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RESEARCH ARTICLE

Analysis of Valproic Acid-Induced Gene Expression Changes in Human Neuron Cells

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ABSTRACT: Over the past two decades, the prevalence of Autism Spectrum Disorder (ASD) has become a very crucial issue. Therefore, understanding the causes and risk factors of ASD is important, and many researchers have focused on analyzing its factors. Valproic Acid (VPA), a drug used for treating neural and psychiatric disorders, has been implicated in increasing ASD risk when exposure occurs *in utero*. Even though there is evidence linking VPA to ASD-like behaviors in animal models, the biological mechanisms remain still unclear. This study aims to elucidate these mechanisms by analyzing gene expression changes in human neuron cells treated with VPA. We performed cell viability assays to determine the cytotoxic concentration of VPA. Also, we conducted RNA sequencing (RNA-Seq) to analyze the gene expression of non-treated control and VPA-treated cells. Our experimental results reveal that VPA induces significant changes in gene expression levels in human brain cells. The experimental result provides a new insight into how VPA exposure contributes to ASD phenotypes. By identifying differentially expressed genes and their associated biological processes and cellular functions, this research enhances our understanding of the molecular impacts of VPA on neuronal development. In conclusion, this research paper focused on potentially guiding future therapeutic strategies for ASD.

KEYWORDS: Biomedical and Health Sciences; Autism Spectrum Disorder; Gene Expression; Valproic Acid; Neuron Cells; RNA Sequencing.

Introduction

Over the past two decades, there has been a notable increase in the number of diagnoses of Autism Spectrum Disorder (ASD)—approximately 2.8% of infants in the U.S. being identified with the disorder, a significant rise from 1 in 150 in the year 2000 to 1 in 36 in the year 2020.[1] ASD's etiology is believed to be multifactorial: on the one hand, genetic mutations such as those associated with Rett syndrome may affect the level of brain development, while on the other hand, environmental factors such as viral infections, drug exposures, or air pollution could contribute to its onset.[2] The rising prevalence of ASD poses a serious need to examine its underlying causes and potential risk factors, with particular attention to prenatal chemical exposures.

The research pivots towards Valproic Acid (VPA), a drug with contrasting dual facets: therapeutic and teratogenic.[3] The chemical is mainly used in dealing with neural and psychiatric disorders, including seizures and mood stabilization. Such a role can extend into a prenatal environment; however, an extra amount of exposure may lead to an increased chance of the child's ASD.[4] A recent study conducted on the correlation between *in-utero* exposure of rodents to VPA and their offspring's autism-like behaviors revealed there is a high relevance of overdosing on VPA and having an autistic-behavior-accompanied offspring. Yet, with the current advancements, the biological mechanism behind it remains unknown. Therefore, in the experiment, to further investigate the cause of changes induced by VPA exposure, we used an analysis of the neuron cells' altered gene expression levels.[5]

The importance of analyzing gene expression level patterns of VPA-induced ASD neuron models lies in the fact that it provides a detailed molecular mechanism of how VPA damages neuron cells and results in ASD phenotype.[6] To elucidate the complex process, we specifically focused on the two following methodologies: first—a cell viability assessment after valproic acid was treated on human neuron cells, and second—an RNA-Seq analysis to compare the gene expression pattern between non-VPA-treated neuron cells and VPA-treated neuron cells.[7] The former method was crucial in evaluating which concentrations of VPA exhibit cytotoxic effects, as understanding the threshold of VPA concentration is essential; too high a concentration could lead to cell death, while too low a concentration does not induce any significant effect. The latter method lets us analyze the total gene expression changes. We performed a comparative analysis by conducting RNA-Seq with the control group (non-treated) and the experimental group (VPA-exposed), identifying the genes expressed differently. This gene comparison allowed us to discern which specific human functions or traits VPA exposure alters.

