Research Article

The Ability of Risperidone to Methylate the *St8sia2* and *St8sia4* Genes and Its Effect on Sialic Acid Levels in The Brains of Embryos and Offspring of Albino Rats

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Abstract

Background

Epigenetic modifications, particularly DNA methylation, play an important role in regulating gene expression during brain development. The *St8sia2* and *St8sia4* genes are involved in the biosynthesis of polysialic acid, a molecule that contributes to neuronal development and plasticity. **Aim of the Study**: The present study aimed to investigate the effects of maternal exposure to sulforaphane and risperidone during pregnancy and the early postnatal period on the DNA methylation patterns of the *St8sia2* and *St8sia4* genes and sialic acid levels in the brains of rat fetuses and offspring at different developmental stages. **Methodology**: Eighteen healthy adult female albino rats, aged 11–12 weeks were randomly allocated into three experimental groups: saline control, sulforaphane (SFN), and risperidone (RIS). **Results**: The methylation patterns of both *St8sia2* and *St8sia4* differed among the experimental groups and developmental stages. For *St8sia2*, methylation was detected in 13 of 86 samples (15.1%), including 12 samples from the RIS group and one from the control group, whereas no methylation was detected in the SFN group. The differences among groups were not statistically significant at E14 ($P = 0.085$), but became significant at P0 ($P = 0.028$) and P7 ($P = 0.023$), with a highly significant difference when all developmental stages were considered together ($P < 0.001$). For *St8sia4*, methylation was detected in 9 of 86 samples (10.5%), all of which belonged to the RIS group. No methylation was detected in either the control or SFN groups. The differences were not significant at E14 ($P = 0.307$) or P0 ($P = 0.090$), but a significant difference was observed at P7 ($P = 0.023$). Sialic acid concentrations also varied among the groups. At E14, no significant differences were observed. At P0 and P7, however, sialic acid levels were significantly lower in the RIS group than in both the saline and SFN groups, whereas no significant difference was observed between the saline and SFN groups. **Conclusion**: Maternal exposure to risperidone was associated with alterations in the DNA methylation patterns of the *St8sia2* and *St8sia4* genes in the brains of rat offspring, particularly during the early postnatal period. In contrast, the SFN group maintained an unmethylated pattern for both genes across the examined developmental stages. Risperidone exposure was also associated with reduced brain sialic acid levels at P0 and P7.

Keywords: *St8sia2*; *St8sia4*; DNA methylation; Sulforaphane; Risperidone; Sialic acid.

Introduction

The brain is a complex organ with millions of neurons that control and regulate the body, and the proper functioning of the brain depends on the communication between individual neurons and their interaction with glial cells. Every human cell type, such as endothelial, immune, muscle, blood or neuronal, has a glycocalyx. This architecture encodes a vast amount of information relevant for health and disease and is directly targetable in therapeutic settings. The glycocalyx is made up of a dense layer of associated sugars, proteins and lipids, with a complex composition that varies according to tissue and cell type. Sugars considered the basic building blocks are polysaccharides or monosaccharides, and they link up in different ways to form compounds called 'glycans', which may consist of a few to many thousands of monosaccharide units. Functional specificity of glycans is often determined by the sugar composition at the nonreducing termini of glycan chains. In mammals, sialic acids are essential sugars usually found

at the distal ends of glycans, at the termini of glycoproteins and glycolipids. These sugars are involved in molecular recognition and cell–cell interaction and contribute to interactions of cells with extracellular environment (Schnaar et al., 2014; Wang, 2012). Sialic acids are important players in the interactions of glycans at the cell and molecular scale. Disruption of sialic acid metabolism may have major effects on embryonic and nervous system development, and aberrant sialylation has been linked to a number of human diseases (Schnaar et al., 2014; Yoo et al., 2015; Zhu et al., 2024). Sialic acid is also a key structural and functional component of brain gangliosides and sialylated glycoproteins and contributes to neuronal growth, synaptic connectivity, and cognitive processes (Wang, 2009; Li et al., 2023).

Sialylation is the covalent addition of sialic acid to the ends of glycoproteins. This is an important biological modification which plays a role in embryonic and neuronal development. In this process a family of enzymes called sialyltransferases, attach sialic acid to the terminal ends of glycan chains of cell surface molecules. Among these enzymes, *St8sia2* and *St8sia4*, also called *St8sia*-II and *St8sia*-IV respectively, are specialized polysialyltransferases in charge of the biosynthesis of α2,8-linked polysialic acid (polySia), (Hildebrandt & Dityatev, 2015; Mühlenhoff et al., 2013). In the nervous system polySia is mainly conjugated to the neural cell adhesion molecule (NCAM), but other acceptor molecules have also been described (Mühlenhoff et al., 2013; Galuska et al.,2017). PolySia modification of NCAM decreases cell–cell adhesion and modulates neuronal migration, axonal growth, synaptic plasticity and other developmental processes participating in the establishment and functional organization of the nervous system (Rutishauser, 2008; Hildebrandt & Dityatev, 2015).

Moreover, *St8sia2* and *St8sia4* are expressed in the brain in a developmentally regulated manner. Expression of *St8sia2* and *St8sia4* is dramatically upregulated during embryonic brain development, reaching high levels before birth. *St8sia2* expression declines relatively rapidly after birth, while *St8sia4* declines more gradually and becomes especially important in the mature brain (Mühlenhoff et al., 2013). Further experimental studies using genetically engineered mice showed that *St8sia2* is the main contributor to polySia biosynthesis during early brain development, and *St8sia4* during later developmental and mature stages (Oltmann-Norden et al., 2008; Mühlenhoff et al., 2013).

