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The effects of the ketone body β-hydroxybutyrate on the neuronal transcriptome

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Abstract

The ketogenic diet is emerging as an effective therapeutic option for patients with neurological disorders. The diet results in metabolism of fatty acids to ketone bodies like β-hydroxybutyrates (BHBs), which serve as an alternative fuel source for brain cells. However, the molecular effect of BHB on neurons is not well understood. We hypothesized that BHB administration will induce upregulation of energy metabolism-related processes in neurons. To assess the effect of BHB administration on the neuronal transcriptome, we reanalyzed a publicly available RNAseq dataset (GSE252513) using a bioinformatic "3-pod" approach. We conducted pathway analysis and identified leading edge genes using Gene Set Enrichment Analysis (GSEA) and conducted chemical perturbagen analysis using the iLINCS repository to identify drugs that are concordant and discordant with BHB administration. We identified significantly altered ($p<0.05$) pathways associated with inflammation and immunity such as "regulation of proinflammatory cytokine production" and "modulation of inflammatory responses." We did not identify significant modulation of energy metabolism-related pathways in response to BHB administration. Our results suggest that under normal conditions, BHBs primary actions include modulation of cellular neuronal immune responses.

Keywords: Neuron, Transcriptome, Ketone Body

1. Introduction

The brain is an energy demanding organ. Neurons use a significant proportion (over 75%) of the brain's energy supply, to facilitate signaling-related processes including the maintenance of resting membrane potential, generation of action potentials, and synaptic transmission (1). Neuronal function may be affected in central nervous system (CNS) disorders that are associated with energy metabolism deficits (2). Adenosine triphosphate

(ATP) is the brain's principal energy currency. It is generated through the process of glycolysis in the cytosol and by oxidative phosphorylation in mitochondria (3). When glucose availability is low, the liver produces ketone bodies, which are water soluble molecules produced by the metabolism of fatty acids (4). The two primary types of ketone bodies are acetoacetate (AcAc) and, the focus of our study, β-hydroxybutyrate (BHB). Ketone body generation begins in the hepatic mitochondria, where fatty acids are broken down into acetyl-CoA. While some of this acetyl-CoA is funneled through the Kreb's cycle and ultimately used in the generation of ATP, the excess is used for ketogenesis. Ketone bodies which are produced through ketogenesis are released into the bloodstream where they are taken up by cells of the CNS, including neurons (5). The ketone bodies are metabolized to acetyl-CoA in the brain cell's mitochondria and used in the generation of cellular ATP, thus supplying energy sourced from fats rather than sugar (6). The ketogenic diet is a high-fat, low-carbohydrate diet that supplies the body with fatty acids as an alternative to glucose. It shows promise for the treatment of CNS disorders.

In an assessor-blinded trial studying the role of the ketogenic diet in Alzheimer's patients, significant improvements were seen in the Activities of Daily Living and Quality of Life scale, a commonly used instrument to assess Alzheimer's disease patients (7). Patients with neuropsychiatric disorders including bipolar disorder reported clinical benefits after following the ketogenic diet for 2-3 years, allowing them to discontinue the use of lamotrigine medication. Those same patients saw improved Quality of Life scores (8). Acute administration of the ketogenic diet also results in overall symptom improvement in patients with bipolar disorder. Approximately 70% of bipolar disorder patients on a 4-month ketone diet had a greater than one point improvement on the Clinical Global Scale (9). Patients with other CNS disorders also show benefits from the ketogenic diet. In patients with autism, the ketogenic diet improves social-behavioral deficits, communication, and repetitive behavior patterns (10). Childhood epilepsy patients reported significant improvements following administration of the ketogenic diet; 38% of children had a 50% seizure reduction compared to only 6% of children in the control group (11). Studies have also explored whether the ketogenic diet, which can be unpalatable for patients, is necessary to induce these therapeutic benefits or if administering ketone bodies (BHBs) alone can have the same therapeutic effect. The effect of BHB administration alone was investigated in a mouse model of epilepsy. BHB suppressed inflammatory responses and thus, BHB was proposed as a potential treatment for epilepsy due to its neuroprotective effects (12).

