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RESEARCH ARTICLE

Role Of MECP2 Deficiency In Autism Spectrum Disorder Symptoms: A Pathway Analysis

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ABSTRACT: Methyl-CpG-binding protein-2 (*MECP2*) is a critical gene involved in neural development. Disruptions to the functioning of this gene have been associated with the risk of neurodevelopmental disorders, including Rett syndrome and autism spectrum disorder (ASD), characterized by social and cognitive deficits. In this paper, we test the hypothesis that *MECP2* deficiency may influence gene expression and disrupt specific molecular pathways, leading to ASD-related phenotypes. Using RNA-seq data from male mice *MECP2* mutant versus wild-type excitatory neurons, differential gene expression was mapped on a volcano plot. Genes that were significantly upregulated or downregulated were subjected to a Gene Ontology (GO) analysis to identify over-represented molecular pathways. Based on the findings, it was concluded that a deficiency in *MECP2* led to the dysregulation of vital neurotransmitter signaling pathways, including those for serotonin, dopamine, metabotropic glutamate, and muscarinic acetylcholine receptors, which show a strong correlation to ASD-related phenotypes. By integrating RNA-sequencing data with pathway analysis, this study aims to provide insights into how the reduction in the *MECP2* expression could contribute to the etiology of ASD, paving the way for targeted therapeutic strategies to alleviate the disease's behavioral symptoms.

KEYWORDS: Biomedical and Health Sciences, Genetics and Molecular Biology of Disease, Autism Spectrum Disorder, *MECP2* deficiency, Gene Ontology Analysis.

Introduction

Approximately 1 in every 100 children is diagnosed with Autism Spectrum Disorder (ASD) around the world,[1] making it one of the most common neurodevelopmental disorders. ASD is characterized by a range of communication and behavioral traits, including repetitive movements, avoidance of eye contact, and anxiety.[2] Due to the "anti-social" nature of ASD, patients with this disorder often face challenges with social interactions and slower processing speed,[3,4] making it notably difficult for them to adapt to social and environmental changes. Despite widespread awareness about ASD, its etiology remains complex and poorly understood, involving a range of environmental, genetic, and epigenetic factors.[5] (Figure 1)

Clinical trials suggest that one of the most substantial genetic risk factors for the disease is the presence of siblings with autism, with the recurrence rate of autism being approximately 2% to 8%.[6] Apart from familial clustering, other genetic and epigenetic contributors include advanced parental age, maternal immune activation (MIA), and neuroinflammation.[7–10] Environmental risk factors, such as heavy metal toxicity,[11] parental drug abuse,[12] and air pollutants,[13] are also gaining recognition for their potential implications on autism in the offspring.

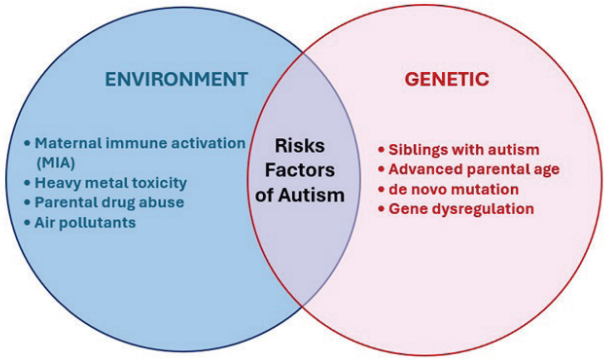

Figure 1: Venn Diagram of the genetic and environmental risk factors involved in the development of ASD. Image created using BioRender.com.

