Differential microRNA expression in the prefrontal cortex of mouse offspring induced by glyphosate exposure during pregnancy and lactation

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Abstract. Glyphosate is the active ingredient in numerous herbicide formulations. The role of glyphosate in neurotoxicity has been reported in human and animal models. However, the detailed mechanism of the role of glyphosate in neuronal development remains unknown. Recently, several studies have reported evidence linking neurodevelopmental disorders (NDDs) with gestational glyphosate exposure. The current group previously identified microRNAs (miRNAs) that are associated with the etiology of NDDs, but their expression levels in the developing brain following glyphosate exposure have not been characterized. In the present study, miRNA expression patterns were evaluated in the prefrontal cortex (PFC) of 28 postnatal day mouse offspring following glyphosate exposure during pregnancy and lactation. An miRNA microarray detected 55 upregulated and 19 downregulated miRNAs in the PFC of mouse offspring, and 20 selected deregulated miRNAs were further evaluated by quantitative polymerase chain reaction (PCR). A total of 11 targets of these selected deregulated miRNAs were analyzed using bioinformatics. Gene Ontology (GO) terms associated with the relevant miRNAs included neurogenesis (GO:0050769), neuron differentiation (GO:0030182) and brain development (GO:0007420). The genes *Cdkn1a*, *Numbl*, *Notch1*, *Fosl1* and *Lef1* are involved in the Wnt and Notch signaling pathways, which are closely associated with neural development. PCR arrays for the mouse Wnt and Notch signaling pathways were used to validate the effects of glyphosate on the expression pattern of genes involved in the Wnt and Notch pathways. *Nr4a2* and *Wnt7b* were downregulated, while *Dkk1*, *Dixdc1*, *Runx1*, *Shh*, *Lef-1 and Axin2* were upregulated in the PFC of mice offspring following glyphosate exposure during pregnancy and lactation. These results indicated abnormalities of the Wnt/β-catenin and Notch pathways. These findings may be of particular interest for understanding the mechanism of glyphosate-induced neurotoxicity, as well as helping to clarify the association between glyphosate and NDDs.

Introduction

Glyphosate is a phosphonomethyl amino acid derivative that is the active ingredient in a number of herbicides (1). Glyphosate-based herbicides are the most highly utilized agrochemicals in the world, particularly on genetically modified plants (2). Extensive evidence demonstrates that the large scale application of glyphosate causes high amounts of residue in water and soil (3). This residue was not previously considered to pose any risks to human health (4). However, recently, glyphosate has been demonstrated to induce embryo-toxic and neurotoxic effects in *in vitro* and *in vivo* studies (5,6). The teratogenic effect of glyphosate on early morphogenesis in embryos raises concerns regarding the clinical phenomenon including, birth defects and behavior disorders, seen in children exposed to glyphosate in the countryside (2,7). Therefore, the safety of glyphosate remains controversial.

In vitro studies have revealed that glyphosate can pass through the blood brain barrier and placental barrier (8). Evidence has also indicated that glyphosate causes widespread apoptotic neurodegeneration, as well as effects on neuronal development and axon growth (9). Results from the Childhood Autism Risks from Genetics and Environment study provide further evidence for an association between neurodevelopmental disorders (NDDs) and gestational organophosphate exposure, particularly glyphosate (10). However, the detailed mechanism of glyphosate neurotoxicity in the developing brain is not well understood. Similarly, while the exact etiology of NDDs remains unknown, novel studies have provided insight into the possible role of environmental and epigenetic factors in the etiology of NDDs (11,12).

MicroRNAs (miRNAs) are small, non-coding RNAs that are recognized as endogenous regulators of post-transcriptional gene expression (13). They are involved in numerous biological processes, including the cell cycle, cell proliferation, the cellular response to stress (14) and the regulation of gene expression (15). Increasing evidence indicates that miRNAs are dysregulated in numerous diseases, including NDDs such as attention deficit hyperactivity disorder (ADHD) and autism

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spectrum disorder (ASD). Multiple circulating miRNAs have been demonstrated to be differentially expressed in child patients with a range of NDDs compared with healthy children (16). Development of the prefrontal cortex (PFC), which is the brain region most affected in ADHD (17), may be associated with regulation of gene expression by miRNAs, which are numerous in the brain (18). A previous study by the current group indicated that miRNA let-7d was elevated in the serum of ADHD subjects (19), as well as in the PFC of spontaneously hypertensive rats, which were used as an ADHD model (20).