Ketone bodies have shown positive effects when studied in people with neurodevelopmental and neurodegenerative diseases (13). The presence of BHB plays a vital role in keeping the brain's neurons healthy. The mechanisms by which BHB contributes to maintaining brain health are an important area of study. Impaired glucose metabolism is reported in many CNS disorders (14). Providing the brain with ketones, such as with the ketogenic diet, may provide an alternative source of ATP production in the brain by supplying ketones through fatty acid oxidation (15). Although earlier studies considered the need for glucose starvation in combination with supply of ketone bodies, supplementing the brain's energy supply with BHB may potentially overcome the energy deficits associated with these disorders, leading to improvement of symptoms. Anti-epileptic treatments were reduced during dietary intervention in a case study of a subject with autism in her adolescent years (10). At puberty, the subject developed seizures and was treated with a gluten free, casein-free ketogenic diet. This resulted in high ketosis. The ketogenic diet reduced her seizures, weight, and autism symptoms (10). This raises the question, could BHB be a leading factor in these results?

In addition to its role as an energy source, BHB also modulates inflammation. BHB has significant effects on the enzyme histone deacetylase (HDAC). HDAC catalyzes the deacetylation of histones and is involved in the epigenetic regulation of inflammatory gene expression (16). BHB is a specific inhibitor of HDACs (17). When HDAC activity is impaired, it leads to increased inflammation in the brain (18). BHB administration restored histone deacetylation in a murine model of Huntington's disease (19). BHB induces ramification of the brain's primary immune cells, microglia, promoting anti-inflammatory effects. BHB administration increases anti-inflammatory

cytokine IL-10 expression via AKT signaling (20). HDAC inhibition also induces similar changes in microglial ramification (20), suggesting that BHB anti-inflammatory activity is mediated by its role as an endogenous HDAC inhibitor. Studying the effects of BHB is pertinent to CNS disorders as a growing body of studies show its therapeutic potential. The underlying mechanisms of BHBs therapeutic effects are still unclear. Thus, in this study we address the effects of administering BHB on human neurons. We hypothesize that BHB treated neurons will display significant changes in 1) energy metabolism related gene expression and 2) immune related gene expression. We test our hypothesis by reanalyzing a published RNAseq dataset using our bioinformatic "3-pod" approach.

2. Methods

RNAseq data was generated as described in Nomura et.al (21). Raw RNAseq (fastq) files were downloaded from the Gene Expression Omnibus (GSE252513). Briefly, human neurons (ScienCell) cultured in neuron media (ScienCell) were administered 10 µM BHB (Sigma Aldrich) and harvested at 24 hours for RNA extraction and RNAseq analysis (21). Control group neurons were cultured in neuron media with no addition of BHB (**Figure 1**).The experiment was conducted using n=3 replicates for each group. For data analysis, the aligned RNAseq reads were analyzed for differential expression between BHB treated cells and controls. Differential expression analysis was performed using the R programming language (22) with edgeR dependencies limma (23) and voom (24); covariates were ignored due to a lack of data provided in the Gene Expression Omnibus entry. Any genes which did not have corresponding HGNC symbols were removed from the analysis. Duplicate genes were combined using the mean of the log2 fold change (log2FC) and the maximum of the p-value, ensuring a conservative approach to the data. Following this, the resultant differentially expressed gene list was submitted to a three-prong analysis including gene set enrichment analysis (GSEA) (25) and iLINCS analysis (26). The resulting pathways were clustered using Pathway Analysis Visualization with Embedding Representations (PAVER) (27). The 3-pod workflow (https://doi.org/10.5281/zenodo.8190833) is described in Figure 1. Venn diagrams and heatmaps used to visualize shared clusters or pathways were generated by the 3-pod bioinformatic workflow.

3. Results

In total, 4,822 genes were significantly differentially expressed ($p \leq 0.05$) in BHB-treated neurons compared to neurons in the control group (**Figure 2**). GDF15 is a gene that regulates food intake, energy expenditure, and body weight. The upregulation of this gene is associated with disease states like inflammation, injury, and oxidative stress. Immune-related pathway clusters were significantly enriched in BHB-treated neurons compared to controls (**Figure 3**).Upregulated immune-related pathway clusters include pathways associated with positive regulation of cytokine production, positive regulation of leukocyte mediated immunity, regulation of T-cell proliferation, cell-cell adhesion regulation, adaptive immune response, regulation of mononuclear cell migration, regulation of phagocytosis, and response to molecule of bacterial origin. Downregulated enriched pathways following BHB administration are associated with cell division processes and include mitotic sister chromatid segregation, chromosome segregation, and DNA conformation change (**Figure 3)**. Other enriched pathway clusters include those associated with cholesterol metabolism and cellular signaling processes like Wnt signaling and phospholipase C activity. Energy-metabolism related pathways were not significantly enriched following GSEA analysis. Leading-edge (LE) genes are the genes that are most frequently identified in significant ($p<0.05$) GSEA pathways but are not necessarily significantly differentially expressed.