Due to the complexities of ASD, there is no curative or standard treatment plan for the disorder. The existing treatments for ASD are limited to early-year intervention with occupational, speech, and behavioral therapies to regulate the disorder's phenotypic characteristics, including aggression, self-injurious behavior (SIB), and severe tantrums.[14,15] However, medications can also be prescribed to children with comorbid challenging behaviors to reduce the intensity of these symptoms and improve social functioning.[16] To date, there are only two drugs that have been FDA-approved for the treatment of the behavioral symptoms of ASD in children: aripiprazole and risperidone.[17,18]

Ongoing research is spearheading advancements in targeted therapeutic strategies to expand the treatment landscape for ASD. One such drug that is gaining traction in the scientific community is buspirone (Buspar®),[19] an azapirone drug that

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acts as a partial agonist of the serotonin 5-HT_{1A} receptor. Buspirone works by binding to presynaptic 5-HT_{1A} receptors, a type of inhibitory autoreceptor, and partially activating them. (Figure 2) Over time, the drug causes increased serotonergic activity in the neurons by preventing the direct binding of serotonin and inducing desensitization of the somatodendritic autoreceptors.[20] Thus, the drug rebalances the levels of serotonin in the amygdala and other parts of the brain where 5-HT_{1A} receptors are prevalent, contributing to its anxiolytic effects. Buspirone is mainly used to treat generalized anxiety disorders (GADs) but also appears to be useful in other neurological disorders, including attention-deficit hyperactivity disorder and ASD.[21]

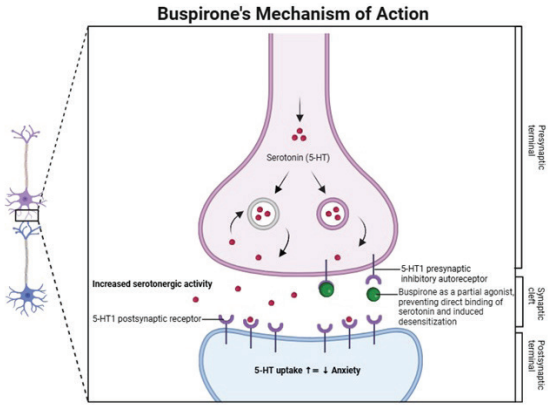

Figure 2: Mechanism of action of buspirone, a potent drug for anxiety in autism. Image created using BioRender.com.

Another novel therapeutic strategy includes positive allosteric modulators (PAMs) targeting the M4-muscarinic acetylcholine receptor (CHRM4). (Figure 3) The PAMs act as ligands that bind to an allosteric site of CHRM4, resulting in the potentiation of acetylcholine-induced responses and amplified downstream effects, including the inhibition of dopamine release.[22,23] A promising advantage of allosteric modulators is that the potentiation of the muscarinic acetylcholine receptors takes place only when and where the neurotransmitter acetylcholine is released, making sure to preserve the time and space pattern of acetylcholine action.[24,25] This drug mechanism is being explored for target therapeutic strategies in neurodevelopmental disorders like schizophrenia and ASD, where dysregulated dopamine signaling translates into typical phenotypes, including repetitive behavior and anxiety.[24,26,27]

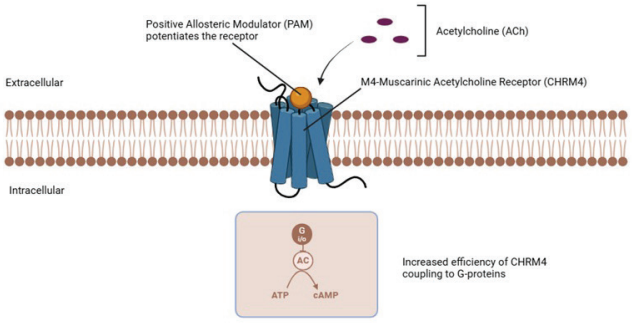

Figure 3: Mechanism of action of positive allosteric modulators (PAMs) for the M4-Muscarinic Acetylcholine Receptor (CHRM4). Image created using BioRender.com.