The functional importance of these enzymes is also underscored by animal studies, in which loss of both *St8sia2* and *St8sia4* results in a virtually complete loss of polySia and manifests in severe brain development abnormalities, including neuronal migration and axonal organization defects (Weinhold et al., 2005). Moreover, changes in polySia–NCAM signaling can influence synaptic plasticity and neuronal connectivity, thus highlighting the importance of tightly controlled polysialylation during brain development (Hildebrandt & Dityatev, 2015; Mühlenhoff et al., 2013).

Research into the microstructure and connectivity of brain cells has shown a dysregulation in the connectivity between different areas of the cerebral cortex, which is implicated in the neurodevelopmental abnormalities observed in disorders such as Autism Spectrum Disorder (ASD). These changes can include disruption of epigenetic mechanisms such as DNA methylation that can affect the regulation of genes during critical periods of neuron development (Ecker et al., 2015).

Study Samples

This study utilized 18 healthy adult female albino laboratory rats, obtained from the laboratory animal breeding unit at the College of Veterinary Medicine, Tikrit University. The rats ranged in age from 11 to 12 weeks and in weight 225 ± 10 grams. They were housed under standard laboratory conditions, including a temperature of 22–25°C and a 12-hour light/12-hour dark cycle, with continuous access to food and water. All experimental procedures involving animals were reviewed and approved by the Animal Ethics Committee, College of Veterinary Medicine, Tikrit University (Approval No.: Tu.Vet.161; Date: December 17, 2025).

Experimental Design

Mating was achieved by pairing each female with an adult male; the day a vaginal plug was observed was designated as Gestational Day 1. Following confirmation of pregnancy, the females were randomly assigned to three main groups and housed in clean plastic cages. Each group received a different solution via oral gavage; this treatment continued from Gestational Day 7 through Postnatal Day 7. The rats were monitored for weight and general health to detect any potential adverse effects of the drug.

Materials Administered to Rats

Physiological saline (NaCl) was used as the dosing agent for the first group (the control group) at a standard concentration of 0.9%. Pregnant dams were administered a daily oral dose of 1 ml, from Gestational Day 7 through Postnatal Day 7. The raw material used was DL-sulforaphane 97% (Cat. No. D818039; Macklin Biochemical Co., Ltd., Shanghai, China; Macklin Biochemical, 2025a). Pregnant laboratory rats were administered a daily dose of 1 ml of the solution containing 0.5 mg of raw sulforaphane per rat**.** Pregnant rats were administered a dose of 1 ml per rat, containing 0.5 mg of raw risperidone 99% purity (Cat. No., R832185; Macklin Biochemical Co., Ltd., Shanghai, China; Macklin Biochemical, 2025b). The dose was selected based on previous developmental studies in rats (Singh et al., 2016; Singh & Singh, 2017; Okab et al., 2025).

Sample Collection Method

A total of 142 samples were collected across three distinct time points to investigate molecular and biochemical developmental changes in the three experimental groups. Animals were anesthetized using a chloroform solution.
Fetal brains were collected on embryonic day 14 (E14): Pregnant dams (two per group) were dissected under sterile conditions; the uterus was opened, and fetuses were carefully extracted. Brains were then isolated using appropriate, precision dissection tools under conditions designed to preserve sample integrity. Neonatal rat brains were collected immediately after birth postnatal day 0 (P0), half of the pups from each group were separated from their mothers, anesthetized, and euthanized, while the remaining half were kept until one week after birth. Brains were then isolated from the neonates and preserved. Brains from young rats were collected one week after birth postnatal day 7 (P7) (comprising the second half of the pups, which had been reared and suckled by mothers that continued to receive the three experimental substances). These pups were separated from their mothers, anesthetized, and euthanized; their brains were then isolated using appropriate dissection tools under conditions ensuring sample preservation.

Sample Distribution According to Type of Analysis

Following extraction from the fetuses and newborns, the brains were divided into sections for the required tests, as follows: Some brain sections were preserved in a DNA stabilization solution (500 µL of DNA Shield in Eppendorf tubes); a total of 86 samples were collected, then transferred to a deep freezer and stored at temperatures between -20 and -80 °C for subsequent DNA extraction and epigenetic analysis (specifically, DNA methylation patterns). Other brain sections were preserved in liquid nitrogen for direct tissue freezing; prior to this, the tissue was rinsed with saline, and excess saline was removed using filter paper. A total of 56 samples were collected, then transferred to a deep freezer and stored at temperatures between -20 and -80 °C for biochemical testing, specifically the measurement of sialic acid levels.

Molecular Analysis

We conducted molecular assays to investigate the genetic and epigenetic alterations resulting from the experimental treatments applied to the genes under study. Specific primers were designed to detect methylated and unmethylated samples—as well as the genetic impact of methylation on the target genes (*St8sia4* and *St8sia2*)—via

polymerase chain reaction (PCR); these primers were synthesized by Macrogen Inc. (Seoul, South Korea), as detailed in Table (1).

Table (1) Primer sequences used in the study, obtained using the Primer-BLAST tool available on the NCBI website.

Annealing	Size	Length	Sequence (5´-3´ direction)	Target Gene
Methylation Specific and Unmethylated Specific Primers				
57	181	25	F- GTTGTTCGTGGTTTTTTTTATTTTC	*St8sia2* M
57	181	24	R- ACACTATCCCTCCAACTAAACGAC	
56	158	24	F- AGTTGGGATAATTTGGATTTTTTC	*St8sia4* M
56	158	25	R- ATACATTTACCCGATAAATTACGTC	
57	181	26	F- TTGTTTGTGGTTTTTTTTATTTTTGT	*St8sia2* U
57	181	25	R- AACACTATCCCTCCAACTAAACAAC	
56	158	25	F- GAGTTGGGATAATTTGGATTTTTTT	*St8sia4* U
56	158	26	R- CATACATTTACCCAATAAATTACATC	

DNA Methylation

DNA Extraction

Brain samples were collected from rat fetuses and newborns following dissection and placed in Eppendorf tubes containing DNA Shield preservation solution. They were immediately transported from the dissection site at the College of Veterinary Medicine's animal facility to a deep freezer in the Central Laboratories Department, where they were stored at temperatures ranging from -20 to -80 °C pending molecular analysis and DNA extraction. DNA extraction was performed using the Presto Mini gDNA Kit (Geneaid). Subsequently, DNA concentration and purity were measured using a NanoDrop spectrophotometer; the extracted samples were then analyzed to verify the presence of DNA in each sample.