Overall, the top LE genes (**Figure 4A**) are related to inflammation and immune activity. They include: the chemokine C-C motif chemokine ligand 5 (CCL5), a component in immunoregulatory and inflammatory processes, PYD and CARD domain containing (PYCARD), which is involved in the activation of the inflammasome, annexin A1 (ANXA1), which is involved in innate immune response and has anti-inflammatory activity and interleukin 23 receptor (IL23R), the receptor for the pro-inflammatory cytokine IL23. Many of the bottom

10 LE genes (**Figure 4B**) are involved in cell division processes. Polo like kinase 1 (PLK1) is related to mitosis and downregulation is associated with inhibited cancer cell growth, APC regulator of WNT signaling pathway (APC) is a tumor suppressor and regulates cell division, zw10 kinetochore protein (ZW10) is associated with cell division checkpoint, aurora kinase B (AURKB) is a serine/threonine kinase involved in mitosis regulation and semaphorin 5A (SEMA5A) is an axonal growth cone guidance protein implicated in neural development. Histone deacetylase (HDAC) genes (**Figure 4C**) were predominantly downregulated, an expected outcome as BHB is a known inhibitor of HDAC enzymes.

Concordant drugs (**Figure 5A**) like HDAC inhibitors play important roles in epigenetic or non-epigenetic regulation, inducing death, apoptosis, and cell cycle arrest in cancer cells (28) which could help treat neurological disorders through their gene regulatory properties. HDAC inhibitors exert an anti-inflammatory effect by modulating the acetylation of both histone and non-histone proteins, which alters the expression of inflammatory genes and pathways, though their precise role can vary depending on the specific cell type and inflammatory stimulus (29). CDK inhibitors block the production of CDK enzymes, which are partially responsible for cell division; because of this, increased CDK inhibitors may decrease the risk of cancer and other neurological diseases (30). Discordant drugs (Figure 5B) like KIT inhibitors inhibit KIT which can sometimes develop into tumors (31). FLT3 inhibitors, another downregulated drug MoA, reduce neuropathic pain (32). Similarly, PDGFR tyrosine kinase receptor inhibitors are also used to treat tumors as they block receptors that mediate autocrine tumor growth, use fibroblast-rich tumor stroma, and regulate tumor vasculature. KIT inhibitors, FLT3 inhibitors, and PDGFR tyrosine kinase receptor inhibitors each influence blood-brain barrier cell processes by altering receptor signaling pathways that impact tumor growth, neuropathic pain, and tumor vasculature, thereby affecting the cellular interactions and permeability of the blood-brain barrier.

4. Discussion

BHB can keep the brain's neurons healthy and has previously improved various neurological conditions. We hypothesized that BHB administration modulates metabolism-related gene expression and immune response in neurons, which may contribute to its therapeutic effects. In order to test this hypothesis, we analyzed a publicly available RNAseq dataset using a “3-pod” bioinformatic workflow to determine the effect of BHB treatment on the transcriptome of human neurons. Surprisingly, we did not identify a significant effect of BHB on energy metabolism in neurons under the studied experimental conditions. However, DEG and LE gene analysis implicated immune-related genes, particularly proinflammatory cytokines and chemokines like CCL5, PYCARD, ANXA1, IL23R, and IL23, following BHB treatment. These genes were predominantly upregulated; interestingly anti-inflammatory related genes like GNG10 were significantly downregulated, suggesting increased immune activation in response to BHB treatment. BHB treatment is a mediator of systemic and brain inflammation (9), but less is known about its role in different cell types. Nomura and colleagues showed that inflammatory gene response is muted in unstimulated, BHB-treated microglia (21). In response to lipopolysaccharide (LPS) administration, inflammatory gene activation is significantly upregulated in untreated cells, but not in BHB-treated microglia. This supports an anti-inflammatory role for BHB following LPS activation but a mild proinflammatory response under other conditions. Our results suggest that neurons respond in a similar manner to microglia under non stimulated conditions as BHB appears to have a proinflammatory effect in our analysis. How neurons respond to a combination of LPS and BHB treatment will form the basis of future studies and will be important to understanding the potential role of BHB in disease conditions. Our results also suggest a potential mechanism for the inflammatory effects of BHB administration. iLINCS analysis identified HDAC inhibitors as drugs that induce gene signatures that are concordant with BHB administration. HDACs are potent regulators of neuroinflammation and HDAC inhibitors are being explored as anti-inflammatory agents for the treatment of CNS disorders (33).