Considering the pivotal role of miRNAs in the regulation of gene expression and neurodevelopment dysfunction, a miRNA microarray method was used in the present study to investigate miRNA expression changes in the PFC of mouse offspring following glyphosate exposure during pregnancy and lactation. Furthermore, certain significantly altered miRNAs associated with brain development were selected to perform bioinformatics analysis. This included target gene prediction, Gene Ontology (GO) term enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The aim of the present study was to reveal the potential function of these miRNAs in the PFC of mice offspring, as well as the mechanism of glyphosate neurotoxicity in the developing brain.

Materials and methods

Sample preparation. All procedures were approved by the Institutional Animal Care and Use Committee of Hangzhou Medical College (Hangzhou, China) and conformed to the guidelines for ethical treatment of animals. Experiments required collecting RNA samples from PFCs isolated from postnatal day (PND) 28 male mice, with the day of birth considered as PND 0. The pesticide used in the present study was a commercial formulation marketed in China as Roundup® (Monsanto Company, St. Louis, MO, USA), containing 48 g glyphosate isopropylamine salt per 100 cm^3 of product (equivalent to 35.6% w/v of Glyphosate acid).

All experiments were performed in accordance with the China Council of Animal Care and approved by the Hangzhou Medical College Animal Care Committee. A total of 18 pregnant ICR mice (age, 9-11 weeks; weight 40-50 g; Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were randomly divided into two groups, with each group consisting of 8 pregnant mice. All mice were given free access to food and water and were maintained in a 12 h light/dark cycle in a temperature-controlled breeding room (21°C) with 45-60% humidity and <66±2 dB room noise level. Each group was used for the miRNA microarray assay and the polymerase chain reaction (PCR) array. In the control group, pregnant mice were provided with purified water. In the glyphosate-treated group, pregnant mice were provided with drinking water containing 0.38% glyphosate (1% Roundup®) during pregnancy and lactation, equivalent to 50 mg of glyphosate/kg/day. This dose corresponded with 1/20th of the glyphosate no-observed-adverse-effect level, as described previously (4). The mothers received treatment from embryonic day (E) 14 to PND 7 and were then provided with normal drinking water. The offspring received it indirectly via pregnancy and lactation and weaning occurred on PND 21, they were then provided with normal drinking water. A total

of 8 offspring (4 females and 4 males) from each group were sacrificed on PND 28, the brains were quickly removed, and the PFC was isolated on an ice pad.

Total RNA extraction. Total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and purified with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer'sprotocol. The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) by a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies; Thermo Fisher Scientific, Inc.), the value of A260/A280 provided an estimate of the purity of RNA. When the RNA samples complied with an A260/A280 ratio of 1.8-2.0, the RNA analysis could proceed and RNA integrity was determined by 1.2% agarose gel electrophoresis.

miRNA microarray hybridization. Profiling of miRNA expression was performed using miRCURY LNA™ microRNA Array v19.0, 7th generation, hsa, mmu & rno (Exiqon, Inc., Woburn, MA, USA). The microarray contained 3,100 capture probes, which cover all human, mouse and rat miRNAs annotated in the miRBase (release 19) (mirbase.org/). The total isolated RNA was taken from pooled samples of each group. They were then labeled using the miRCURY™ Power Labeling kit (Exiqon, Inc.) and hybridized to miRCURY LNA™ miRNAs Array v19.0, according to the manufacturer's protocol. Hybridization image scanning was performed using the Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA).

miRNA microarray analysis. Scanned images were imported into GenePix Pro 6.0 software (Molecular Devices, LLC) for grid alignment and data extraction. Replicated miRNAs were averaged, and miRNAs with intensities ≥30 in all samples were selected for calculating a normalization factor. Expressed data were normalized using the median normalization. Then, significant, differentially expressed miRNAs between the two groups were identified using fold change and P-values. Differentially expressed miRNAs between two samples were filtered through fold change. The value of the miRNAs was the foreground intensity of each probe. The normalized ratio of the miRNA's foreground and background and other data were deposited in the NCBI Gene Expression Omnibus and are accessible online (accession no. GSE100079; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100079). Filtering was performed to identify differentially expressed miRNAs with fold changes ≥2.0 and P-values ≤0.05.

