways involved in proliferation, migration, and apoptosis signaling. The results from this study show oleic acid to have a concentration dependent increase in hypothalamic neuronal proliferation followed by cell death. These results are in line with studies showing prenatal oleic acid exposure to increase overall brain neurogenesis [86] while high concentrations stimulate cell death [87]. Palmitic acid has also previously been shown to induce cell death in PC12 cells [20] and is similar to the findings in this study. In hippocampal neural progenitor cells, there is an increase in the production of fatty acids and a decrease in fatty acid oxidation during proliferation while the reverse is true during cell quiescence [16, 88]. This suggests that changes in fatty acid metabolism and levels impacts neuronal proliferation processes and lead to changes in the phenotypic development of neurons.

There is limited evidence that supports or refutes oleic and palmitic acid's role on neuronal migration. Both fatty acids can either stimulate or inhibit migration and may be dependent on the concentration and the cellular subtype examined [89-91]. The results from this study show oleic acid to reduce neuronal migration with no effect by palmitic acid. These results suggest the importance of oleic acid in promoting hypothalamic neuronal proliferation but not on migration. Palmitic acid has previously been shown to be cytotoxic to neuronal stem cells while low

concentrations can stimulate differentiation (Wang Z, Hao A, 2014). The effect of palmitic acid on hypothalamic neurogenesis processes is not as clear but may involve differentiation. This last aspect of neurogenesis could not be examined in this study because the hypothalamic neurons have already differentiated.

4.3 Evidence for oleic and palmitic acid involvement in orexigenic neuropeptide signaling pathways

The RNA-seq did not detect any changes to the typical fat-sensing hypothalamic orexigenic neuropeptides, such as enkephalin and galanin. The hypothalamus typically begins to express these neuropeptides at embryonic day 17-18 and may be too early to detect the fat-sensing neuropeptides [21]. However, the enrichment of genes involved in Wnt signaling pathways may be upstream effectors of neuropeptide expression. The three concentrations of oleic acid treatment had impacted gene expression changes in proteins downstream of Wnt signaling, SWI/SNF and Arid1 (**Table 2; Figure 3**). The inactivation of SWI/SNF and Arid1 leads to the activation of the YAP/TAZ/TEAD transcriptional regulator complex to increase proliferation [92]. Meanwhile, prenatal high-fat diet exposure decreases the activity of the transcriptional regulators YAP and TEAD, with this decrease to stimulate the expression of the orexigenic neuropeptide, enkephalin [93]. This evidence suggests high-fat diet induced neurogenesis events may be mediated by fatty-acid effects on the Wnt signaling pathway, presumably to increase proliferation of hypothalamic orexigenic peptide neuronal precursors and to later increase the neuropeptide levels.

Treatment of 10 and 100 μM oleic and palmitic acids also impacted the GnRH signaling pathway. The hypothalamic GnRH neurons coexpress or contact with orexigenic peptide neurons, such as neuropeptide Y and galanin, [75-78]. These hypothalamic hormone pathways regulate

endocrine function and are impacted by dietary changes. There is abundant evidence that obesity negatively impacts successful fertility outcomes and prenatal obesity and high-fat diet exposure to influence offspring pubertal development and fertility [94-96]. The results of this study also suggest endocrine programming is in part mediated by high fatty acid levels that are increased during obesity and excessive dietary fat intake.

While activation of CCKR signaling pathway leads to changes in gene transcription involving cell survival, proliferation and migration, CCKR activation is also heavily linked to satiety and decreased eating [97]. Genetic knockout of CCKR1 in rats have an obesogenic and hyperphagic phenotype [98]. There is also evidence linking direct activation of CCKRs to stimulate orexigenic peptide neurons in addition to coexpression with feeding neuropeptides [99, 100]. During the fed-state, CCK is released by the gut to signal satiety in the hypothalamus [99, 100]. The differential expression of genes involved in the CCKR signaling pathway suggests high concentrations of fatty acids lead to dysfunctional gut-brain signaling in the intact organism.

4.4 Oleic and palmitic acids mediate high-fat diet changes in signaling pathways associated with metabolism

Other signaling pathways that were uncovered by oleic and palmitic acid treatment on hypothalamic neurons overlap with outcomes from *in vivo* high-fat diet studies. The Wnt signaling pathway has been previously shown to be involved in high-fat diet effects on several physiological systems, including bone, adipose tissue, vascular tissue, and brain (Bagchi DP, Macdougald OA, 2020; Chen N, Wang J, 2018). Changes or mutations with the Wnt signaling cascade leads to metabolic disorders, including targets uncovered in this study (Chen N, Wang J, 2018). Integrin signaling in adipose tissue has also been associated with metabolic effects resulting from high-fat

diet intake, particularly in the development of obesity-induced diabetes (Williams AS, Wasserman DH, 2016; Ruiz-Ojeda FJ, Ussar S, 2021). Excessive ingestion of high-fat diets has been linked to increased activation of apoptosis signaling pathways in the hypothalamus (Moraes JC, Velloso LA, 2009). Excessive high-fat diet ingestion and metabolic changes are also mediated by receptor tyrosine kinases, with changes in PDGF signaling pathway as one example (Zhao M, Svennson KJ, 2020). These results highly suggest high-fat diet effects are primarily mediated by oleic and palmitic acids.

4.5 Conclusions

The results from this study reveal several potential methods of oleic and palmitic acid effects on neurogenesis events, particularly involving signaling pathways that intersect with orexigenic neuropeptides and cell survival. Further studies examining each distinct pathway on neuropeptides may further elucidate the molecular mechanisms of prenatal high-fat diet exposure on neurogenesis events.

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Figure 1. ***HCL analysis.*** Hierarchical clustering analysis of 1, 10 and 100 μM oleic acid (top) and palmitic acid (bottom) show differences between the 100 μM concentrations (left) and 1 and 10 μM concentrations (right). Many genes that are expressed in the lower concentrations are not expressed at 100 μM concentrations (black). The upregulated (green) and downregulated (red) genes do not show any further clustering.

Figure 2. ***Gene ontology analysis.*** Gene ontology analysis of 1, 10 and 100 μM oleic acid (A) and palmitic acid (B) show significant enrichment of genes involved in cellular component, biological process, and molecular function. The top 10 significant gene sets are labeled for each process.

Figure 3. A potential mechanism of oleic and palmitic acid effects on the genes involved in the Wnt signaling pathway.

Figure 4. A potential mechanism of oleic and palmitic acid effects on the genes involved in CCKR signaling pathway.

Figure 5. A potential mechanism of oleic and palmitic acid effects on the genes involved in the apoptosis signaling pathway.