Unsurprisingly, the immune-modulatory effect of HDAC inhibitors is well-studied in microglia. For example, HDAC inhibition drives changes in the functional ramification of microglia to an anti-inflammatory phenotype (34). It has been proposed that BHBs effect on microglial ramification is mediated by HDAC inhibition (20). Interestingly, our results suggest that BHBs immune modulatory effects in neurons may also be mediated via HDAC regulation.

Our initial hypothesis proposed that BHB treatment would induce significant changes in energy metabolism, as BHB is a ketone body that can be used by neurons as an alternative source of fuel. However, we found few pathways related to energy metabolism and mitochondria in our analysis. Further experiments, for example comparing the effects of BHBs on energy metabolism under glucose starvation conditions or under other stressor conditions, will be necessary to determine whether energy substitution is a primary mechanism of BHBs in neurons. Limitations to be considered include the time course of this study. RNAseq data was generated after 24hrs of BHB administration, which can obscure the longer-term effects of BHB with the ephemeral effect of inflammation. The longer-term effects of BHB on the neuronal transcriptome have yet to be studied. Cell culture offers an excellent way to study the effects of BHB directly on human neurons. However, these findings do not necessarily reflect the effects of BHB in the human brain, which is a complex tissue composed of diverse and interdependent neuronal and glial cell types. Overall, our results indicate that BHB-treatment induces significant changes in immune and inflammatory gene expression in neurons.

To date, studies about BHBs effects on neurons largely focused on its role as an energy substrate but our study suggests that changes in bioenergetic processes is not a major effect of acute BHB administration. This is reflected by the data, as the primary DEG, pathway and LE findings are predominantly linked to immune response. While BHBs role as a systemic and brain immune modulator are established, much less is known about its effect on neurons specifically. Our results reveal further insight into this topic. We found BHB has mild proinflammatory effects on neurons under normal growth conditions. Our data suggests that these immune effects may be regulated via BHBs action as a mediator of HDACs. As for future applications, these findings suggest BHB administration as a possible method for regulating inflammation in neurons and support further exploration of this ketone body in the treatment of neurological disorders.

5. Conflicts

The authors have no conflicts to declare.

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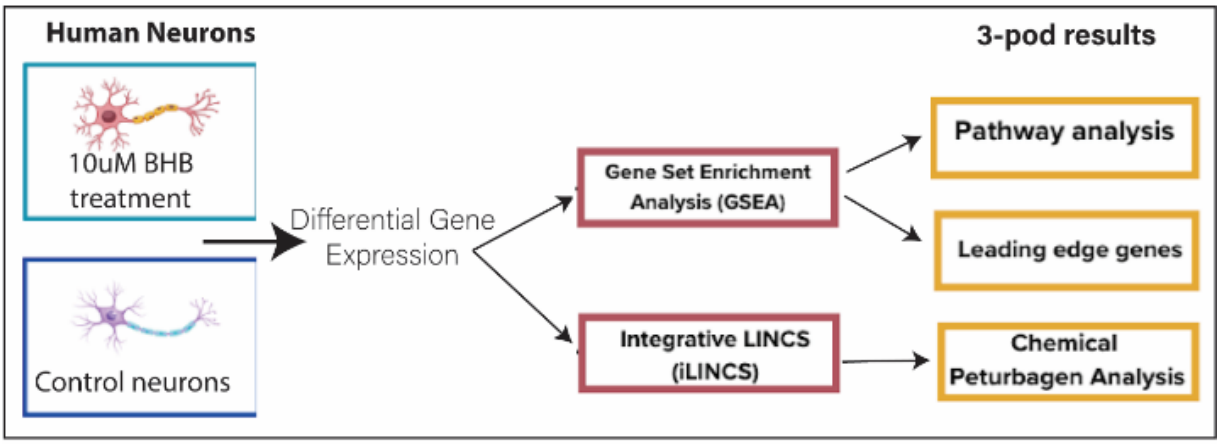

Figure 1. Experiment and bioinformatic analysis workflow.