Reverse transcription-quantitative PCR (RT-qPCR). Based on previous research (21,22), 20 miRNAs (mmu-miR-322-5p, mmu-miR-376b-5p, mmu-miR-592-5p, mmu-miR-142a-5p, mmu-miR-540-5p, mmu-miR-181a-5p, mmu-miR-183-3p, mmu-miR-470-5p, mmu-miR-19b-3p, mmu-let-7a-5p, mmu-miR-376a-5p, mmu-miR-381-3p, mmu-miR-3475-3p, mmu-miR-34b-5p, mmu-miR-320-3p, mmu-miR-484, mmu-miR-93-5p, mmu-miR-375-5p, mmu-miR-34c-5p, mmu-miR-3098-3p) were selected that are considered to be relevant to brain development. To validate the accuracy of the miRNA microarray data, the RNAs were polyadenylated

through a poly (A) polymerase reaction using the MystiCq® microRNA cDNA Synthesis mix (Sigma-Aldrich; Merck, KgaA, Darmstadt, Germany) and then reverse transcribed into cDNA by ReadyScript™ reverse transcriptase and oligo-dT adapter primers (Sigma-Aldrich; Merck KGaA). Individual miRNAs were quantified using SYBR Green qPCR ReadyMix™. Reverse primers were MystiCq® Universal PCR Primer (Sigma-Aldrich; Merck KGaA) and the forward primers were the specific MystiCq miRNA qPCR assay primers, mmu-miR-34b-5p, 5'-AGGCAG TGTAATTAGCTGATTGT-3'; mmu-miR-322-5p, 5'-CAG CAGCAATTCATGTTTTGGA-3'; mmu-miR-376b-5p, 5'-GTGGATATTCCTTCTATGGTTA-3' purchased from Sigma-Aldrich (Merck KGaA) and Wcgene Biotechnology Corporation (Shanghai, China) synthetic primers as follows: mmu-let-7a-5p, 5'-TGAGGTAGTAGGTTGTATAGTT-3'; mmu-miR-19b-3p, 5'-TGTGCAAATCCATGCAAAACT GA-3'; mmu-miR-34c-5p, 5'-AGGCAGTGTAGTTAGCTG ATTGC-3'; mmu-miR-93-5p, 5'-CAAAGTGCTGTTCGT GCAGGTAG-3'; mmu-miR-142a-5p, 5'-CATAAAGTAGAA AGCACTACT-3'; mmu-miR-181a-5p, 5'-AACATTCAACGC TGTCGGTGAGT-3'; mmu-miR-183-3p, 5'-GTGAATTAC CGAAGGGCCATAA-3'; mmu-miR-320-3p, 5'-AAAAGC TGGGTTGAGAGGGCGA-3'; mmu-miR-375-5p, 5'-GCG ACGAGCCCCTCGCACAAAC-3'; mmu-miR-376a-5p, 5'-GGTAGATTCTCCTTCTATGAGT-3'; mmu-miR-381-3p, 5'-TATACAAGGGCAAGCTCTCTGT-3'; mmu-miR-470-5p, 5'-TTCTTGGACTGGCACTGGTGAGT-3'; mmu-miR-484, 5'-TCAGGCTCAGTCCCCTCCCGAT-3'; mmu-miR-540-5p, 5'-CAAGGGTCACCCTCTGACTCT GT-3'; mmu-miR-592-5p, 5'-ATTGTGTCAATATGCGAT GATGT-3'; mmu-miR-3098-3p, 5'-TTCTGCTGCCTGCCT TTAGGA-3'; mmu-miR-3475-3p, 5'-TCTGGAGGCACAT GGTTTGAA-3'; U1, 5'-CTTACCTGGCAGGGGAGATA-3'. The protocol of miRNA RT-qPCR array analysis was as previously described (23) and as specified on the Wcgene website (wcgene.com). The mouse U1 small nuclear rna gene were used to normalize expression. The $2^{-\Delta\Delta Cq}$ method (24) was used to determine differences in expression level between the glyphosate and the control group. P<0.05 was considered to indicate a statistically significant difference. The miRNA RT-qPCR array experiments were conducted at Wcgene Biotechnology Corporation (Shanghai, China).