Figure 6. A potential mechanism of oleic and palmitic acid effects on the genes involved in the PDGF signaling pathway.

Figure 7. A potential mechanism of oleic and palmitic acid effects on the genes involved in the integrin signaling pathway.

Figure 8. ***HCL analysis.*** Hierarchical clustering analysis of genes that are expressed under all three conditions of oleic acid treatment (top) or palmitic acid treatment (bottom). Four clusters are present with oleic acid and three with palmitic acid treatment.

Figure 9*. Proliferation and migration.* Proliferation and migration analysis of hypothalamic neurons reveals significant effects by oleic and palmitic acid. A. Representative images of the scratch assay used to measure migration of the hypothalamic neurons over 24 hr. B. The 1 and 10 μM oleic acid significantly increased proliferation. 50 μM and higher concentrations of oleic acid induced significant cell death. Similar reduction in neuronal number was found at 50, 200 and 400 μM palmitic acid. C. Treatment with all concentrations of oleic acid and only at 100 μM palmitic acid significantly reduced migration of neurons. $*p < 0.05$.

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3 Overlapping Positive Gene Expression		13			11		
		Oleic Acid (μM)			Palmitic Acid (μM)		
2 Overlapping Gene Expression		*1*	*10*	*100*	*1*	*10*	*100*
Oleic Acid (μM)	*1*	169 66	49	31	29	44	23
	10	71	150 73	23	32	31	17
	100	36	42	191 254	16	16	16
Palmitic Acid (μM)	*1*	49	59	31	172 292	32	35
	10	50	71	34	45	290 148	17
	100	37	92	30	37	41	321 171
3 Overlapping Negative Gene Expression		16			15		

Table 1. The number of differentially expressed genes under each fatty acid condition, across two conditions, and across three conditions were determined. The boxes and numbers in red represented downregulated genes while the boxes and numbers in green represent upregulated genes.

	GENE ID	Gene name	1 μM OA	10 μM OA	100 μM OA	1 μM PA	10 μM PA	100 μM PA
WNT	SMARCd3	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily D, Member 3			-2.63			
	SRCAP	Snf2 Related CREBBP Activator Protein			-3.34			
	Ppp3cc	Protein Phosphatase 3 Catalytic Subunit Gamma			-1.17			
	Pcdhga2	Protocadherin Gamma Subfamily A, 2			-3.39			
	Apc	APC Regulator Of WNT Signaling Pathway			-1.94			
	Csnk2b	Casein Kinase 2 Beta		-1.32				
	Prkcd	Protein Kinase C Delta		-2.72			-0.94	
	Arid1b	AT-Rich Interaction Domain 1B		-4.58				
	Gng12	G Protein Subunit Gamma 12	-6.27					
	Tle2	TLE Family Member 2, Transcriptional Corepressor	-4.53					
	PP3cb	UNKNOWN					-0.73	
	Dvl2	Dishevelled Segment Polarity Protein 2					-2.7	
	Gnb1	G Protein Subunit Beta 1					-8.17	
	Ppp3ca	protein phosphatase 3 catalytic subunit alpha		5.83				
	Itpr3	inositol 1,4,5-trisphosphate receptor, type 3		1.1675943				
	Lrp6	LDL Receptor Related Protein 6		0.8253357				
	Smarcc2	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin Subfamily C Member 2		1.1361862				
Apoptosis	Tmbim6	Transmembrane BAX Inhibitor Motif Containing 6			-0.73			
	Bak1	BCL2 Antagonist/Killer 1			-0.71			
	LOC680121	UNKNOWN			-0.2			
	Pik3ca	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha			-1.4			-1.28

	Hspa2	Heat Shock Protein Family A (Hsp70) Member 2		-11.4				
	Prkcd	Protein Kinase C Delta		-2.72				
	Jdp2	Jun Dimerization Protein 2						-1.42
	Bcl2l2	BCL2 Like 2						-4
	Map4k2	Mitogen-Activated Protein Kinase Kinase Kinase 2						-1.13
CCKR	Eif4e	Eukaryotic Translation Initiation Factor 4E						-0.6
	Hbegf	Heparin Binding EGF Like Growth Factor					-1.16	-1.05
	Men1	Menin 1						-0.42
	Yes1	YES Proto-Oncogene 1, Src Family Tyrosine Kinase					-7.64	-10.16
	Cdc42	Cell Division Cycle 42					-3.77	
	Gnb1	G Protein Subunit Beta 1					-8.16	
	Prkcd	Protein Kinase C Delta					-0.95	
	Akap1	A-Kinase Anchoring Protein 1					4.3918232	
	Camkk1	Calcium/Calmodulin Dependent Protein Kinase Kinase 1			4.7658107		4.0322283	
	Eif4e	Eukaryotic Translation Initiation Factor 4E					1.2645321	
	Mef2d	Myocyte Enhancer Factor 2D			12.618943		13.610386	
	Egr1	Early Growth Response 1			0.2956026			
	AABR07045405	UNKNOWN			9.3047935			
	Hbegf	Heparin Binding EGF Like Growth Factor			1.1625983			
Integrin	Col4a2	Collagen Type IV Alpha 2 Chain	-22.62			-23.04		
	Rap2c	RAP2C, Member Of RAS Oncogene Family	-7.15					
	Mapk1	Mitogen-Activated Protein Kinase 1				-0.77		
	Itga3	integrin subunit alpha 3				-0.6		
	AABR07005533	Pseudogene, high in nervous system				-7.35		
	Tln1	talin 1				-0.46		