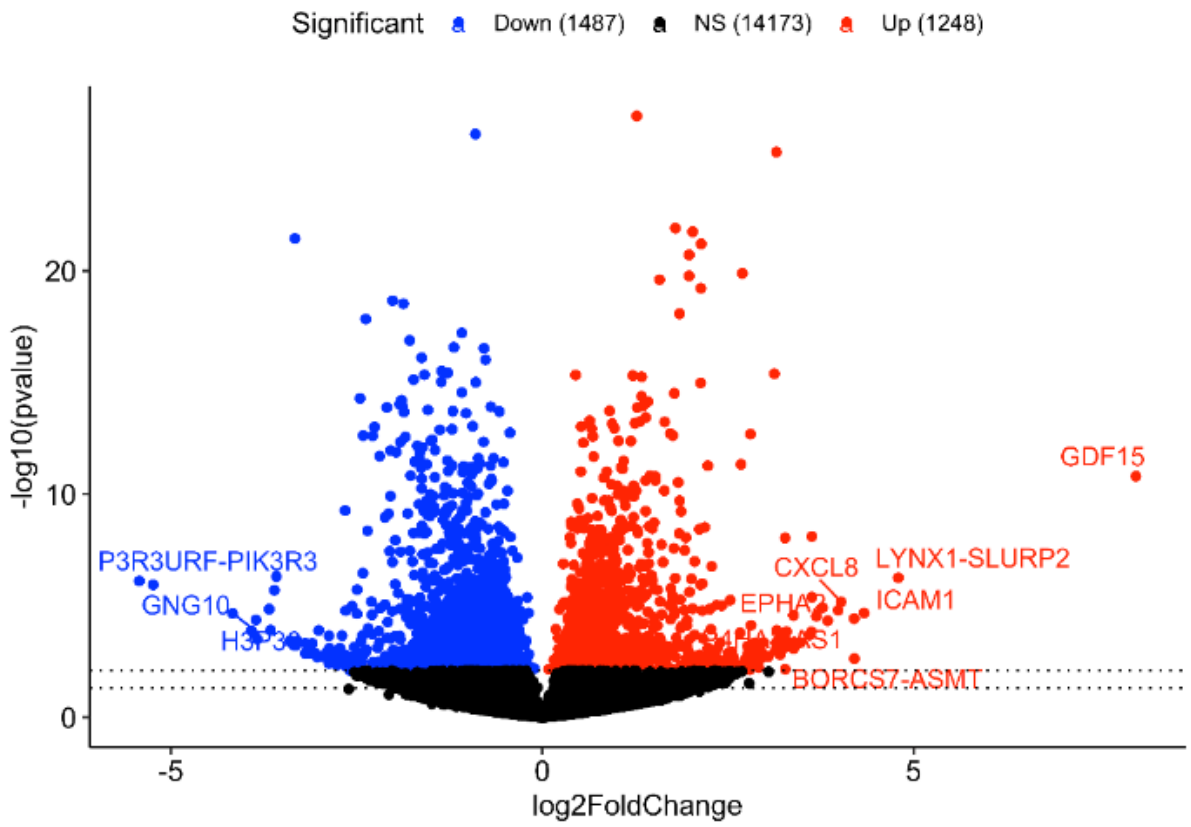

Figure 2. RNAseq analysis of β-hydroxybutyrate (BHB)-treated primary human neurons. Data are expressed as a volcano plot of log2 fold change (x-axis) and -log10 (p-value) (y-axis). Positive values (red dots) indicate genes with significantly increased expression, while negative values (blue dots) indicate genes with significantly decreased expression. The first dotted line (from bottom to top) indicates an unadjusted threshold of significance $p \leq 0.05$, whereas the second dotted line above the first one indicates an adjusted threshold of significance, adjusted $p \leq 0.05$. Annotated genes are those which were both significant by their p-value after adjustment for multiple testing and were the top 10 genes with the greatest absolute log-fold change, defining them as the "most" differentially expressed genes found in the dataset.