Target gene prediction and bioinformatics analysis. Based on existing research and the RT-qPCR results the potential function of 11 miRNAs was explored (mmu-miR-142a-5p, mmu-miR-181a-5p, mmu-miR-19b-3p, mmu-miR-322-5p, mmu-miR-470-5p, mmu-miR-540-5p, mmu-miR-320-3p, mmu-miR-324-5p, mmu-miR-34b-5p, mmu-miR-484, mmu-miR-93-5p), TargetScan (targetscan. org/vert_71/), miRanda (microrna.org/) and PicTar (pictar. org/) software was used to predict target mRNAs. Then, miRNA function was explored further using the GO database (geneontology.org/) as an analysis tool for target genes of the predicted miRNAs. The pathways of the miRNA targets were then explored using the KEGG functional annotation analysis (genome.jp/kegg/). The results indicated that a large number of the target genes were involved in the Wnt and Notch signaling pathways.

Wnt and Notch signaling pathway PCR array. On the basis of the KEGG functional annotation analysis, the mouse Wnt and Notch signaling pathway RT² profiler™ PCR array plates (Wcgene Biotechnology Corporation) were used, which contained 84 key genes involved in the Wnt pathway and 26 key genes involved in the Notch pathway. The reaction was performed according to the manufacturer's protocol. Real-time PCR was performed using SYBR-Green Master mix (Qiagen GmbH) and processed in the GeneAmp 5700 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific). The data was exported to and analyzed by Wcgene Biotechnology Corporation.

Statistical analysis. SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. Data are presented as the mean ± standard error of the mean. Aspin-Welch's t-test was applied to identify genes and miRNAs that demonstrated a significant differential expression upon exposure to glyphosate. P<0.05 was considered to indicate a statistically significant difference.

Results

miRNA expression analysis. The results of the miRCURY LNA™ miRNA microarray assay identified 74 miRNAs that were differentially expressed in the two groups with P<0.01 and signal values >500. In the glyphosate group, 55 miRNAs were upregulated and 19 miRNAs were downregulated compared with the control group (Table I and Fig. 1).

Single validation of miRNA by qPCR. To confirm the accuracy of the miRNA microarray, 20 differentially expressed, representative miRNAs, which were considered relevant to brain development were selected based on previously published studies and used for qPCR, (25,26). The results demonstated that the expression levels of 12 miRNAs, including miR-322-5p and miR-19b-3p, were upregulated in the glyphosate group. The expression levels of 7 miRNAs, including miR-34b-5p and miR-320-3p, were downregulated (Fig. 2). These results were in accordance with the miRNA microarray data.

miRNA predicted targets and relevant bioinformatic analysis. To better recognize the role of miRNA expression in biosynthesis, three software packages (TargetScan, PicTar and miRanda) were used for target prediction, GO enrichment analysis and KEGG annotation analysis. A total of 11 putative target genes from miRNAs in the two groups were identified (Table II). These genes are associated with neuronal development, suggesting that miRNAs are involved in regulating the pathological processes of glyphosate-induced neurotoxicity through the targeting of these genes.

These putative miRNA targets were used as inputs to perform the GO functional enrichment. The 26 GO terms identified to be associated with neuronal development are shown in Table III, including neurogenesis (GO:0050769), neuron differentiation (GO:0030182) and brain development (GO:0007420). These results demonstrate that the target genes of miRNAs in mouse PFC may be involved in a wide variety of pathophysiological processes following glyphosate exposure.

Table I. Differentially expressed miRNAs in the prefrontal cortex between the glyphosate and control groups analyzed by microarray at a signal value >500 and P<0.01.