To strengthen evidence for these treatments, further molecular exploration of neurogenetic pathways is warranted. Among the numerous genes implicated in ASD pathway dysregulation, methyl-CpG-binding protein 2 (MECP2) emerges as a potential focus for understanding the molecular mechanisms underlying the symptoms of this disorder.[28,29] The *MECP2* gene, located on the X chromosome, is a critical epigenetic regulator that binds to methylated DNA and regulates gene expression and synaptic function, playing a central role in the postnatal development of the human brain.[30]

Recent studies have drawn correlations between mutations in *MECP2* and neurodevelopmental disorders, including Rett syndrome and broader ASD phenotypes such as social interaction deficits.[31] However, little research has been directed towards understanding the molecular correlations between *MECP2* gene deficiency and ASD symptoms. Therefore, this study seeks to address these gaps by investigating how *MECP2* deficiency can disrupt the functioning of specific molecular pathways, leading to the manifestation of ASD phenotypes. Furthermore, this study will explore how *MECP2* gene dysfunction alters potential molecular pathways relevant to ASD therapeutics and evaluate the impact of *MECP2* within the clinical landscape.

To fulfill the scope of this study, a secondary data analysis approach was employed, comprising of three main steps - 1) differential gene expression analysis of RNA-sequencing data from *MECP2*-deficient versus wild-type excitatory neurons in mice, 2) a subsequent Gene Ontology analysis for pathway enrichment, 3) exploration of how *MECP2* pathway dysregulation relates to therapeutic targets like buspirone and PAMs for CHRM4.

Methods

For this study, the RNA sequencing data were sourced from Supplementary Table 2 of an article published in Nature,[32] *"Characterization of human mosaic Rett syndrome brain tissue by single-nucleus RNA sequencing."* The respective dataset contained RNA-seq profiles from excitatory neurons of male mice *MECP2*–/y (1,230 cells) versus *MECP2*+/y (1,230 cells). Data normalization was not performed separately as the dataset was pre-processed and included identifiable columns for the mean expression in wild-type genes (meanWT) and knockout genes (meanKO), Log_2FC value, and p-value for each gene, allowing for minimal subsequent filtering. Next, the Benjamini-Hochberg procedure was used to calculate the false discovery rate (FDR) of the genes, which is a statistical measure that calculates the percentage likelihood of false positives in the gene set.[33] Genes with an FDR value of zero were marked as insignificant and removed from the data set using the 'DATA, FILTER' function on an Excel spreadsheet. This resulted in the reduction of the number of input genes from 25,284 to 8032. All the above-mentioned calculations and filtering were performed using Microsoft Excel.

Next, a volcano plot was created using the web-based tool Galaxy, usegalaxy.org,[34] to visualize the differential gene expression from the RNA-seq dataset. A volcano plot is a type of scatter graph that displays the magnitude of change in gene

expression due to a particular condition, which, in this study, is the absence of the *MECP2* gene in excitatory neurons.[35]

Thresholds were applied to the volcano plot to establish a focus on the respective data. A Log Fold Change threshold of $|Log_2FC| = 0.35$ was set, meaning genes with changes below this value were excluded from further analysis, denoted as the grey-dotted area of the graph. Log_2FC represents the magnitude and type of expression changes in a particular gene; a negative value displays down-regulation (cells are producing less of the coded protein), and a positive value displays up-regulation (cells are producing more of the coded protein). Additionally, a significance threshold of p-value < 0.0106 was applied to identify the genes with statistically robust differences. For further clarity, genes were colored if they passed these established thresholds – red for significantly upregulated genes and blue for significantly downregulated genes. Within this subset of colored genes, the top 15 genes with the smallest p-values were identified for further analysis. These genes were selected because they exhibited the most substantial changes in their expression due to MECP2 deficiency, serving as potent candidates for the following Gene Ontology (GO) analysis.[36]

Next, Gene Ontology (GO) analysis of the top 15 most significant genes was performed using the PANTHER Pathway toolkit available at geneontology.org. GO analysis is a method within bioinformatics that is used to identify cellular and molecular pathways that have been significantly over-represented within a selected set of genes. The criterion for pathway significance was 'adjusted p-value < 0.01'. The result of the GO analysis was then studied through literature reviews to draw connections between affected molecular pathways due to *MECP2* gene deficiency and behavioral symptoms of ASD.