In this study, we aim to analyze the effect of valproic acid on human neuron cells, specifically with the following methods. First, we analyzed cell viability after treating valproic acid on human neuron cells. Next, we performed RNA-Seq analysis to compare the gene expression pattern between non-VPA-treated and VPA-treated neuron cells. Analysis of the gene expression level pattern of the VPA-induced ASD neuron model would provide the detailed molecular mechanism of how VPA damages neuron cells and results in ASD phenotype.

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Methods

Cell culture and maintenance:

SHSY5Y neuronal cell line was used for this research. RPMI1640 cell culture media supplemented with 10% FBS and 1% antibiotic substances of penicillin/streptomycin was used for the growth medium. The cells were cultured and maintained at 37°C in a CO_2 incubator.

Preparation of valproic acid stock solution:

The final concentration of 145.5mM valproic acid (CAT# PHR1061, Sigma) stock solution was prepared with 29.1 mg powder with 1.20 mL of PBS solution. The solution was vortexed until the valproic acid was all solubilized into PBS. The stock solutions were then aliquot into several tubes and stored in a -70°C freezer.

Cell imaging:

The fluorescent inverted microscope (Nikon) was used to capture the image of the cells. The 40x and 200x magnitudes of the images were captured after SHSY5Y cells were treated with various concentrations of valproic acid. Valproic acids were treated for seven days.

Cell viability analysis:

The cell viability analysis was performed using a LUNA-FL automatic live cell counter device. The cells were stained with the Acridine orange and propidium iodide. The live cells were green, and dead cells were stained with red fluorescence.

RNA-Seq analysis:

Two cells (control and valproic acid-treated cells) were cultured. After the cells were harvested, total RNA was extracted using the Trizol method according to the manufacturer's instructions. RNA quality and concentration were assessed using a NanoDrop spectrophotometer and Agilent Bioanalyzer, ensuring RNA Integrity Number (RIN) values above 7.0 for all samples to proceed with library preparation. RNA libraries were prepared using the TruSeq Stranded mRNA Library Preparation Kit (Illumina), following the manufacturer's protocol. Briefly, poly-A mRNA was purified from 1 µg of total RNA using oligo-dT magnetic beads, followed by fragmentation. First-strand cDNA synthesis was performed using random primers and reverse transcriptase, followed by second-strand synthesis to generate double-stranded cDNA. The cDNA was then end-repaired, adenylated at the 3' ends, and ligated to TruSeq-indexed adapters. The adapter-ligated cDNA fragments were amplified by PCR to enrich the library fragments. The final libraries were purified using AMPure XP beads and quantified using a Qubit fluorometer and Agilent TapeStation. Then, the prepared libraries were sequenced on the Illumina platform (HiSeq 2500) at Macrogen (Seoul, South Korea). Paired-end sequencing was performed with a read length of 150 bp, generating approximately six million reads per sample. The raw sequencing data were initially processed for quality control using FastQC. Low-quality reads and adapter sequences were removed using Trimmomatic. Cleaned reads were aligned to the human reference genome (GRCh38) using HISAT2. The alignment was performed with default parameters, and the alignment quality was assessed using SAMtools. Aligned reads were counted using featureCounts to generate gene-level expression counts. Transcript abundance was quantified using StringTie and normalized as Fragments Per Kilobase Million (FPKM). Differential gene expression analysis between the two cell types was performed using DESeq2. Genes with a fold change of ≥ 2 and an adjusted p-value of < 0.05 were considered significantly differentially expressed.

Result and Discussion

Evaluation of VPA concentrations on cell viability:

Sample #	Final concentration of VPA (mM)	Cell viability
1	0	100%
2	0.025	99.9%
3	0.050	97%
4	0.110	91%
5	0.220	95%
6	0.450	96%
7	0.900	97%
8	1.810	81%
9	3.625	71%
10	7.250	64%
11	14.500	15%
12	29.000	5%

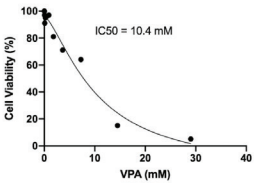

Figure 1: Evaluation of VPA concentrations for RNA-Seq analysis. Twelve different concentrations of VPA were tested on human neuron cells over seven days to determine the optimal concentration for RNA-Seq analysis. Cell viability was assessed using an automatic cell counter, which used fluorescence to differentiate between live (green) and dead (red) cells. Based on this result, 3.62 mM was selected for RNA-seq analysis.