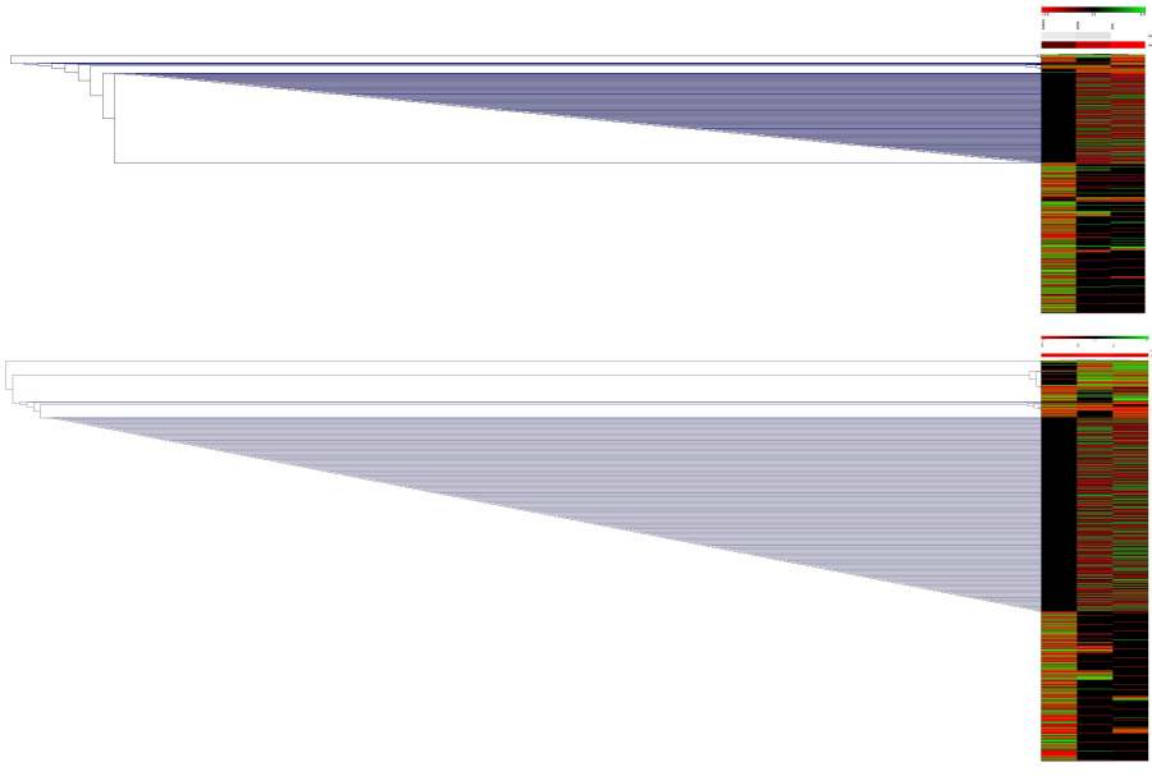
	Vasp	Vasodilator Stimulated Phosphoprotein				-1.27		
PDGF	Vav2	Vav Guanine Nucleotide Exchange Factor 2	2.9239659			3.5405431		
	Mknk1	MAPK Interacting Serine/Threonine Kinase 1	7.2235186			6.3759186	5.197433	
	Pik3r3	Phosphoinositide-3-Kinase Regulatory Subunit 3	2.586125			1.422323		
	Itpr3	Inositol 1,4,5-Trisphosphate Receptor Type 3	1.3193731			1.2044036		
	Elf2	E74 Like ETS Transcription Factor 2					0.8254755	
	Erf	ETS2 Repressor Factor					0.9405986	
	Akt2	AKT Serine/Threonine Kinase 2					8.8919959	
	Eif4	Eukaryotic Translation Initiation Factor 4E				3.1943711		
	Stard13	StAR Related Lipid Transfer Domain Containing 13				0.4648321		
	Srgap2	SLIT-ROBO Rho GTPase Activating Protein 2				0.8136659		
	Mapk6	Mitogen-Activated Protein Kinase 6 or ERK3						0.611919
	Srgap2	SLIT-ROBO Rho GTPase Activating Protein 2						1.4861218
	Nin	Ninein						1.8965917
Gonadotropin	Prkab1	Protein Kinase AMP-Activated Non-Catalytic Subunit Beta 1			-0.67			
	Sdf4	Stromal Cell Derived Factor 4			-0.61			
	Irs2	Insulin Receptor Substrate 2			-1.24			
	Ksr1	Kinase Suppressor Of Ras 1			-3.09			
	Prkab2	Protein Kinase AMP-Activated Non-Catalytic Subunit Beta		-9.6				
	Prkcd	Protein Kinase C Delta		-2.72			-0.95	
	Map4k2	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 2						-1.13
	Smad4	SMAD Family Member 4						-3.15
	Per1	Period Circadian Regulator 1						-1.13

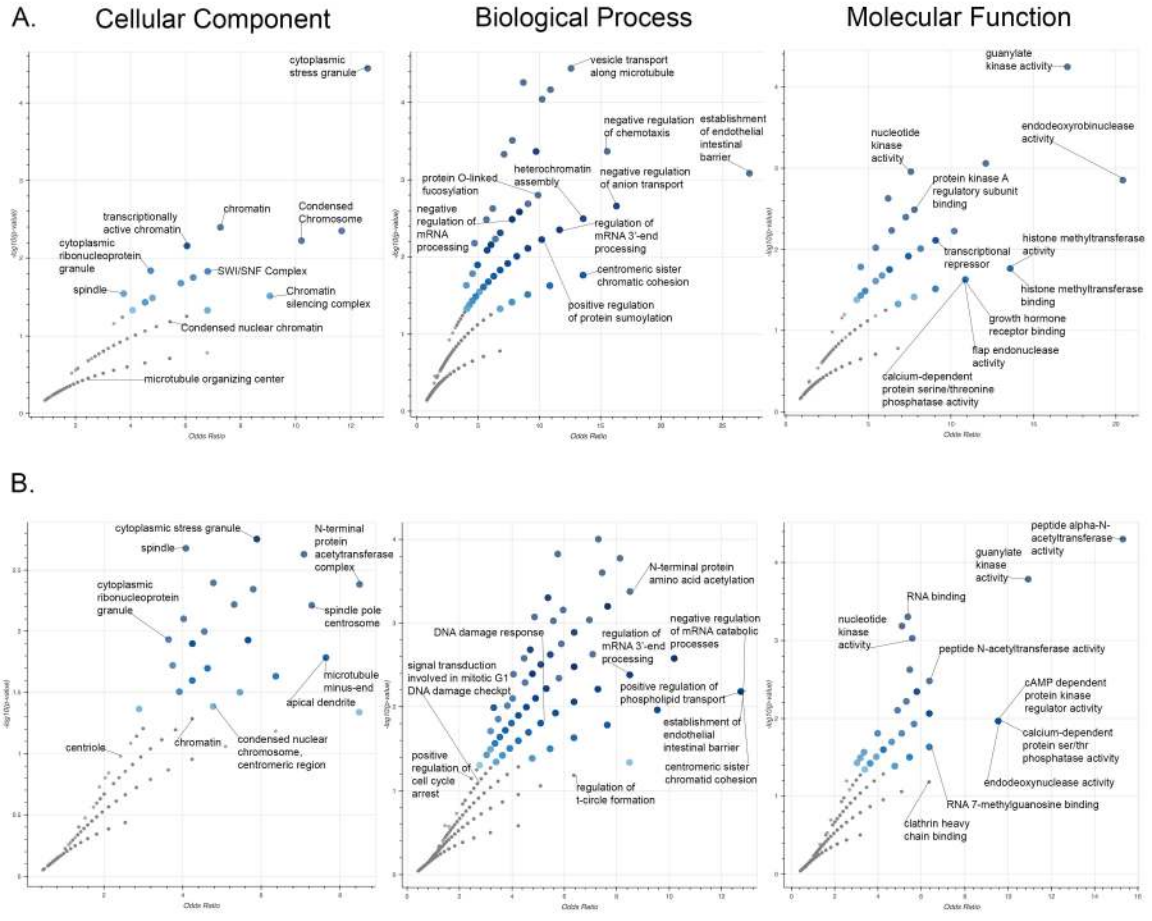
Bmpr2	Bone Morphogenetic Protein Receptor Type 2					-2.9	
Cdc42	Cell Division Cycle 42					-3.77	
Gnb1	G Protein Subunit Beta 1					-8.17	
Smad2	SMAD Family Member 2					-1.41	
Bmpr2	Bone Morphogenetic Protein Receptor Type 2						1.8583023
Map4k5	Mitogen-Activated Protein Kinase Kinase Kinase Kinase 5						9.9520574
Smad5	SMAD Family Member 5						3.9190466