Result and Discussion

The resultant volcano plot, generated using the web-based tool Galaxy, identified 547 differentially expressed genes (DEGs) between wildtype and *MECP2* knockdown mice from the total 8,032 genes, 254 downregulated and 293 upregulated. (Figure 4) With the established significance thresholds of $|Log_2FC| = 0.35$ and p-value < 0.0106 determined via FDR correction, 6.8% of the total gene collection was colored either red or blue, indicating increased or reduced gene expression, allowing for a focused analysis of only these genes that showed significant change in their expression. Among these, the top 15 genes with the most significant changes were identified and labelled on the graph: *Mecp2, Snap25, Junb, Arc, Nap1l5, Dynll1, Dynlrb1, Tsc22d1, Zc3hc1, Dynll2, Tspyl4, Camta1, Prkacb, Calm2, and Tmsb4x.* (Table 1)

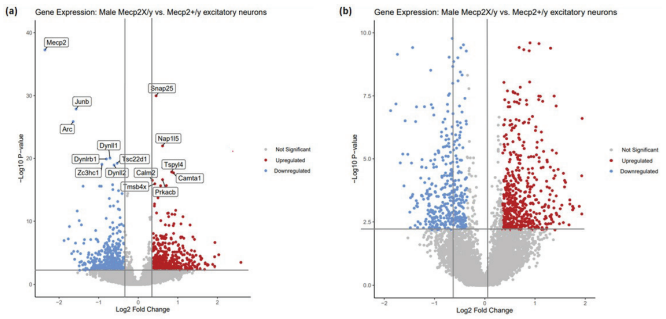

Figure 4: Volcano plot of differentially expressed genes between *MECP2*-deficient (MECP2–/y) and wild-type (MECP2+/y) excitatory neurons (1,230 cells per condition); (a) Full-scale volcano plot with Log_2FC on the x-axis and $-log_{10}$ P-value on the y-axis (0-40 range with top 15 most significantly differentially expressed genes (DEGs) labelled, (b) Volcano plot with y-axis range limited to 0-10 for clear visualization of DEG distribution and significance thresholds. Plots were generated using a web-based tool, Galaxy.

Table 1: Top 15 differentially expressed genes (DEGs) with the most significant upregulation or downregulation due to *MECP2* deficiency. The genes demonstrated the lowest p-values and highest fold changes, serving as primary candidates for subsequent gene ontology analysis. Table created using Microsoft Excel.

Sr No.	Gene Name	Log2FC (Fold Chain)	P-Value	Upregulated or Downregulated
1	Mecp2	-2.357881683	5.45E-38	Downregulated
2	Snap25	0.451994671	1.09E-30	Upregulated
3	Junb	-1.570122765	1.4E-28	Downregulated
4	Arc	-1.644369311	1.4E-26	Downregulated
5	Nap1l5	0.614110018	1.04E-22	Upregulated
6	Dynll1	-0.712450204	9.31E-21	Downregulated
7	Dynlrb1	-0.80382455	1.25E-20	Downregulated
8	Tsc22d1	-0.532035733	6.07E-20	Downregulated
9	Zc3hc1	-0.917429069	8.71E-20	Downregulated
10	Dynll2	-0.611337221	1.14E-19	Downregulated
11	Tspyl4	0.844798449	1.47E-18	Upregulated
12	Camta1	0.888635289	1.96E-18	Upregulated
13	Prkacb	0.614047402	2.44E-17	Upregulated
14	Calm2	0.354589978	3.18E-17	Upregulated
15	Tmsb4x	0.41942864	1.12E-16	Upregulated

Using the GO analysis and PANTHER toolkit, five pathways were identified as significantly over-represented by the top 15 genes. (Table 2)

Table 2: Molecular pathways were significantly enriched among the top 15 most significantly differentially expressed genes (DEGs). Pathways were identified using Gene Ontology enrichment analysis and the PANTHER pathway toolkit. All five identified pathways are significantly over-represented and are associated with key signaling systems disrupted by MECP2 deficiency. Table created using Microsoft Excel.