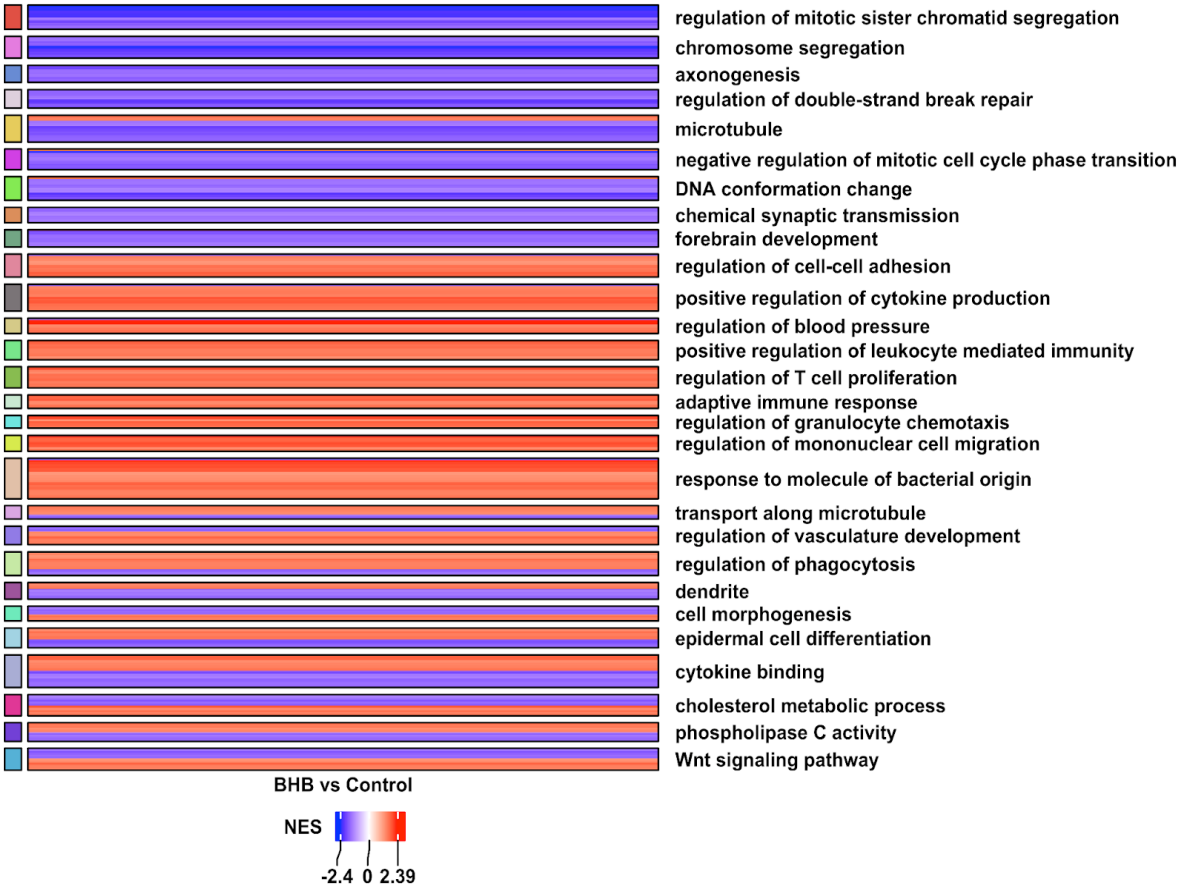

Figure 3. Heatmap of gene set enrichment analysis (GSEA) identifying differentially expressed pathways ($p \leq 0.05$). Cluster analysis was conducted using PAVER. Pathways are presented by normalized enrichment score (NES). A positive NES (red) indicates over-represented or enriched pathways in BHB treated neurons. A negative NES indicated pathway under-representation in BHB treated neurons.