Figure 1 aims to identify the optimal concentration of VPA for RNA-Seq analysis. Finding the optimal concentration was performed by evaluating the viability of human neuron cells across a range of VPA concentrations. Such a procedure was necessary as excessively high concentrations would result in the cells' death, while insufficiently low concentrations would disable to elicit a discernible effect.

In this study, twelve different concentrations of VPA were tested and incubated with human neuronal cells for seven days. Cell viability was assessed using the automatic cell counter device, differentiating live cells using fluorescence: live ones are shown in green, and dead ones are in red. The automated cell counter machine also calculated the total number of cells by summing up the number of live and dead cells, and the percentage of viable cells was calculated as the ratio of live cells to the total number of cells multiplied by 100.

The results mainly indicate a clear inverse relationship between VPA concentration and cell viability—as the concentration of VPA increases, the cell viability decreases. Moreover, the 1.81 mM of VPA showed a noticeable decrease in cell viability compared to that of 0.9 mM of VPA. The viability drastically declined for a 14.5 mM sample by 15%. The concentration at which cell viability was reduced to exactly 50%—so-called IC50—came out to be approximately 10.4 mM.

In conclusion, 0 mM of VPA was set to be a control condition, and based on the results, a VPA concentration of 3.62 mM was ultimately selected for a subsequent RNA-Seq analysis. This decision was based on considering the abovementioned

issues associated with concentrations that are either too high or too low.

Effect of VPA on cell morphology and confluency:

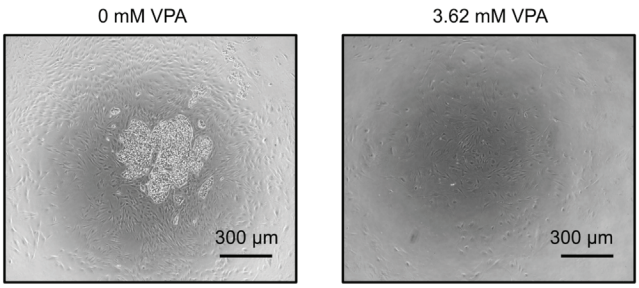

Figure 2: Comparative analysis of cellular morphology and confluency between control and 3.62 mM VPA-treated cells. Human neuron cells were treated with 3.62 mM VPA for seven days, and their morphology and confluency were documented using an inverted microscope. VPA (3.62 mM) treatment showed healthy cell condition with a slight decrease in confluency.

Figure 2 analyzes the differences in morphology and confluency between control and 3.62 mM-VPA cells. The methodology of the experiments was as follows: first, the cells were treated with VPA for seven days, and second, the cellular morphology and confluency were then observed and documented using an inverted microscope, which enabled a more detailed visual analysis of the cells' physical changes and growth patterns as well as for capturing images.

Multiple significant findings were gained through such comparative analysis. The 3.62 mM VPA-treated cells exhibited less live cell number than 0 mM VPA control cells. Also, the control cells tended to be more clustered and accumulated in the center, ultimately showing a multi-layer growth pattern. We speculated that this pattern indicates overpopulation since it is highly probable that as the cells replicate excessively, they spill over from the original mono-layer structure. On the other hand, overpopulation was not observed in the 3.62mM VPA-treated cells; it did not show any overpopulated morphology due to a decreased live cell number.

Based on their relatively regulated and sustainable growth pattern, 3.62 mM VPA-treated cells seem to have healthy cell conditions—albeit a slight decrease in confluency. Thus, we analyzed the RNA-Seq using the control and 3.62 mM cells.