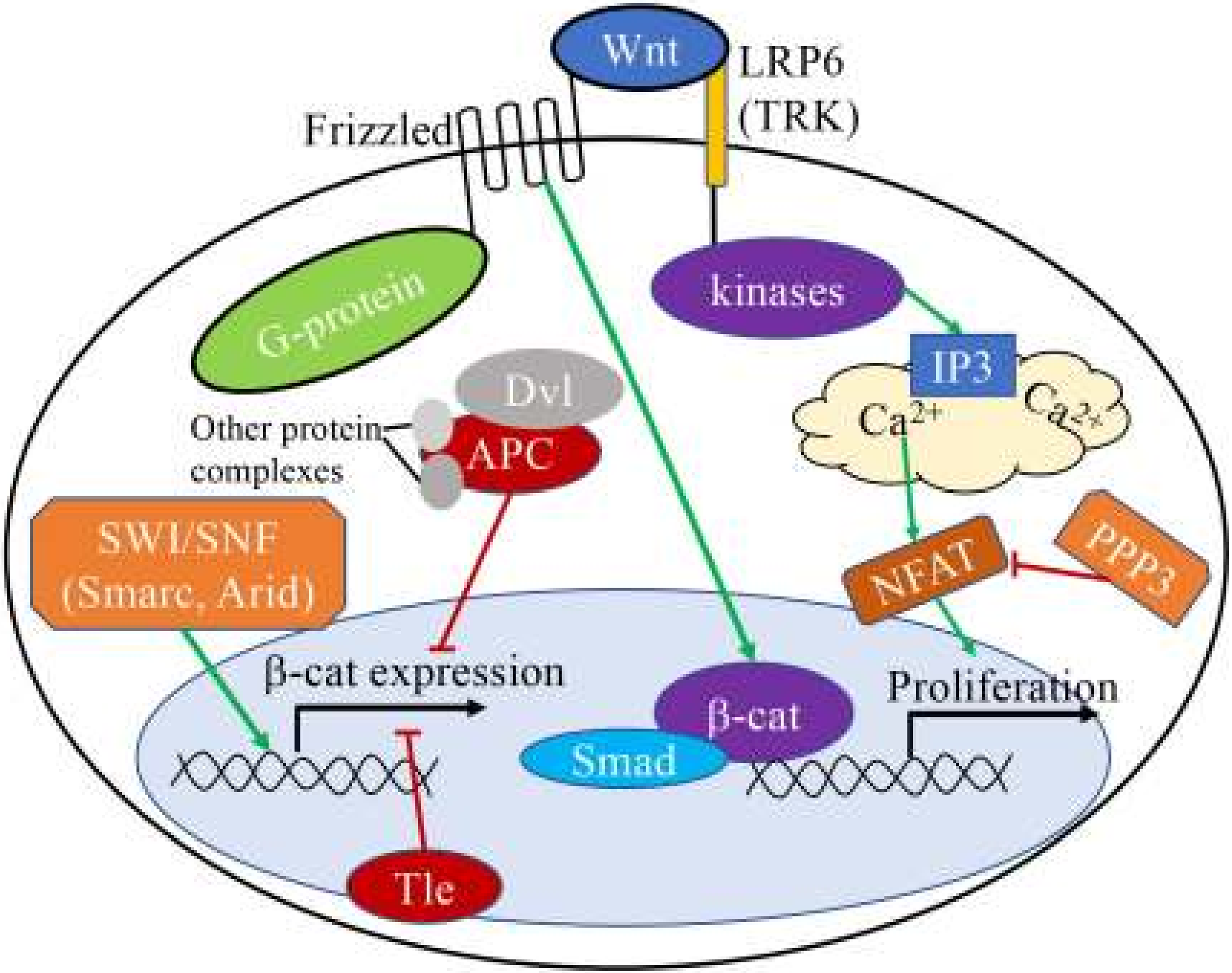
Table 2. Gene Ontology (GO) analysis uncovered significant enrichment in several cellular pathways under conditions of oleic or palmitic acid ($p < 0.05$). Green are upregulated genes while orange are downregulated genes.