Measurement of DNA methylation in the promoters of the *St8sia2* and *St8sia4* genes.

DNA methylation analysis was performed according to the following steps: DNA was subjected to bisulfite modification using a DNA methylation kit from TINZYME. Polymerase Chain Reaction (PCR) was carried out using a thermal cycler, following specific reaction protocols; the primer annealing temperatures were 57°C for M*St8sia2* and U*St8sia2*, and 56°C for M*St8sia4* and U*St8sia4*.

Biochemical Analysis by ELISA Technique

The concentration of sialic acid was determined using the Rat Sialic Acid (SA) ELISA Kit manufactured by the Chinese company SUNLONG, following the accompanying instructions.

Statistical Analysis

The analysis of the methylation test results was based on the presence or absence of bands corresponding to methylated and unmethylated DNA following agarose gel electrophoresis; samples were classified accordingly as either methylated or unmethylated. The data were then organized into contingency tables, and Fisher's Exact Test was used to compare methylation frequencies across the three experimental groups and the three time points. This analysis was performed using Jamovi (version 2.7.37) software, with statistical significance set at $P < 0.05$. Sialic acid concentration data were analyzed using GraphPad Prism (version 10) software. One-way ANOVA was employed to

compare experimental groups at each specific time point, while two-way ANOVA was used to analyze differences across experimental groups and the three developmental stages. Tukey's multiple comparisons test was conducted to identify specific differences between groups. Results are presented as the mean ± standard deviation (Mean ± SD), with statistical significance defined as $P < 0.05$.

Results

Figure (1) illustrates the results of Methylation-Specific PCR (MSP-PCR) for the *St8sia2* gene following agarose gel electrophoresis, revealing DNA methylation patterns in brain tissue samples from rat fetuses and newborns. A clear difference in band appearance is observed among the groups: some samples showed amplification using methylation-specific primers, while others exhibited distinct amplification using unmethylation-specific primers.

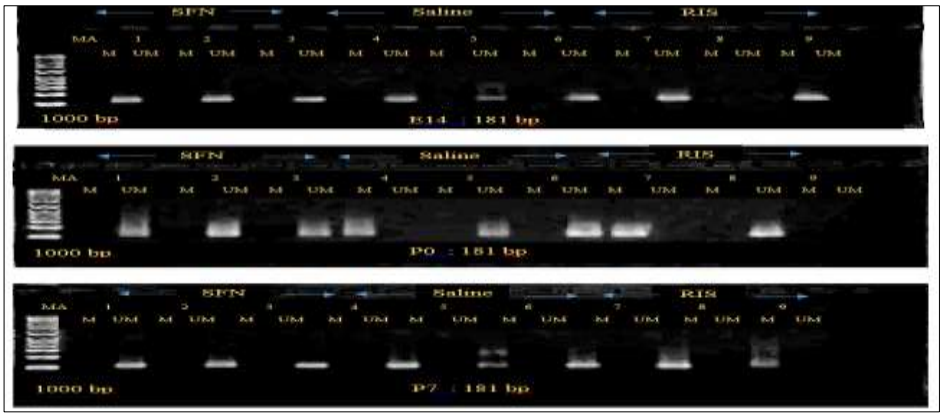

Figure (1): PCR results showing the DNA methylation pattern of the *St8sia2* gene for the three groups (RIS, SFN, Saline) across three developmental stages (100-1000 bp DNA ladder).

The results were analyzed using Fisher's Exact Test. As shown in Table (2), the distribution of the methylation pattern for the *St8sia2* gene (positive methylation) comprised 13 samples (15.1% of the total): 12 samples (40.0%) were from the group of rats whose mothers were administered Risperidone (RIS), 1 sample (3.6%) was from the group whose mothers received saline (control group), and 0 samples (0%) were from the group whose mothers received Sulforaphane (SFN). Regarding the unmethylated pattern (negative methylation), there were 73 samples (84.9% of the total): 18 samples (60.0%) were from the RIS group, 27 (96.4%) from the control group, and 28 (100%) from the SFN group. The results indicate a sharp decrease in methylation levels in both the control and SFN groups. In the control group, a minimal methylation rate of only 3.6% was observed at the second stage (P0), whereas methylation was completely absent (0.0%) at both the first (E14) and third (P7) stages. Similarly, the SFN group showed a complete absence of methylation (0.0%) across all three developmental stages, indicating a stable maintenance of the unmethylated state throughout these time points. In contrast, a notable and gradual increase in methylation levels was observed in the RIS group across the developmental stages; the rate rose from 33.3% at the first stage (E14) to 40.0% at the third stage (P7), with the highest level—45.5%—recorded at the second stage (P0).

Table (2) Percentage of DNA methylation status of the *St8sia2* gene for the experimental groups (RIS, SFN, Saline) across developmental stages (P7, P0, E14).