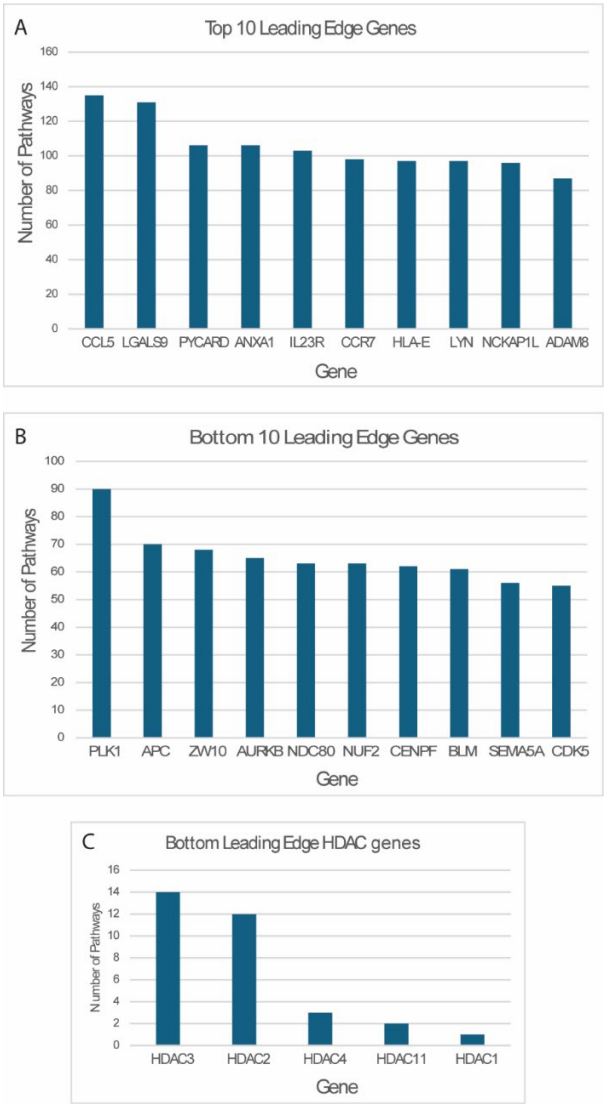

Figure 4. The top (A) and bottom (B) leading edge (LE) genes in β-hydroxybutyrate (BHB)-treated primary neurons were identified by gene set enrichment analysis (GSEA). Several histone deacetylases (HDAC1- 4, 11) genes were also identified in LE gene analysis in downregulated biological pathways (C). LE genes are the genes that are most frequently identified in significant ($p \leq 0.05$) GSEA pathways but are not necessarily significantly differentially expressed. Data represented on column graphs by LE gene symbol (x-axis) and number of genes (y-axis).

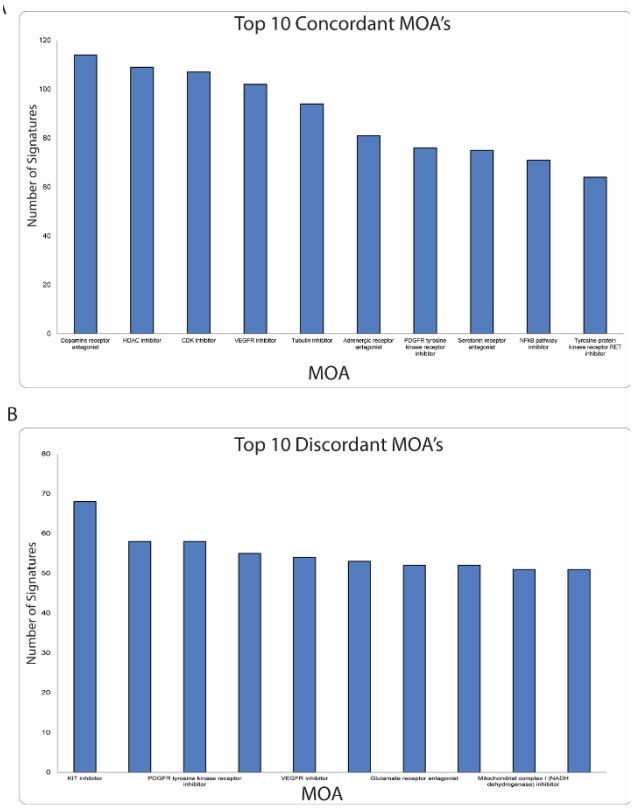

Figure 5. (A) The top mechanisms of action (MoAs) for drugs that induce a gene expression signature similar (concordant) to the gene expression induced by BHB are dopamine receptor antagonists, HDAC inhibitors, and CDK inhibitors. (B) The top MoAs of drugs that induce a gene expression signature that are dissimilar (discordant) to BHB are KIT inhibitors, FLT3 inhibitors, and PDGFR tyrosine kinase receptor inhibitors.