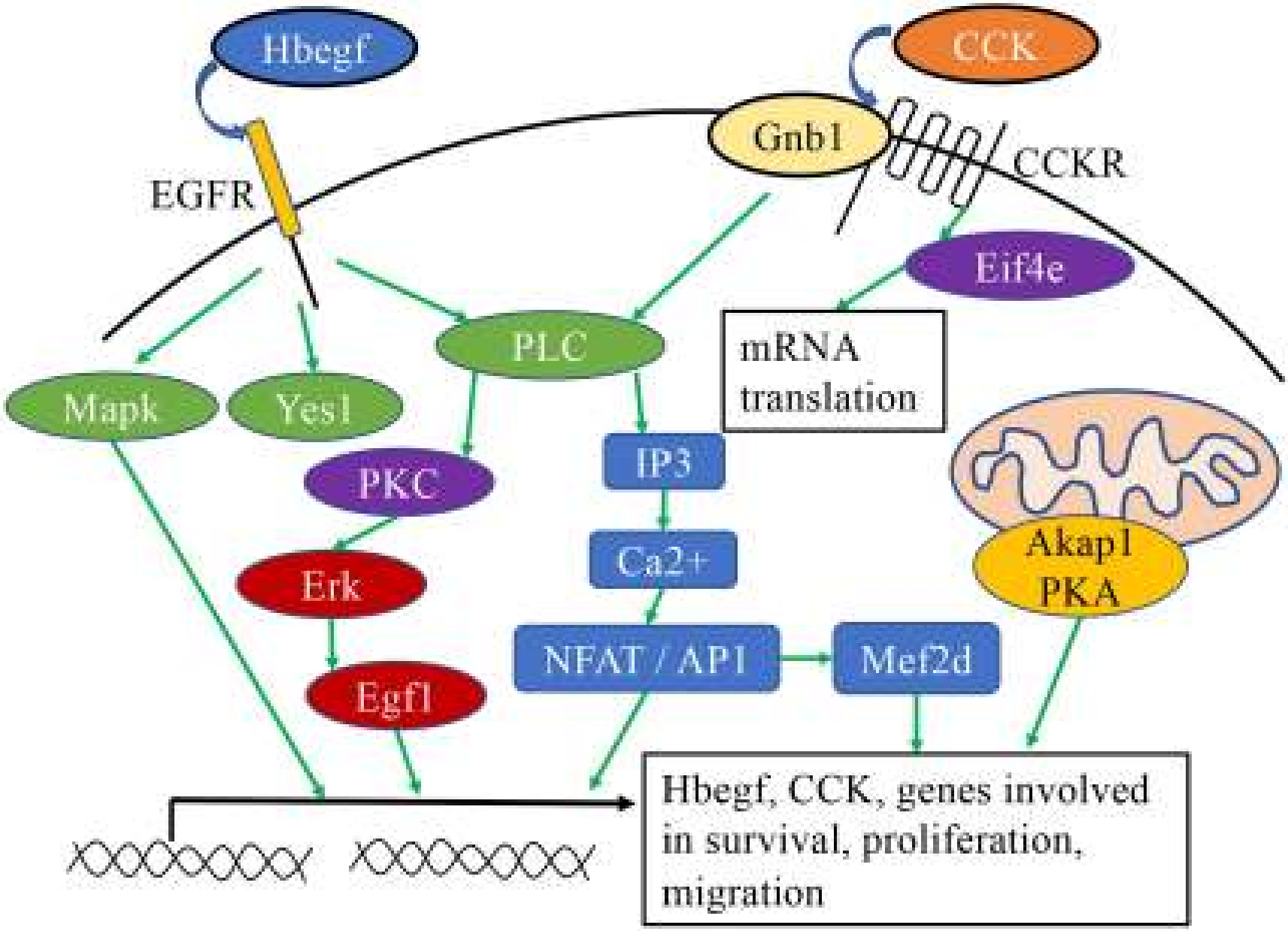
	Proliferation		Migration	
Treatment	***t*(14) = value**	***p*-value**	***t*(14) = value**	***p*-value**
1 mM OA	7.96	< 0.01	3.67	< 0.05
10 mM OA	3.57	< 0.05	5.55	< 0.01
50 mM OA	7.22	< 0.01	4.34	< 0.01
100 mM OA	8.50	< 0.01	3.9	< 0.01
200 mM OA	9.38	< 0.01	-	-
400 mM OA	10.64	< 0.01	-	-
1 mM PA	4.94	< 0.01	0.84	1
10 mM PA	0.30	1	1.35	1
50 mM PA	3.04	< 0.05	2.18	1
100 mM PA	0	1	5.63	< 0.01
200 mM PA	4.01	< 0.01	-	-
400 mM PA	2.96	< 0.05	-	-

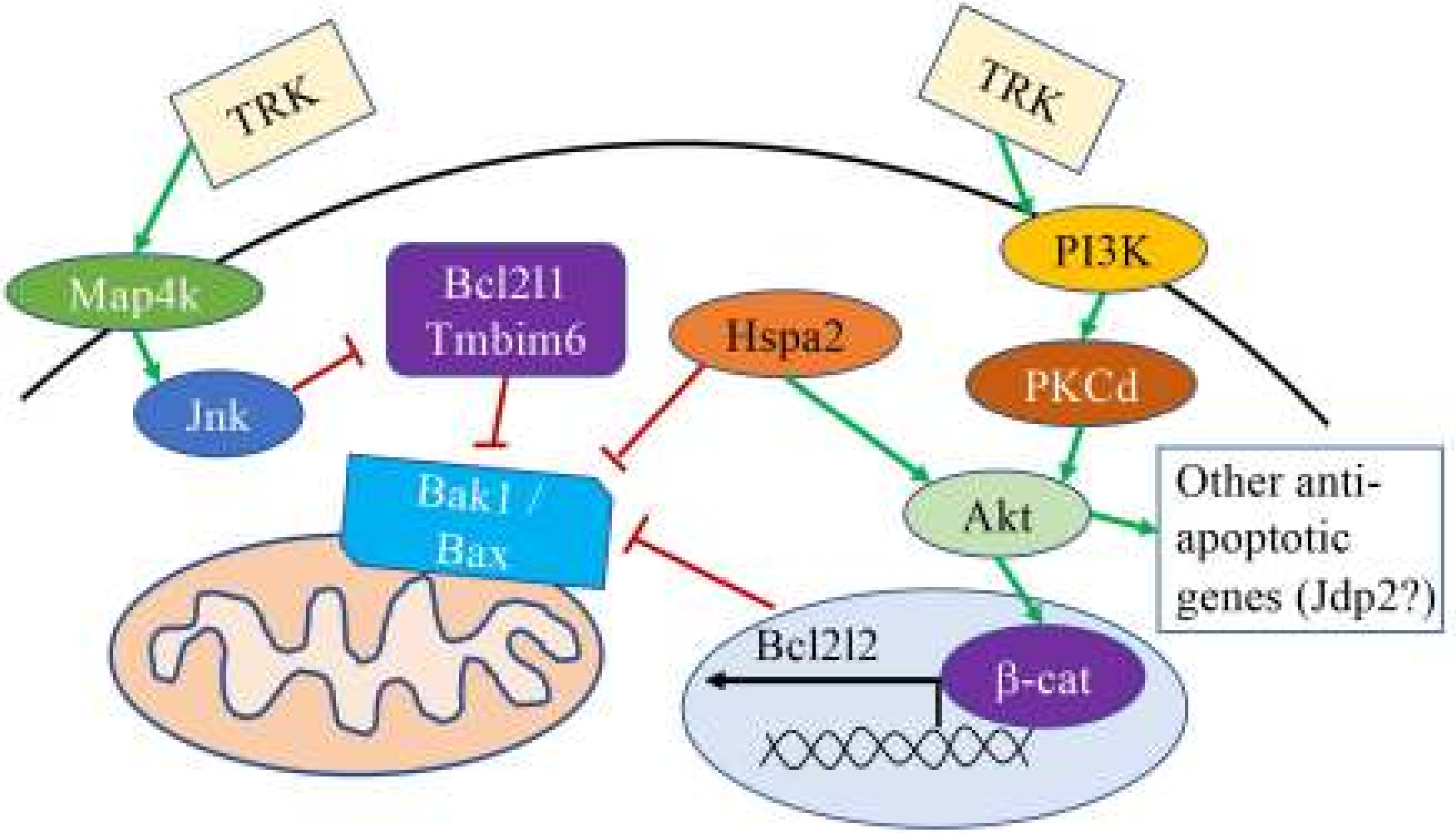
Table 3. Bonferroni *post hoc* test for the proliferation assay reveal statistical significance on proliferation across all concentrations of oleic acid (OA) and palmitic acid (PA) treatment except at 10 and 100 μM palmitic acid.