			Methylation Status		
Developmental stage	Experimental groups		0	1	Total
	Saline	Observed	8	0	8

Embryonic 14		% within row	100.0%	0.0%	100.0%
	SFN	Observed	8	0	8
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	6	3	9
		% within row	66.7%	33.3%	100.0%
	Total	Observed	22	3	25
		% within row	88.0%	12.0%	100.0%
Postnatal 0	Saline	Observed	9	1	10
		% within row	90.0%	10.0%	100.0%
	SFN	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	6	5	11
		% within row	54.5%	45.5%	100.0%
	Total	Observed	25	6	31
		% within row	80.6%	19.4%	100.0%
Postnatal 7	Saline	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	SFN	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	6	4	10
		% within row	60.0%	40.0%	100.0%
	Total	Observed	26	4	30
		% within row	86.7%	13.3%	100.0%
Total	Saline	Observed	27	1	28
		% within row	96.4%	3.6%	100.0%
	SFN	Observed	28	0	28
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	18	12	30
		% within row	60.0%	40.0%	100.0%
	Total	Observed	73	13	86
		% within row	84.9%	15.1%	100.0%

The number (0) indicates the unmethylated state, and the number (1) indicates the methylated state.

To evaluate the impact of three different treatments on epigenetic changes and the methylation pattern of the *St8sia2* gene in rat embryonic and neonatal brains, Fisher's Exact Test was conducted to compare methylation rates across the three experimental groups at three distinct time points. Statistical analysis revealed variation linked to the animal's developmental stage; specifically, differences between the three groups—particularly between the risperidone group and the control and sulforaphane groups—did not reach statistical significance at the first stage (E14) ($P = 0.085$). In contrast, statistically significant differences were observed at the second stage—immediately after birth (P0)—with a p-value of ($P = 0.028$). At the third stage (P7), this significant effect of the experimental groups on methylation status and epigenetic changes persisted, showing clear statistical significance ($P = 0.023$), as illustrated in Figure (2). Furthermore, a highly significant statistical result was observed when pooling data across all time points ($P < 0.001$).

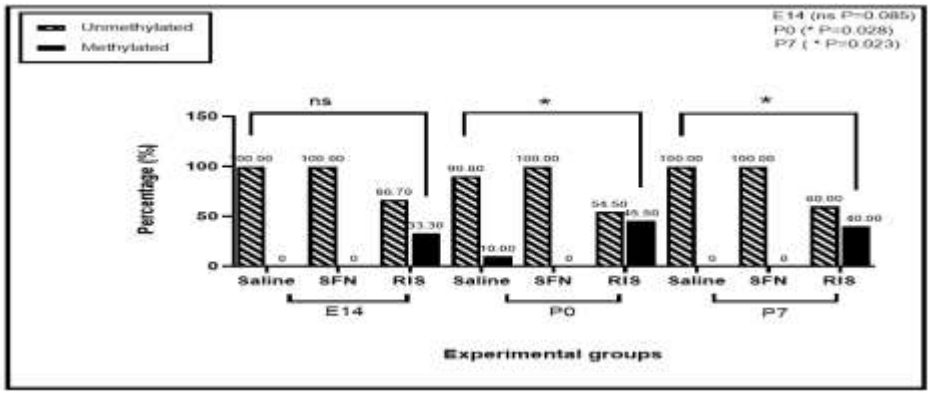

Figure (2) Percentage distribution of *St8sia2* gene methylation patterns across the experimental groups (Saline, SFN, RIS) and Developmental stages (E14, P0, P7).

Figure (3) illustrates the results of Methylation-Specific PCR (MSP-PCR) for the *St8sia4* gene following agarose gel electrophoresis. It displays the DNA methylation patterns in brain tissue samples from rat fetuses and newborns. While differences in band appearance across groups are evident—with some samples showing amplification using methylation-specific primers and others showing clear amplification with unmethylated-specific primers, this indicates a variation in methylation patterns among samples and groups throughout developmental stages. Sample classification as methylated or unmethylated was based on the presence or absence of bands in the reactions corresponding to each primer type.

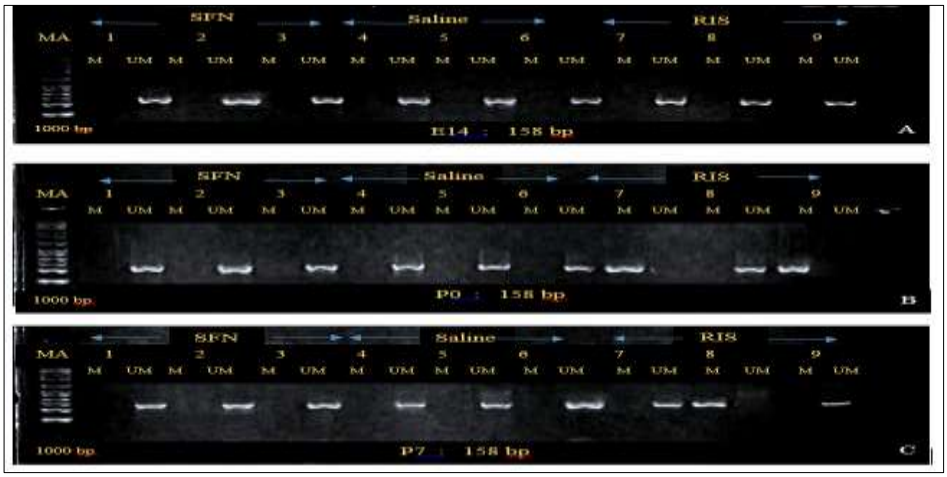

Figure (3): PCR results showing the DNA methylation pattern of the *St8sia4* gene for the three groups (RIS, SFN, Saline) across three developmental stages (50-1000 bp DNA ladder).

The results were analyzed using Fisher's Exact Test. As shown in Table (3), the distribution of the methylated pattern (positive methylation) for the *St8sia4* gene involved a total of 9 samples (10.5%): all 9 samples (10.5%) originated from the group of rats whose mothers were administered Risperidone (RIS), while 0 samples (0.0%) came from the group whose mothers received saline (control group), and 0 samples (0.0%) came from the group whose mothers received Sulforaphane (SFN). Regarding the unmethylated pattern (negative methylation), there were 77 samples (89.5%) in total: 21 samples (70.0%) from the RIS group, 28 (100%) from the saline group, and 28 (100%) from the

SFN group. A sharp decrease in methylation levels was observed in both the control and SFN groups; methylation was absent (0.0%) across all three developmental stages, indicating that the saline and SFN groups stably maintained an unmethylated state throughout these time points. In contrast, a marked and gradual increase in methylation levels was observed in the RIS group across the developmental stages: the rate rose from 22.2% at the first stage (E14) to 27.3% at the second stage (P0), reaching a peak of 40.0% at the third stage (P7). The number (0) indicates the unmethylated state, and the number (1) indicates the methylated state.