PANTHER Pathways	Set Size	Fold Enrichment	P-Value	Adjusted P-Value (FDR)
5HT1 type receptor mediated signaling pathway	2	60.66	4.89E-04	1.97E-02
Metabotropic glutamate receptor group II pathway	2	58.07	5.34E-04	1.72E-02
Dopamine receptor mediated signaling pathway	2	48.74	7.58E-04	2.03E-02
Muscarinic acetylcholine receptor 2 and 4 signaling pathway	2	45.49	8.69E-04	2.00E-02
Metabotropic glutamate receptor group III pathway	2	39.56	1.15E-03	2.31E-02

Notably, all five pathways showed a commonality in their involvement in G protein-coupled receptor (GPCR) signaling and neurotransmitter systems. This could indicate a potential shared mechanism of neurotransmitter dysregulation amongst the affected pathways, leading to the behavioral and neurological phenotypes of ASD patients. (Table 3) 5HT1 (serotonin), dopamine, metabotropic glutamate, and muscarinic acetylcholine receptors are GPCRs that trigger heterotrimeric G proteins to inhibit the adenylyl cyclase (AC) enzyme, which

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leads to reduced production of the second messenger molecule cyclic adenosine monophosphate (cAMP).[37] A reduction in these cAMP levels can lead to the dysfunction of protein kinase A (PKA) mediated biological processes in the brain, including disruptions in synaptic transmission and plasticity, neurogenesis, and apoptosis inhibition.[38] PKA is known for its role in phosphorylating multiple target proteins, including ion channels and transcription factors like cAMP response element-binding protein (CREB), which are crucial for long-term potentiation (LTP) and memory formation.[39,40] Ongoing research suggests a potential correlation between cAMP signaling dysregulation and ASD. For instance, studies have shown that cAMP production was found to be reduced in the platelets of patients with fragile X syndrome, a disease that often coexists with ASD.[41]

Table 3: Overview of the over-represented GPCR-mediated neurotransmitter pathways identified from the top DEGs. Dysregulation of these pathway mechanisms is implicated in ASD pathophysiology, drawing a potential correlation between MECP2 deficiency and the risk of ASD. Table created using Microsoft Excel.

Pathway	Receptor Family	Primary Molecule	Possible Contribution to ASD Phenotypes
5-HT1 Type Receptor Mediated Pathway	GPCR (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT1F)	Serotonin (5-HT)	Anxiety, impaired social behavior
Dopamine Receptor Mediated Pathway	GPCR (D1, D2, D3, D4, D5)	Dopamine	Social deficits, reduced reward processing, repetitive behaviors
Metabotropic Glutamate Receptor Pathway (Group II and III)	GPCR (mGluR2, mGluR3, mGluR4, mGluR6-8)	Glutamate	Learning deficits, anxiety
Muscarinic Acetylcholine Receptor Pathway (M2 and M4)	GPCR (M2 and M4)	Acetylcholine	Learning deficits, anxiety, weak memory capacity

Apart from the commonality of AC inhibition and cAMP reduction, these pathways also display other vital roles in the human nervous system. Dysregulation of the aforementioned pathways due to *MECP2* deficiency can result in the development of emotional and behavioral abnormalities often seen in ASD.

Serotonergic Pathway in ASD:

As indicated in Table 3, the GO enrichment analysis identified a Fold Enrichment value of 60.66 and a corrected p-value of 0.0172 for the 5HT1 type receptor-mediated signaling pathway, making it the most significantly overrepresented pathway from the input gene set. The 5-HT1 serotonin receptors are a group of receptors, mainly found in the brain, that bind to serotonin, a neurotransmitter that is known to regulate mood, memory, and learning.[42] During brain maturation at early stages of life, 5-HT1 plays a key role as a neurotransmitter and a growth factor that regulates neural development, synapse formation, and cortical arrangement.[43] A study done by the Center for Neurobiology and Behavior, Columbia University, concluded that 5-HT_{1A} receptor knockout mice exhibited increased anxiety-like behaviors in a series of behavioral tests, suggesting that 5-HT_{1A} receptors are involved in the regulation of exploratory and fear-related behaviors.[44] Thus, it can be deduced that disruptions in the 5-HT1 type receptor-mediated signaling pathway could lead to behavioral deficits such as anxiety and impaired social interactions, phenotypes often seen in ASD patients.