A, Upregulated miRNAs in the glyphosate group

miRNA	Control group	Glyphosate group	Fold-change
mmu-miR-711	68	82	2.0276
mmu-miR-27b-3p	126.5	274.5	3.55381
mmu-miR-381-3p	65.5	111	4.75526
mmu-miR-425-5p	69	89.5	2.20282
mmu-miR-872-5p	79.5	99	2.13158
mmu-miR-592-5p	62.5	96	10.3658
mmu-miR-434-3p	891.5	1,777	2.12625
mmu-miR-181a-5p	223	985.5	5.69772
mmu-miR-130a-3p	354	772.5	2.49714
mmu-miR-30a-5p	457	1,319.5	3.24703
mmu-miR-27a-3p	80.5	100	2.45888
mmu-miR-374b-5p	65	87	2.58045
mmu-miR-181c-5p	85.5	170	4.27632
mmu-miR-136-5p	466	1,436.5	3.49801
mmu-miR-29b-3p	736	2512	3.70614
mmu-miR-204-3p	76	98.5	2.07237
mmu-miR-7b-5p	70.5	94.5	3.6098
mmu-miR-128-3p	2263	5,590	2.56915
mmu-miR-1958	78.5	100	2.14266
mmu-miR-466m-5p	64	73.5	2.2359
mmu-miR-126a-5p	158.5	466	4.10526
mmu-miR-98-5p	319	1,104	4.10139
mmu-miR-540-5p	64	94.5	6.7352
mmu-miR-376b-5p	58.5	167	16.8316
mmu-miR-9-5p	6,291.5	12,794	2.09456
mmu-miR-129-2-3p	292.5	694.5	2.66427
mmu-miR-344b-3p	83	150	2.93836
mmu-miR-410-3p	166	364.5	2.93628
mmu-miR-470-5p	64.5	72	5.21053
mmu-miR-204-5p	81	113	2.58348
mmu-miR-219a-5p	290	741.5	2.97483
mmu-miR-29c-5p	68.5	103	3.48947
mmu-miR-19b-3p	86	220	5.00505
mmu-miR-183-3p	62	78	5.57143
mmu-miR-677-5p	77	150	3.72895
mmu-miR-19a-3p	128.5	233.5	2.6063
mmu-miR-1971	78	110	2.82237
mmu-miR-142a-3p	95	199.5	4.26947
mmu-miR-148a-3p	66	107.5	3.90662
mmu-miR-142a-5p	59.5	99.5	9.88995
mmu-miR-383-5p	76.5	101.5	3.37218
mmu-miR-693-5p	64	84	2.25219
mmu-let-7a-5p	171.5	590.5	4.88616
mmu-miR-21a-5p	173	396	3.18842
mmu-miR-488-3p	67	95	3.68058
mmu-miR-181b-5p	92	177.5	3.88215
mmu-miR-145a-5p	73	98.5	3.3676

Table I. Continued.

A, Upregulated miRNAs in the glyphosate group

miRNA	Control group	Glyphosate group	Fold-change
mmu-miR-376a-5p	57.5	78	4.81579
mmu-miR-691	962	2,353.5	2.62274
mmu-miR-3068-3p	232	413	2.14251
mmu-miR-322-5p	67.5	159.5	23.8105
mmu-miR-30b-3p	101	124	2.22819
mmu-miR-365-3p	132.5	264	3.29501
mmu-miR-28a-5p	75.5	106	3.66541
mmu-miR-380-3p	89	116.5	3.35885

B, Downregulated miRNAs in the glyphosate group

miRNA	Control group	Glyphosate group	Fold-change
mmu-miR-744-5p	239	131.5	0.47581
mmu-miR-34c-5p	176.5	89.5	0.3677
mmu-miR-486a-5p	85.5	57	0.41863
mmu-miR-132-5p	1,478.5	698.5	0.47413
mmu-miR-500-3p	90.5	62	0.49263
mmu-miR-484	80	53	0.33676
mmu-miR-467b-5p	86	57.5	0.40789
mmu-miR-3098-3p	364.5	166	0.40067
mmu-miR-342-3p	187	100	0.47039
mmu-miR-34b-5p	240.5	86	0.26139
mmu-miR-3072-3p	194	113	0.49684
mmu-miR-320-3p	216.5	93.5	0.33023
mmu-miR-195a-5p	1,096.5	487.5	0.43865
mmu-miR-375-5p	86	57	0.36154
mmu-miR-133b-3p	111	71	0.49221
mmu-miR-93-5p	170	84	0.34644
mmu-miR-1839-3p	379	193	0.48888
mmu-miR-3475-3p	96	48	0.08435
mmu-miR-324-5p	155	79.5	0.40297

miRNA, microRNA.

KEGG functional annotation analysis. To evaluate the biological pathways involved in glyphosate-induced neurotoxicity, KEGG pathway annotation of the miRNA targets (Table IV) was performed. KEGG pathway analysis revealed certain biological processes that may be involved in glyphosate-induced neurotoxicity, and provided useful insights for further investigation of the role of targeted miRNAs in the neurotoxic effects of glyphosate on the developing brain.