Table (3) Percentage of DNA methylation status of the *St8sia4* gene for the experimental groups (RIS, SFN, Saline) across developmental stages (P7, P0, E14).

			Methylation Status		
Developmental stage	Experimental groups		0	1	Total
Embryonic 14	Saline	Observed	8	0	8
		% within row	100.0%	0.0%	100.0%
	SFN	Observed	8	0	8
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	7	2	9
		% within row	77.8%	22.2%	100.0%
	Total	Observed	23	2	25
		% within row	92.0%	8.0%	100.0%
Postnatal 0	Saline	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	SFN	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	8	3	11
		% within row	72.7%	27.3%	100.0%
	Total	Observed	28	3	31
		% within row	90.3%	9.7%	100.0%
Postnatal 7	Saline	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	SFN	Observed	10	0	10
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	6	4	10
		% within row	60.0%	40.0%	100.0%
	Total	Observed	26	4	30
		% within row	86.7%	13.3%	100.0%
Total	Saline	Observed	28	0	28
		% within row	100.0%	0.0%	100.0%
	SFN	Observed	28	0	28
		% within row	100.0%	0.0%	100.0%
	RIS	Observed	21	9	30
		% within row	70.0%	30.0%	100.0%

	Total	Observed	77	9	86
		% within row	89.5%	10.5%	100.0%

To evaluate the impact of three different treatments on epigenetic changes and the methylation pattern of the *St8sia4* gene in the brains of rat fetuses and newborns, Fisher's Exact Test was conducted to compare methylation rates across the three experimental groups at three distinct time points. Statistical analysis revealed variation linked to the animal's developmental stage; specifically, differences between the three groups—particularly between the risperidone group and the control and sulforaphane groups—did not reach statistical significance at the first stage (E14) (*P* = 0.307). Similarly, at the second stage (immediately after birth, P0), no statistically significant differences were observed (*P* = 0.090). In contrast, a significant effect of the experimental groups on methylation status and epigenetic changes was evident at the third stage (P7), reaching statistical significance (*P* = 0.023), as illustrated in Figure (4).

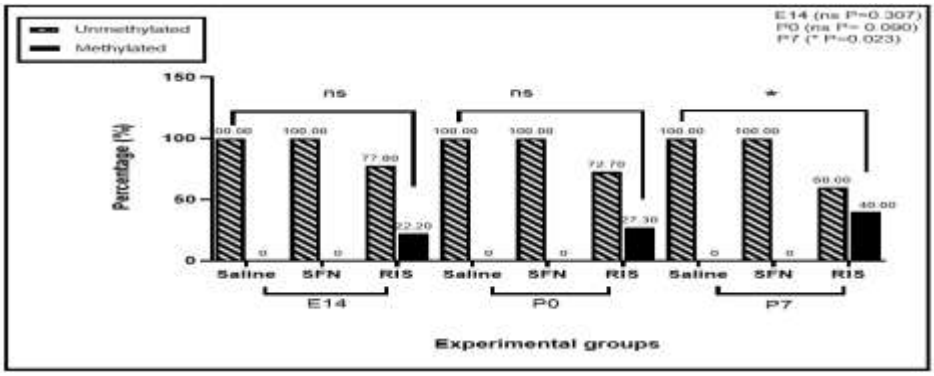

Figure (4): Percentage distribution of *St8sia4* gene methylation patterns across the experimental groups (Saline, SFN, RIS) and Developmental stages (E14, P0, P7).

Sialic acid levels were measured using the ELISA technique across three different groups (RIS, SFN, and Saline) and at three distinct developmental stages (E14, P0, and P7), with a total sample size of 56. Descriptive statistical analysis for the embryonic day 14 (E14) stage showed that the mean sialic acid concentration was 1553.04 ± 156.85 in the Saline group, 1634.45 ± 210.80 in the Sulforaphane (SFN) group, and 1455.48 ± 128.37 in the Risperidone (RIS) group. Statistical analysis using One-Way ANOVA followed by Tukey's test for multiple comparisons revealed no statistically significant differences in sialic acid levels among the study groups; non-significant differences were recorded between the Saline and SFN groups (*P* = 0.5982), the Saline and RIS groups (*P* = 0.4892), and the RIS and SFN groups (*P* = 0.1488), as shown in Figure (5). Descriptive statistical analysis for the day-of-birth stage (P0) revealed that the mean sialic acid concentration was 1941.47 ± 286.37 in the Saline group, 2152.71 ± 178.36 in the Sulforaphane (SFN) group, and 1515.37 ± 374.75 in the Risperidone (RIS) group. Statistical analysis using One-Way ANOVA followed by Tukey's test for multiple comparisons showed no statistically significant difference in sialic acid levels between the Saline and Sulforaphane groups (*P* = 0.3436). However, a significant difference was observed between the Saline and Risperidone groups (*P* = 0.0214) and between the Risperidone and Sulforaphane groups (*P* = 0.0001), as illustrated in Figure (6). Regarding the postnatal day 7 (P7) stage, descriptive statistical analysis showed that the mean sialic acid concentration was 1778.74 ± 268.06 in the saline group, 2011.14 ± 78.13 in the sulforaphane (SFN) group, and 958.75 ± 874.42 in the risperidone (RIS) group; this indicates significant variation and dispersion in the rats' response at this stage. Statistical analysis using One-Way ANOVA followed by Tukey's test for multiple comparisons revealed no statistically significant difference in sialic acid levels between the saline and sulforaphane groups (*P* = 0.7062), whereas a clear significant difference was observed between the saline and risperidone groups (*P* = 0.0221) and between the risperidone and sulforaphane groups (*P* = 0.0025), as shown in Figure (7).