RNA-seq result analysis with volcano plot:

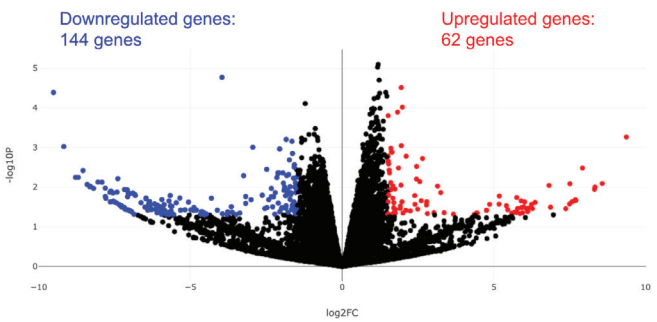

Figure 3: Volcano plot of VAP-treated brain cells compared to control cells without VAP treatment. This figure illustrates the impact of VPA (valproic acid) on gene expression in human brain cells through the identification of differentially expressed genes (DEGs) using RNA sequencing (RNA-Seq). A total of 14,550 genes were analyzed, with DEGs identified based on the criteria of $p < 0.05$ and $|\log2FC| \geq 1$.

Figure 3 aims to find the impact of VPA on gene expression in human brain cells. It does this by identifying Differentially Expressed Genes (DEGs)—both upregulated and downregulated genes—through RNA sequencing. VPA-dependent DEGs were analyzed using RNA-Seq, and the data was subsequently plotted and visualized using volcano plots. VPA treatment significantly changed the gene expression level in human brain cells.

A total of 14550 gene expression levels were analyzed in the process. The following methodology compared these levels with those of the control group without VPA. All 14550 genes were plotted in the volcano plot, and DEGs were identified based on the following criteria: $p < 0.05$ and $|\log2FC| \geq 1$. This was to ensure the selection of genes to portray statistical, gene-expressional changes that were significant enough, thus assuring the data's biological meaningfulness in terms of fold change.

The main result revealed that, out of all 14550 VPA-treated samples, 144 genes were downregulated (highlighted in blue), and 62 genes were upregulated (highlighted in red). Therefore, in conclusion, about 1.416% of the genes analyzed exhibited changes in expression levels due to VPA treatment, with more than double the number of genes being downregulated. Figure 3 demonstrates the substantial impact of VPA on gene expression in human brain cells.

RNA-seq data analysis with gene ontology enrichment analysis on biological function:

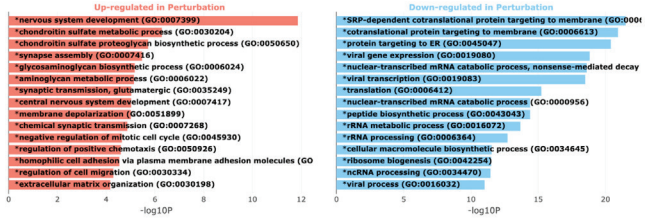

Figure 4: Gene ontology analysis was performed based on the biological processes of upregulated and downregulated genes. VPA treatment affects many biological processes related to the phenotype of ASD children.

Figure 4 shows a gene ontology analysis based on the biological processes of upregulated and downregulated genes. Its objective is to explore which biological processes are affected by these upregulated and downregulated genes, thus identifying the processes most influenced by VPA treatment and understanding its potential mechanisms within the context of ASD.

The analysis used lists of names for both upregulated and downregulated genes. It examined their associated biological processes with the EnrichR program, which provided a detailed gene ontology analysis. The program ranked these processes using the log10 p-value, then displayed the top 15 processes for each upregulated and downregulated gene.

The gene ontology analysis revealed a significant upregulation in genes associated with "nervous system development" and "synapse assembly." This result highly likely indicates that VPA may promote enhanced neural connectivity and synaptic formation—demonstrating its potential therapeutic benefits in neurodevelopmental disorders. Moreover, the analysis has

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shown a significant increase in "chondroitin sulfate (CS) metabolic process." CS plays a critical role in neural development and synaptic plasticity.[8] Previous research revealed an association between reduced levels of CS in urine and ASD, suggesting that the upregulated genes that are related to the CS metabolic process can be linked to VPA-dependent ASD development in children.[9] Also, the other previous research indicated a great increase in the total concentration of Glycosaminoglycan was observed in ASD children's urine.[9] Interestingly, we found that some of the upregulated genes are associated with the glycosaminoglycan biosynthesis process. This result indicates that upregulated genes of glycosaminoglycan biosynthesis may be linked to increased glycosaminoglycan concentration in ASD children's urine.