The pathway that was most enriched was Ras, followed by mitogen-activated protein kinase and the cell cycle. Thus, KEGG analysis revealed certain biological processes that may be involved in glyphosate-induced neurotoxicity. In addition, it
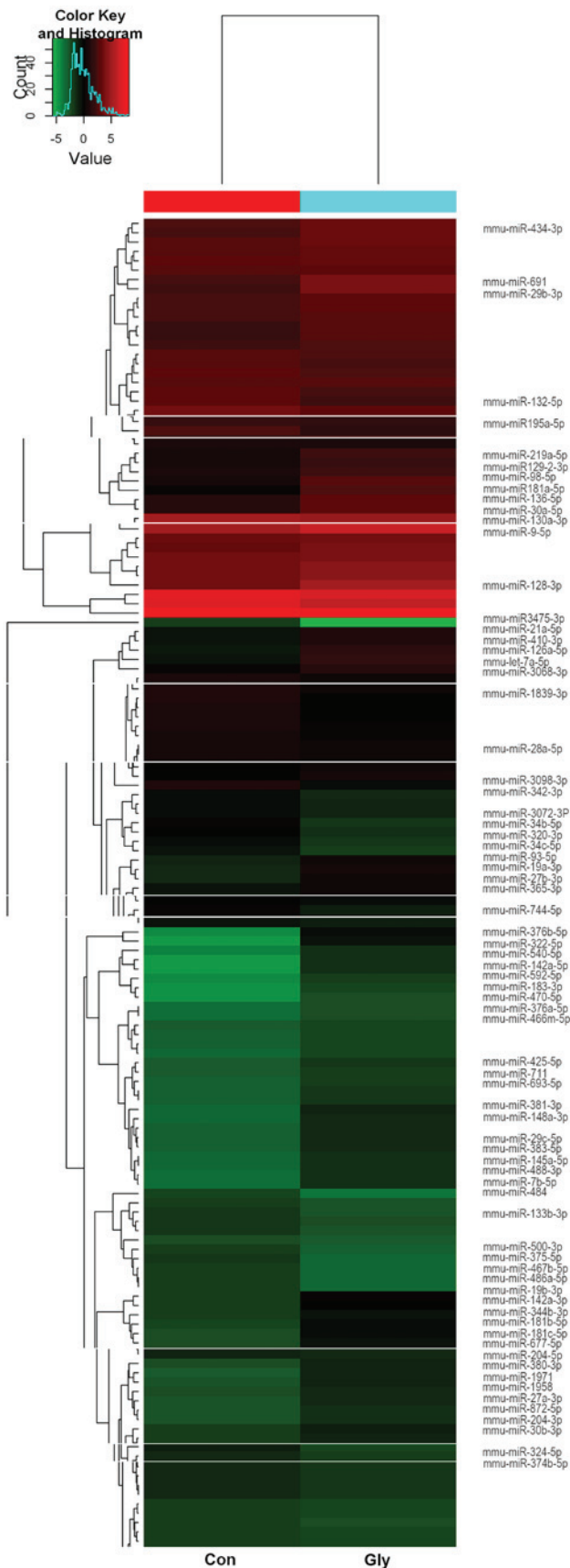
Figure 1. Heat map for the analysis of differentially-expressed miRNA. Each column represents a sample; each row represents an miRNA. Red color indicates upregulated miRNAs; green color indicates downregulated miRNAs in the glyphosate group. The heat map for 74/569 detected miRNAs (13.01%) that were differentially expressed, with P<0.01 and signal value >500, is depicted. Con, control group; Gly, glyphosate-treated group; miRNA, microRNA.

was identified that the genes *Cdkn1a*, *Numbl*, *Notch1* and *Lef1* were involved in the Wnt and Notch signaling pathways. The Wnt signaling pathway serves a key role in cell cycle progression and differentiation during dopaminergic neurogenesis in the midbrain, which is thought to be associated with normal motor behavior (27). Notch signaling also appears to regulate dopaminergic neuronal development (28). Therefore, the two pathways may potentially contribute to neurodegenerative diseases and NDDs.

To validate the effects of the Wnt and Notch signaling pathway, the mouse Wnt and Notch signaling pathway RT² profiler™ PCR array was used in the present study. Fig. 3 presents the differential expression profile of the Wnt and Notch signaling pathways in the two groups. Ten transcripts, including *Nr4a2* and *Wnt7b*, were downregulated by glyphosate exposure, while 25 transcripts, including *Dkk1*, *Dixdc1*, *Runx1*, *Shh*, *Lef1* and *Axin2*, were upregulated by glyphosate exposure (Fig. 3).