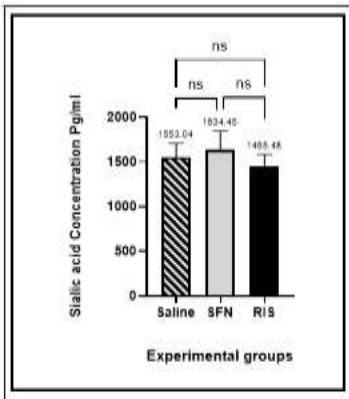

Figure (5) Sialic acid concentration (pg/ml) in the experimental groups at stage E14

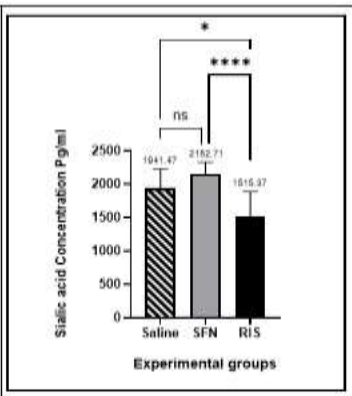

Figure (6) Sialic acid concentration (pg/ml) in the experimental groups at stage P0.

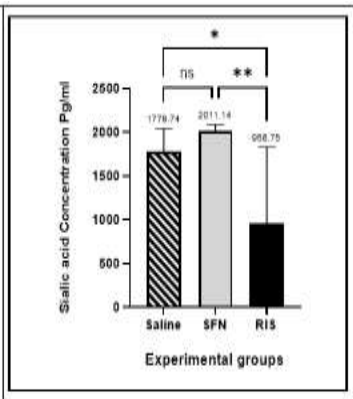

Figure (7) Sialic acid concentration (pg/ml) in experimental groups at stage P7 .

Two-way ANOVA, followed by Tukey's test for multiple comparisons, revealed statistically significant differences among the three developmental stages (E14, P0, and P7) depending on the study group. In the Saline and Sulforaphane (SFN) groups, no statistically significant changes in sialic acid levels were recorded across the different developmental stages; all differences fell within the non-significant range ($P > 0.05$). In contrast, the Risperidone (RIS) group exhibited a marked, significant decrease in sialic acid levels as development progressed. Specifically, a significant decrease was observed between stages E14 and P7 ($P = 0.0373$) and between stages P0 and P7 ($P = 0.0067$), whereas no statistically significant difference was found between stages E14 and P0 ($P = 0.9460$). As shown in Figure (8), the graph displays a gradual rise in sialic acid levels in the Saline and SFN groups. This slight increase is attributed to the natural physiological role of sialic acid in supporting vital developmental processes— such as cell membrane formation, neural tissue differentiation, and the establishment of connections prior to birth. However, statistical analysis indicates that this rise represents a biological trend rather than a statistically significant change, suggesting the maintenance of relatively balanced levels across all developmental stages. In contrast, in the Risperidone (RIS) group, it caused a sharp, significant drop in sialic acid levels during the late postnatal stage compared to the early stages of the rats' development (specifically E14 and P0).

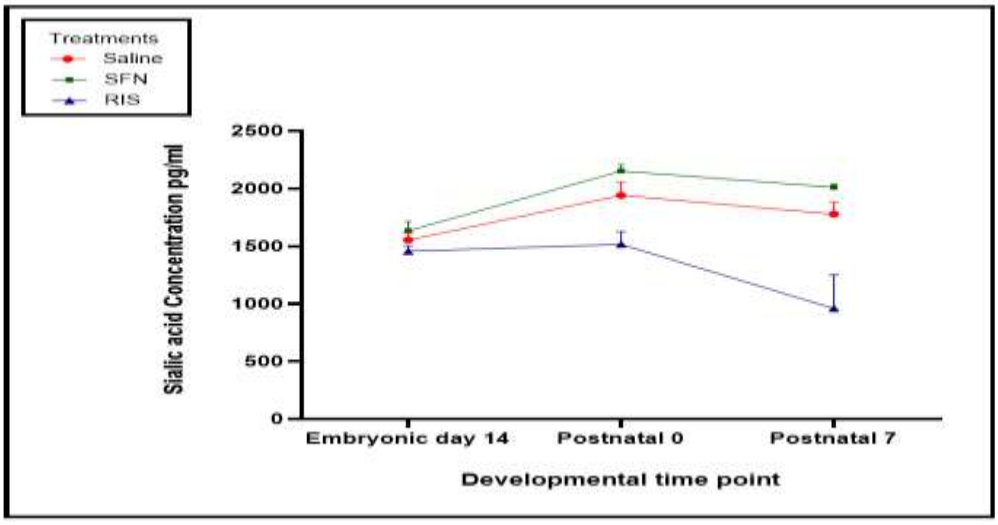

Figure (8) Changes in sialic acid concentration (pg/ml) for the study groups across developmental stages.

Discussion

Maternal exposure to environmental or pharmacological agents during pregnancy often results in the reprogramming of methylation patterns that can subsequently affect brain development and function; the intrauterine environment represents a critical period during which external environmental factors can induce long-lasting epigenetic changes in the fetus. Risperidone (RIS) is a commonly prescribed antipsychotic; however, its placental transfer during pregnancy raises growing concern regarding its potential impact on fetal neurodevelopment, despite its therapeutic efficacy. The results suggest that Risperidone has an adverse effect on fetal growth and physical development during both the prenatal and postnatal periods. Growth retardation may be due to the drug's interference with normal neurodevelopmental processes, alteration of placental function, oxidative stress, and disruption of nutrient transport to the developing fetus, which can indirectly affect overall physical growth. Our results are in agreement with a recent study reported that the administration of Risperidone to pregnant rats led to growth retardation and degenerative changes in the fetal cerebellum, placenta and skeletal system (Okab et al., 2025).