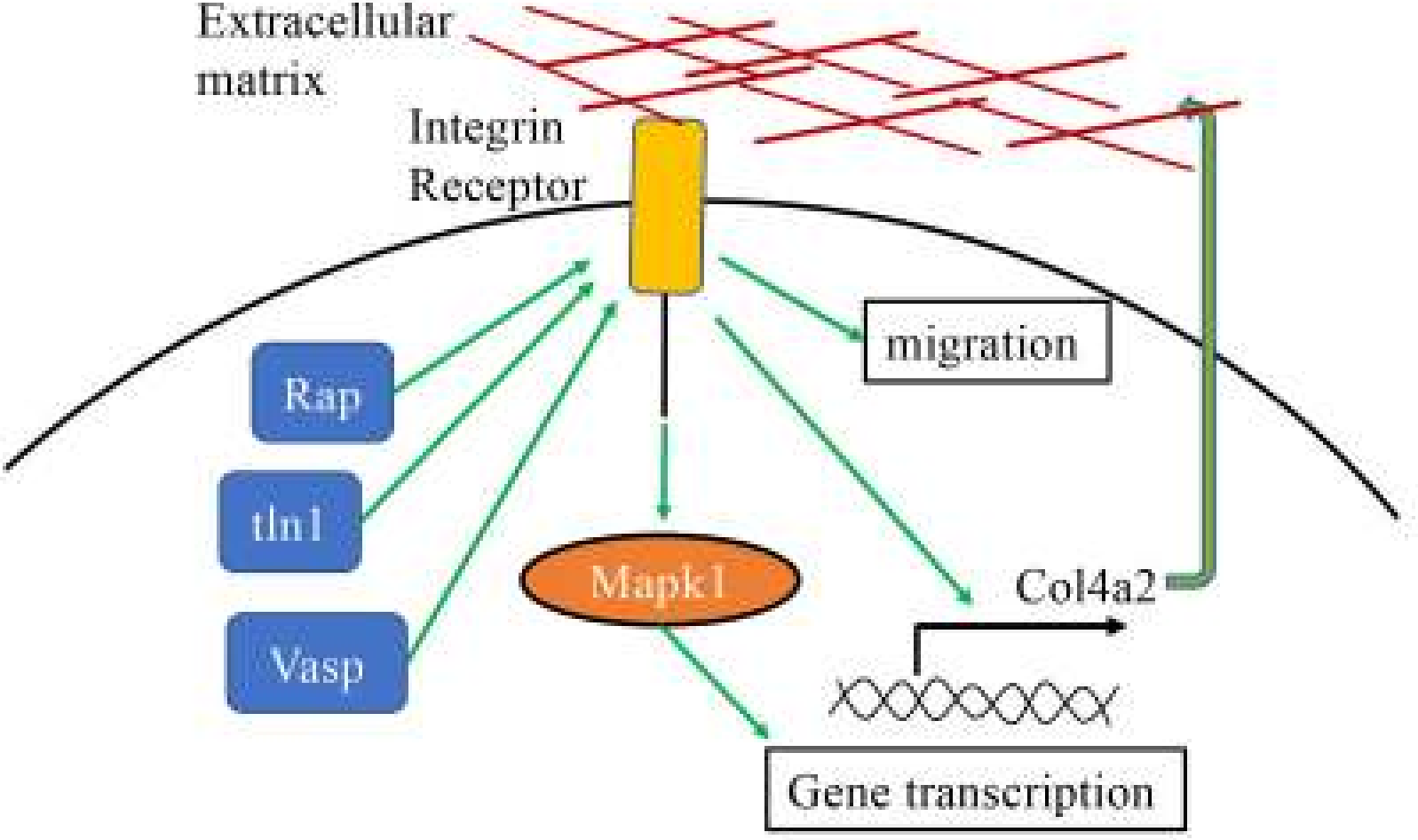


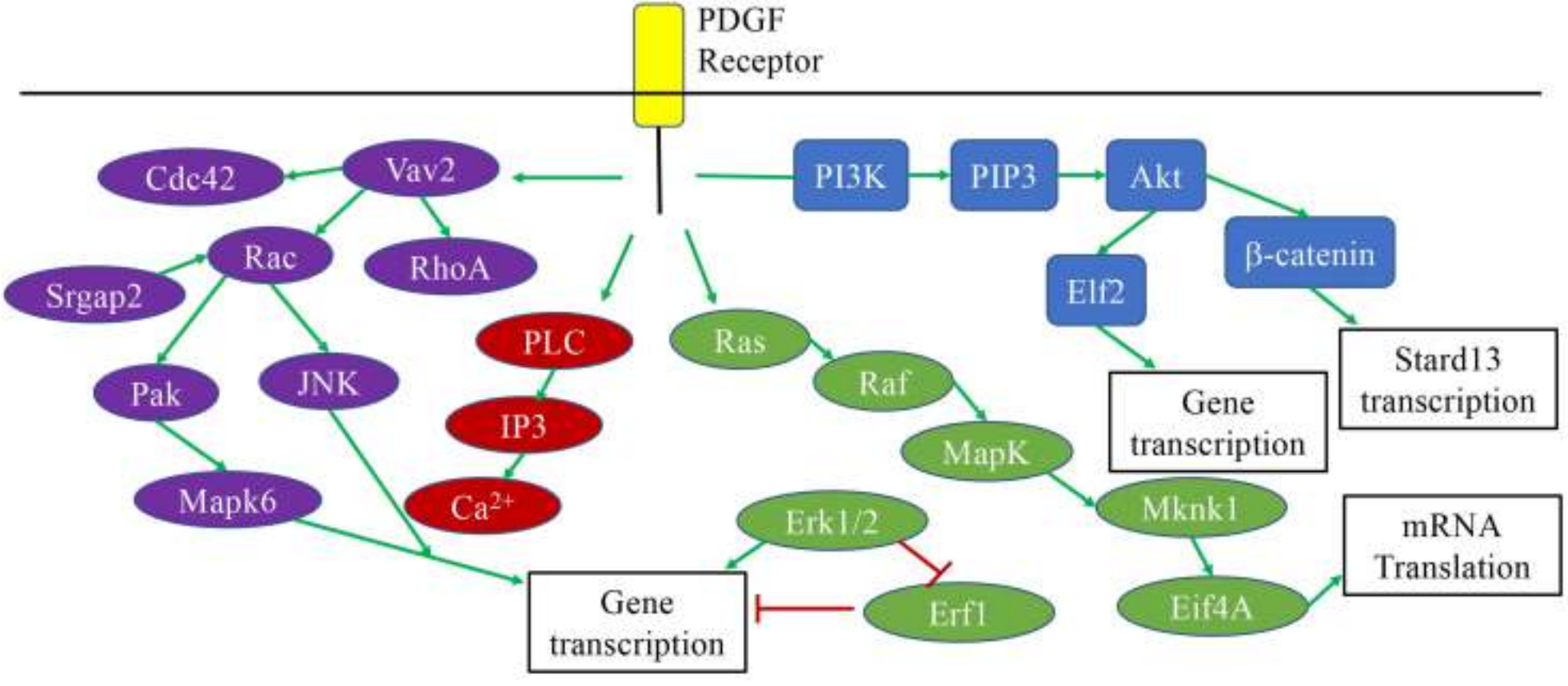

A.
Cellular Component
Biological Process
Molecular Function
cytoplasmic stress granule
transcriptionally active chromatin
chromatin
Condensed Chromosome
cytoplasmic ribonucleoprotein granule
SWI/SNF Complex
spindle
Chromatin silencing complex
Condensed nuclear chromatin
microtubule organizing center
vesicle transport along microtubule
negative regulation of chemotaxis
establishment of endothelial intestinal barrier
protein O-linked fucosylation
heterochromatin assembly
negative regulation of anion transport
negative regulation of mRNA processing
regulation of mRNA 3'-end processing
centromeric sister chromatic cohesion
positive regulation of protein sumoylation
guanylate kinase activity
endodeoxyribonuclease activity
nucleotide kinase activity
protein kinase A regulatory subunit binding
histone methyltransferase activity
transcriptional repressor
histone methyltransferase binding
growth hormone receptor binding
flap endonuclease activity
calcium-dependent protein serine/threonine phosphatase activity
-log10(p-value)
Odds Ratio
B.
cytoplasmic stress granule
spindle