Discussion

Glyphosate neurotoxicity is known to be associated with glutamate excitotoxicity and oxidative stress (29). Acute glyphosate exposure in adult rats causes behavioral changes as well as alterations in dopaminergic markers (1). Previous epidemiological studies have identified that organophosphate pesticide exposure may have deleterious effects on neurodevelopment, including an increased risk of ADHD in Taiwanese children (30) and young Mexican-Americans (31). Shelton *et al* (10) also reported evidence linking NDDs with gestational organophosphate exposure, particularly with glyphosate. Preliminary experiments by the current authors indicated that glyphosate induces neural behavior abnormalities in mice offspring at PND 28 and PND 42 (data not shown). However, the mechanism for these abnormalities is not clear.

Growing evidence indicates that the pathogenesis of NDDs is linked to the interaction between genetics and the environment (32). It has also been demonstrated that exposure to environmental risk factors at an early age could induce NDDs, a process mediated by epigenetics (33). Epigenetics refers to heritable changes in gene function that do not change the nucleotide sequence or alter the gene products' function, but do affect the gene's spatiotemporal expression (34). The genome is known to be shaped throughout the life cycle by environmental factors (35).

Recently, miRNAs have been recognized as critical regulators of neuronal activity and function (36). They can also silence genes by translational repression or mRNA degradation (37), and dramatically alter certain downstream signaling pathways (38). A previous study by the current group also identified that several miRNAs, including miRNA let-7d, were dysregulated in ADHD subjects (19) and in a rat model of ADHD (20).

Based on these previous findings, it was hypothesized that miRNA expression is involved in glyphosate neurotoxicity. To test this, miRNA expression profiles in the PFC of mouse offspring exposed to glyphosate during pregnancy and lactation were analyzed using the miRNA microarray method. In the present study, 0.38% glyphosate was administered (half the dose regularly used), since a similar dose was used in a

Table II. Predicted miRNA target genes associated with neuronal or brain development.

| miRNA	Count	Target genes
mmu-miR-142a-5p	4	Bnip2, Mnat1, Otx2, Setd2
mmu-miR-181a-5p	11	Ddit4, Dock7, Fos, Grm5, Inpp5e, Phox2b, Plcl2, Sema4c, Six2, Slc9a6, Tgif2
mmu-miR-19b-3p	23	Cntfr, Dlc1, Fkbp1b, Fosl1, Kcnc4, Kif3a, Neurod1, Palb2, Pla2g10, Ppara, Ptprg, Raf1, Pon2, Nr3c2, Rap1a, Rfx4, Scn1b, Slc9a6, Sphk2, Tgif1, Tnfrsf12a, Usp33, Wdr1
mmu-miR-322-5p	17	Adrb2, Atp7a, Atxn2, Cnih2, Dll1, Epha7, Grm7, Katnb1, Kif1b, Lrp6, Omg, Ptprm, Raf1, Rnf10, Sncg, Stxbp3a, Tgif2
mmu-miR-470-5p	3	Bcl11a, Isl1, Tgif1
mmu-miR-540-5p	2	Efna4, Ncoa1
mmu-miR-320-3p	9	Adam10, Kcnip4, Mapk8ip3, Myo10, Pik3ca, Plk3, Prkg1, Ulk1, Vim
mmu-miR-324-5p	3	Pbx1, Slitrk4, Unc5c
mmu-miR-34b-5p	10	Abr, Chl1, Cntnap1, Crhr1, Foxg1, Jag1, Lef1, Nrn1, Numbl, Notch1
mmu-miR-484	6	Crtc2, Dpysl2, Kcnj11, Grik4, Sirt2, Strn
mmu-miR-93-5p	17	Arhgef7, Cdkn1a, E2f1, Epha5, Foxb1, Kif5a, Lhx8, Map3k5, Myh14, Neurog2, Ski, Sorl1, Tnfrsf21, Vegfa, Vldlr, Wee1, Wfs1