In the present study, changes in methylation patterns were observed in the *St8sia2* and *St8sia4* genes in the developmental stages of pregnant rats (225 ± 10 g) treated with Risperidone (RIS) at a dose of 0.5 mg/rat, but to different extents. In comparison, the saline and sulforaphane treated groups exhibited relatively stable methylation patterns throughout all developmental stages, suggesting that the normal epigenetic state for both genes was maintained. In the control group, DNA methylation levels were low during the embryonic stage and at birth, consistent with the physiological need for active transcription of polysialyltransferase genes during early neurodevelopment. The group treated with sulforaphane (SFN) at 0.5 mg/rat kept the gene unmethylated in all developmental stages, with a methylation pattern similar to that of the control group. This confirms the protective role of sulforaphane against epigenetic aberrations and is in line with a study showing that sulforaphane reduces hypermethylation of Inhibitor of Apoptosis Proteins (IAPs) encoding genes by downregulating DNMT3a enzyme activity and expression (Li et al., 2021). Emerging evidence indicates that bioactive dietary compounds, such as sulforaphane, can inhibit DNA methyltransferases (DNMT) enzymes, thereby altering the epigenetic landscape (Campisi et al., 2025). This suggests that sulforaphane acts as a protective dietary nutrient capable of counteracting risperidone-induced epigenetic toxicity.

In contrast, prenatal exposure to risperidone led to a progressive increase in DNA methylation of both genes under study, but with different levels of methylation between the genes. This increase was observed first in the embryonic stage (E14), was more pronounced right after birth (P0) and reached its maximum one week after birth (P7). These results imply that risperidone induces persistent epigenetic changes, rather than transient molecular changes, such that methylation marks set during embryonic development are maintained throughout the early stages of postnatal brain maturation. The consistency of these findings across two independent genes and across three developmental stages strengthens the evidence that risperidone exposure is associated with epigenetic alterations in the developing brains. It is noteworthy that *St8sia2* gene showed significantly higher methylation level than *St8sia4* gene during the developmental period. Biologically, this makes sense as *St8sia2* is the major gene during fetal brain development and is highly expressed in proliferating neural progenitor cells and migrating neurons and its alteration is linked to autism spectrum disorder (Yang et al., 2022). Therefore, genes that are transcriptionally active during embryogenesis are more susceptible to epigenetic disruptions when exposed to harmful environmental or pharmacological agents. Prenatal and perinatal exposure to risperidone seems to preferentially target *St8sia2*, resulting in accumulation of methylation marks and stronger transcriptional repression.

The *St8sia4* gene plays a relatively minor role in embryonic and perinatal development, but its expression increases progressively during postnatal neuronal maturation and the maintenance of synaptic plasticity. Thus, the low methylation levels observed in *St8sia4* during embryonic and perinatal development likely reflect its naturally low transcriptional activity during these developmental periods. The emergence of significant methylation one week

after birth suggests that risperidone-induced epigenetic changes are more apparent when physiological expression of the gene increases during postnatal brain maturation. These results are consistent with a recent report of extensive DNA methylation alterations across various brain regions by prenatal risperidone exposure with particularly enhanced hypermethylation in the hippocampus (Murata et al., 2026). These results were also consistent with another study which conducted a comprehensive analysis of DNA methylation in human neuroblastoma cells treated with low and high doses of risperidone for eight days. The study identified different methylated CpG sites (mostly hypermethylated sites) and suggested that risperidone caused widespread epigenetic changes. Moreover, the functional enrichment analysis showed that a significant number of the affected genes were related to neuronal growth, indicating that epigenetic regulation could be involved in the therapeutic efficacy of the drug and long-term biological effects in neurons (Du et al., 2021).

At P0, the sialic acid concentration in the brain was significantly decreased in the RIS group. This finding suggests that the effect of maternal RIS exposure was more pronounced near birth. The perinatal period is a critical period of brain maturation characterized by extensive changes in neuronal differentiation, cell-surface glycoconjugates and synaptic organization. Previous studies have shown that gangliosides containing sialic acid undergo significant changes during this developmental transition. Total ganglioside associated sialic acid was found to increase significantly approaching birth (Mühlenhoff et al., 2013). Mechanistically, risperidone exposure during critical neurodevelopmental windows may enhance the expression or enzymatic activity of DNA methyltransferases, leading to hypermethylation at the promoter region of *St8sia2* and *St8sia4* and subsequent transcriptional silencing. This epigenetic downregulation reduces the synthesis of polysialyltransferase enzymes, directly limiting the enzymatic conversion and incorporation of sialic acid into neural cell surface glycoconjugates, which biochemically accounts for the marked depletion of brain sialic acid observed at P0 and P7.

Yang et al. (2025) found that early-life supplementation with sialic acid in an autism-model rat altered the expression of *St8sia2* and was associated with changes in neuronal and behavioral outcomes.

Conclusion: Maternal exposure to risperidone was associated with alterations in the DNA methylation patterns of the *St8sia2* and *St8sia4* genes in the brains of rat offspring, particularly during the early postnatal period. In contrast, the SFN group maintained an unmethylated pattern for both genes across the examined developmental stages. Risperidone exposure was also associated with reduced brain sialic acid levels at P0 and P7.