N-terminal protein acetyltransferase complex
cytoplasmic ribonucleoprotein granule
spindle pole centrosome
microtubule minus-end
apical dendrite
centrioles
chromatin
condensed nuclear chromosome, centromeric region
N-terminal protein amino acid acetylation
DNA damage response
regulation of mRNA 3'-end processing
negative regulation of mRNA catabolic processes
signal transduction involved in mitotic G1 DNA damage checkpt
positive regulation of phospholipid transport
establishment of endothelial intestinal barrier
positive regulation of cell cycle arrest
centromeric sister chromatid cohesion
regulation of t-circle formation
peptide alpha-N-acetyltransferase activity
guanylate kinase activity
RNA binding
nucleotide kinase activity
peptide N-acetyltransferase activity
cAMP dependent protein kinase regulator activity
calcium-dependent protein ser/thr phosphatase activity
endodeoxynuclease activity
RNA 7-methylguanosine binding
clathrin heavy chain binding
-log10(p-value)
Odds Ratio

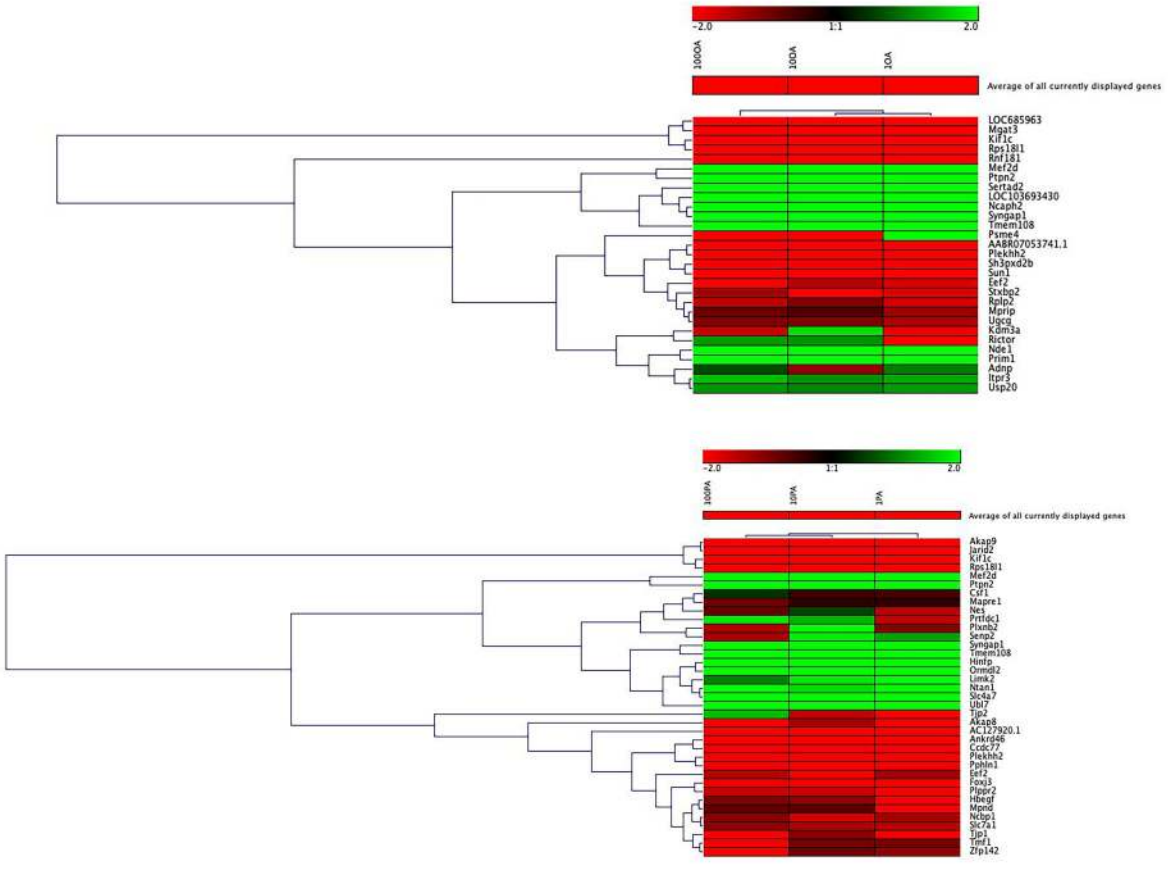

Wnt
LRP6
(TRK)
Frizzled
G-protein
kinases
IP3
Ca2+
Ca2+
Dvl
APC
Other protein
complexes
SWI/SNF
(Smarc, Arid)
NFAT
PPP3
β-cat expression
β-cat
Smad
Proliferation
Tle