Dopaminergic Pathway in ASD:

As indicated in Table 3, the GO enrichment analysis identified a Fold Enrichment value of 48.74 and a corrected p-value of 0.0203 for the dopamine receptor-mediated signaling pathway. Dopamine is a vital neurotransmitter that regulates a list of physiological processes, including motivation, reward, and cognition.[45,46] Dopamine receptors have been recognized as key players in early brain development through their regulation of neuronal differentiation and axonal outgrowth. Recent studies conclude that dopamine receptor activation aids in the trafficking and functioning of AMPA and NMDA receptors, key mediators of excitatory neurotransmission.[47] The dysregulation of dopamine signaling has been implicated in various neurodevelopmental disorders (NDDs), including schizophrenia and autism spectrum disorder. One of the primary outcomes of dopamine receptor dysfunction is the disruption of synaptic plasticity, particularly within the striatum and prefrontal cortex – two regions of the brain that are enriched with dopamine receptors and are responsible for behavioral attributes, including social interaction.[48] Thus, disrupted dopaminergic pathways might contribute to the behavioral actualization of ASD, including low reward value from social stimuli, i.e., social anhedonia and disrupted sensorimotor processing.[49]

Overall, the paper's findings suggest that *MECP2* deficiency disrupts critical pathways that are responsible for neurodevelopment and synaptogenesis, including the regulation of GPCR signaling and cAMP levels. Thus, these molecular dysregulations – influenced by *MECP2* – contribute to ASD phenotypes, including anxiety and stereotypies. These insights align with the mechanism of action of emerging therapeutic strategies such as buspirone, which targets the 5-HT_{1A} serotonin receptors, and CHRM4 positive allosteric modulators, both of which aim to regulate the neurotransmitter systems that were disrupted by *MECP2* deficiency. This strengthens the rationale for further exploring such drugs in the treatment of ASD symptoms.

However, a limitation of this study is that conclusions on the affected molecular pathways are based on the computational analysis of Gene Ontology. The insights provided need to be validated by future experiments with *MECP2* gene knockout mice to assess the likelihood of these results. Moreover, it is noteworthy that it is highly unlikely that a single genetic insult is responsible for all cases of biologically complex and clinically heterogeneous neurodevelopmental disorders such as ASD.

■ Conclusion

In summary, the initial hypothesis of the study stated that reduced *MECP2* gene expression stands as a potential epigenetic factor for the development of ASD and its behavioral phenotypes. The pathway analyses applied in this study serve as a proof-of-concept for this hypothesis, exploring how reduced *MECP2* expression can disrupt the functionality of specific GPCRs and neurotransmitter signaling pathways, contributing to the phenotypic abnormalities of the disorder. Our results align with the mechanisms of novel ASD therapeutics, such as buspirone and CHRM4 positive allosteric modulators, and emphasize the importance of focusing drug targets for ASD in the serotonergic and dopaminergic pathways, which these drugs target. The findings of this study can be further developed upon via experimental validation in *MECP2*-deficient

animal models and large-scale transcriptomic analyses with larger gene sets.

Further studies on potential genetic and epigenetic risk factors, including *MECP2* gene deficiency, are crucial in expanding treatment methods for the disorder's behavioral and neurological phenotypes. Lack of etiological understanding of ASD has restricted treatment options for patients with this neurodevelopmental disorder. Moreover, advancing in target therapeutics through pre-clinical and clinical trials of drug efficacy, including buspirone and PAMs for CHRM4, must also be prioritized to cater to the growing prevalence of ASD worldwide.

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