References

1. Campisi, M., Cannella, L., Visioli, F. & Pavanello, S. (2025). Mechanistic insights and perspectives for healthy aging. *Advances in Nutrition*, 100521.
2. Du, J., Nakachi, Y., Kiyono, T., Fujii, S., Kasai, K., Bundo, M. & Iwamoto, K. (2021). Comprehensive DNA methylation analysis of human neuroblastoma cells treated with haloperidol and risperidone. *Frontiers in Molecular Neuroscience,* 14, 792874.
3. Ecker, C., Bookheimer, S.Y. & Murphy, D.G. (2015). Neuroimaging in autism spectrum disorder: brain structure and function across the lifespan. *The Lancet Neurology*, 14, 1121-34.
4. Galuska, C. E., Dambon, J. A., Kühnle, A., Bornhöfft, K. F., Prem, G., Zlatina, K., Lütteke,T. & Galuska, S. P. (2017). Artificial polysialic acid chains as sialidase-resistant molecular-anchors to accumulate particles on neutrophil extracellular traps. *Frontiers in Immunology*, 8, 1229.
5. Hildebrandt, H. & Dityatev, A. (2015). Polysialic acid in brain development and synaptic plasticity. *Topics in Current Chemistry*, 366, 1–26.
6. Li, D., Lin, Q., Luo, F. & Wang, H. (2023). Insights into the structure, metabolism, biological function and molecular mechanisms of sialic acid. A review. *Foods*, 13(1), 145.

7. Li, Y., Fan, H., Yuan, F., Lu, L., Liu, J., Feng, W., Zhang, H. & Chen, S. (2021). Sulforaphane protects against ethanol-induced apoptosis in human neural crest cells through diminishing ethanol-induced hypermethylation at the promoters of the genes encoding the inhibitor of apoptosis proteins. *Frontiers in Cell and Developmental Biology,* 9, 622152.
8. Macklin Biochemical. (2025a). DL-Sulforaphane 97% (CAS No. 4478-93-7; Cat No. D818039): Technical Specification and Product Data Sheet. Shanghai Macklin Biochemical Co., Ltd., Shanghai, China.
9. Macklin Biochemical. (2025b). Risperidone 99% (CAS No. 106266-06-2; Cat. No. R832185): Technical Specification and Product Data Sheet. Shanghai Macklin Biochemical Co., Ltd., Shanghai, China.
10. Mühlenhoff, M., Rollenhagen, M., Werneburg, S., Gerardy-Schahn, R. & Hildebrandt, H. (2013). Polysialic acid: versatile modification of NCAM, SynCAM 1 and neuropilin-2. *Neurochemical Research*, 38(6), 1134-1143.
11. Murata, Y., Fujii, A., Nakachi, Y., Du, J., Kasai, K., Bundo, M. & Iwamoto, K. (2026). Comprehensive DNA methylation analysis of brain and peripheral tissues following chronic risperidone treatment in common marmosets. *Neuroscience Research*, 222, 105000.
12. Okab, A. M., El-Kattan, A. K., Salem, M. F. & Azzam, A. R. (2025). The potential teratogenicity of atypical antipsychotic drugs olanzapine versus risperidone on the fetuses and placentae of the pregnant female albino rattus: Anatomical and histological study. *Tanta Medical Journal*, 53(3), 386-394.
13. Oltmann-Norden, I., Galuska, S.P., Geyer, R., Mühlenhoff, M., Gerardy-Schahn, R., Hildebrandt, H. & Geyer, H. (2008). Impact of the polysialyltransferases ST8SiaII and ST8SiaIV on polysialic acid synthesis during postnatal mouse brain development. *Journal of Biological Chemistry*, 283(21), 14684–14690.
14. Rutishauser, U. (2008). Polysialic acid in the nervous system: the regulation of cell interactions and plasticity. *Current Opinion in Neurobiology*, 18(5), 507–512.
15. Schnaar, R. L., Gerardy-Schahn, R. & Hildebrandt, H. (2014). Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiological Reviews*, 94(2), 461-518.
16. Singh, K. P., Singh, M. K. & Singh, M. (2016). Effects of prenatal exposure to antipsychotic risperidone on developmental neurotoxicity, apoptotic neurodegeneration and neurobehavioral sequelae in rat offspring. *International Journal of Developmental Neuroscience,* 52, 13-23.
17. Singh, K. P. & Singh, M. K. (2017). In utero exposure to atypical antipsychotic drug, risperidone: effects on fetal neurotoxicity in hippocampal region and cognitive impairment in rat offspring. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 75, 35-44.
18. Wang, B. (2009). Sialic acid is an essential nutrient for brain development and cognition. *Annual Review of Nutrition*, 29, 177–222.
19. Wang, B. (2012). Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Advances in Nutrition*, 3(3), 465S–472S.
20. Weinhold, B., Seidenfaden, R., Röckle, I., Mühlenhoff, M., Schertzinger, F., Conzelmann, S., Marth, J.D., Gerardy-Schahn, R. & Hildebrandt, H. (2005). Genetic ablation of polysialic acid causes severe neurodevelopmental defects in mice. *Proceedings of the National Academy of Sciences*, 102(16), 5676– 5681.
21. Yang, X., Li, L., Chai, X. & Liu, J. (2022). The association between ST8SIA2 gene and behavioral phenotypes in children with autism spectrum disorder. *Frontiers in Behavioral Neuroscience*, 16, 929878.
22. Yang, X., Wei, H., Li, J., Li, G., Zhang, Y. & Li, H. (2025). Efficacy of sialic acid supplementation in early life in autism model rats. *Scientific Reports*, 15(1), 8576.
23. Yoo, S. W., Motari, M. G., Susuki, K., Prendergast, J., Mountney, A., Hurtado, A. & Schnaar, R. (2015). Sialylation regulates brain structure and function. *The FASEB Journal*, 29(7), 3040.
24. Zhu, W., Zhou, Y., Guo, L. & Feng, S. (2024). Biological function of sialic acid and sialylation in human health and disease. *Cell Death Discovery*, 10(1), 415.