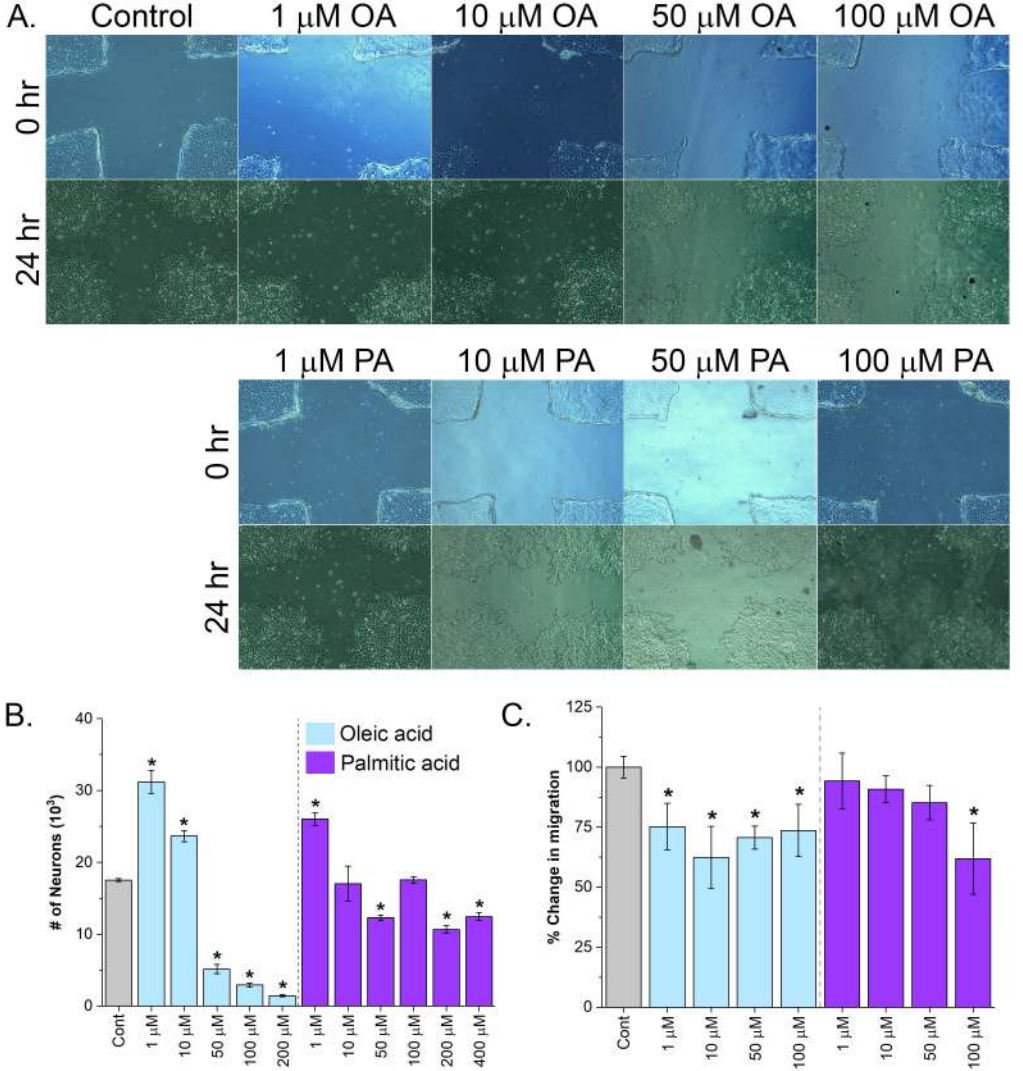

Hbegf
CCK
Gnb1
CCKR
EGFR
Eif4e
PLC
mRNA
translation
Mapk
Yes1
IP3
PKC
Ca2+
Akap1
PKA
Erk
NFAT / AP1
Mef2d
Egf1
Hbegf, CCK, genes involved
in survival, proliferation,
migration


TRK
TRK
PI3K
Map4k
Bcl2l1
Tmbim6
Hspa2
PKCd
Jnk
Bak1 /
Bax
Akt
Other anti-
apoptotic
genes (Jdp2?)
Bcl2l2
β-cat


Extracellular matrix
Integrin Receptor
migration
Rap
tln1
Vasp
Mapk1
Col4a2
Gene transcription


PDGF
Receptor
Cdc42
Vav2
Rac
RhoA
Srgap2
Pak
JNK
Mapk6
PLC
IP3
Ca2+
PI3K
PIP3
Akt
Elf2
β-catenin
Ras
Raf
MapK
Erk1/2
Erf1
Mknk1
Eif4A
Gene transcription
Gene transcription
Stard13 transcription
mRNA Translation


-2.0
1:1
2.0
100OA
10OA
1OA
Average of all currently displayed genes
LOC685963
Mgat3
Kif1c
Rps18l1
Rnf181
Mef2d
Ptpn2
Sertad2
LOC103693430
Ncaph2
Syngap1
Tmem108
Psme4
AAB807053741.1
Plekhh2
Sh3pxd2b
Sun1
Eef2
Stxbp2
Rplp2
Mprip
Ugcg
Kdm3a
Rictor
Nde1
Prim1
Adnp
Itpr3
Usp20
-2.0
1:1
2.0
100PA
10PA
1PA
Average of all currently displayed genes
Akap9
Jarid2
Kif1c
Rps18l1
Mef2d
Ptpn2
Csf1
Mapre1
Nej
Prtidc1
Plxnb2
Sema2
Syngap1
Tmem108
Hintp
Ormdl2
Limk2
Ntan1
Slc4a7
Ubl7
Tjp2
Akap8
AC127920.1
Ankrd46
Ccdc77
Plekhh2
Pphln1
Eef2
Foxj3
Ptpgr2
Hbegf
Mpnd
Ncbp1
Slc7a1
Tjp1
Tmf1
Zfp142


A.
Control
1 μM OA
10 μM OA
50 μM OA
100 μM OA
0 hr
24 hr
1 μM PA
10 μM PA
50 μM PA
100 μM PA
0 hr
24 hr
B.
Oleic acid
Palmitic acid
# of Neurons (10³)
Cont
1 μM
10 μM
50 μM
100 μM
200 μM
1 μM
10 μM
50 μM
100 μM
200 μM
400 μM
C.
% Change in migration
Cont
1 μM
10 μM
50 μM
100 μM
1 μM
10 μM
50 μM
100 μM