Conversely, there was a notable downregulation of genes involved in "protein targeting to ER". This downregulation has a particularly high association with ASD among those of other genes, mainly because ER stress is implicated in the disorder's neuropathology. It is widely known that ER stress hinders neuron development by inhibiting neurite outgrowth and expressing synaptic factors crucial for a proper neural network to form and function.[10] Thus, the high level of downregulation in genes associated with "protein targeting to ER" after VPA treatment suggests how VPA might induce ER stress by under-expression of the genes that encode the proteins targeting ER. Through biological process analysis, we found that many genes significantly changed in gene expression are linked to previous ASD children's phenotypes.

RNA-seq data analysis with gene ontology enrichment analysis on cellular function:

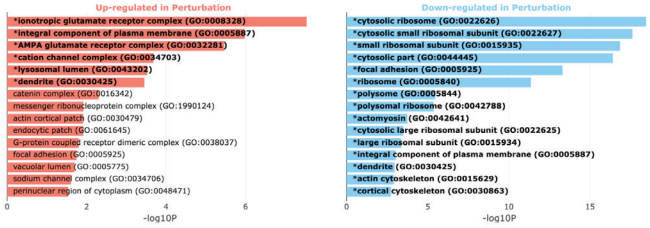

Figure 5: Gene ontology analysis was performed based on the cellular function of upregulated and downregulated genes. VPA treatment affects many different cellular functions that are associated with ASD phenotypes.

The upregulated genes are linked to the ionotropic glutamate receptor complex and AMPA glutamate receptor complex (Figure 5). Earlier studies suggest that changes in how brain cells connect can lead to changes in glutamate receptor expression, damaging the neuron network. The upregulated genes are connected to the cytosolic ribosome, cytosolic small ribosomal subunit, small ribosomal subunit, and ribosome. Earlier research also suggests that rRNA synthesis is decreased in ASD mouse models.[11] This means that making new ribosomes might play a big part in how these models learn and think. These findings could be crucial in understanding how VPA affects how neurons grow and work in ASD.

Conclusion

Our study examined how VPA exposure shakes up gene expression in human neuron cells. Our research paper gives some fresh insights into how VPA affects ASD. We found that VPA causes large changes in gene expression through cell viability tests and RNA sequencing. These genes are all about nervous system development, synapse assembly, and protein targeting of the endoplasmic reticulum. The 144 genes were downregulated, while 62 were upregulated in VPA-treated samples, showing that VPA might be altering the expression of these genes in human brain cells. Therefore, VPA significantly impacts how neurons work. Overall, our findings help us understand how VPA's molecular mechanisms play a part in ASD, which could help us come up with better treatments for ASD in the future.

One limitation of this study is the use of SH-SY5Y cells as a model for human neurons, as these cells are derived from a neuroblastoma line and may not fully replicate the characteristics of primary human neurons. While SH-SY5Y cells are widely used in neurobiology research due to their neuronal differentiation capacity, their cancerous origin and differences in gene expression could limit the direct applicability of the findings to normal human neuronal function. Additionally, the lack of a dose-response analysis for gene expression changes in response to VPA may reduce the granularity of understanding VPA's effects at varying concentrations, which could provide more detailed insights into its mechanism of action on neuronal gene expression. Including this analysis would enhance the study's overall robustness.

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Junhyeok (Jayden) Park, a junior at Korea International School Jeju (KISJ) in South Korea, has a deep passion for biology, driven by his commitment to creating tangible solutions for societal equality. Through further research, he plans to develop innovative ideas that help bridge scientific advancements and societal needs.

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