miRNA, microRNA.
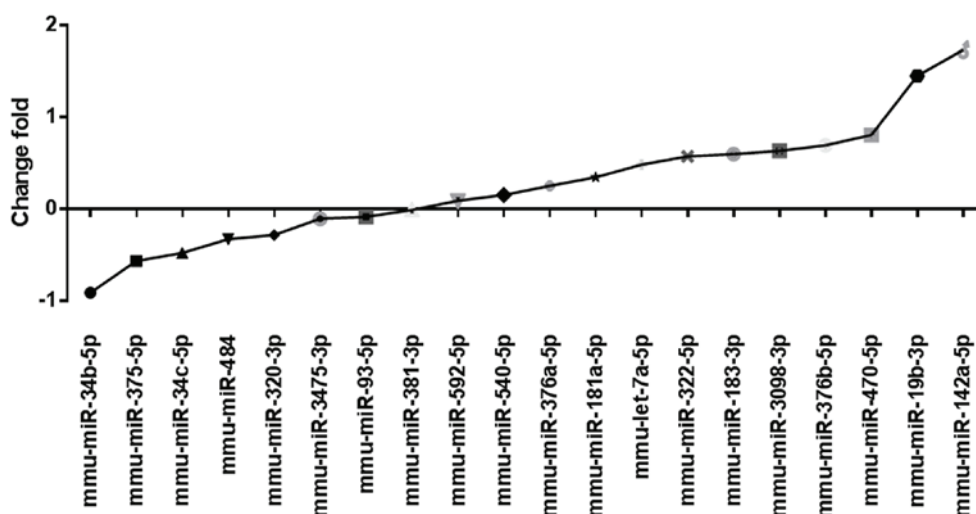
Figure 2. miRNA expression in the prefrontal cortex. A representative sample of 20 differentially-expressed miRNAs were selected and used for quantitative polymerase chain reaction. miRNA, microRNA.

previous study (5). The miRNA microarray is an efficient technique to analyze alternated miRNA expression profiles. The results indicated 53 differentially expressed miRNAs, including 11 miRNAs (e.g., miR-34b-5p, miR-19b-3p, miR-324-5p, miR-320-3p and miR-322-5p) known to be involved in brain development and the pathogenesis of NDDs. This indicated that dysregulated expression of miRNAs may be involved in the mechanism of glyphosate-induced neurotoxicity. Previous research suggests that miR-34b-5p mediates hippocampal astrocyte apoptosis (39) and also affects target genes, including *Numbl* and *Notch1*, which are involved in the Notch signaling pathway (40). The 3'-untranslated regions of β-catenin and Lef-1, which are involved in the Wnt signaling pathway, contain miR-34 binding sites and are sensitive to miR-34b-dependent regulation (41). In addition,

the Tcf/Lef transcription factor was identified to be closely associated with the functionality of the miR-34 family (42). The Myc/FOXO3a/mir-34b feedback inhibition loop has also been demonstrated to be involved in regulating cellular proliferation in mammals (43). Furthermore, miR-324-5p is reportedto act as a control in neuronal progenitor cells (44) and contribute to the shift from self-renewal to neuronal differentiation (45).

The present study also identified several potential miRNA target genes and their possible neurological pathways using GO term enrichment and KEGG pathway analysis. The involvement of predicted targets of miRNAs in these biological processes was also determined. The majority of the predicted genes were enriched in neurogenesis regulation, neuron differentiation and brain development, indicating that these targets

Table III. GO functional enrichment for target genes involved in neuronal or brain development.

GO term	Count	Target genes
GO:0048812, neuron projection morphogenesis	18	IGF1R, NTN1, NUMBL, ULK1, SLITRK4, NRN1, NEUROG2, CHL1, EPHA5, KIF5A, UNC5C, MAPK8IP3, FOXB1, FOXG1, VEGFA, VIM, WEE1, CNTNAP1
GO:0048699, generation of neurons	31	NEUROG2, CHL1, NTN1, PRKG1, FOXG1, VEGFA, NUMBL, IGF1R, ULK1, SLITRK4, NRN1, EPHA5, KIF5A, UNC5C, MAPK8IP3, FOXB1, SKI, FGFR1, VIM, STRN, LEF1, LHX8, CNTNAP1, JAG1, DPYSL2, PBX1, MYCN, TNFRSF21, WEE1, SIRT2, VLDLR
GO:0031175, neuron projection development	22	IGF1R, NTN1, NUMBL, ULK1, SLITRK4, NRN1, NEUROG2, CHL1, EPHA5, KIF5A, UNC5C, MAPK8IP3, FOXB1, FGFR1, VIM, FOXG1, PRKG1, STRN, WEE1, CNTNAP1, VEGFA, VLDLR
GO:0030182, neuron differentiation	27	IGF1R, NTN1, NUMBL, ULK1, SLITRK4, NRN1, NEUROG2, CHL1, EPHA5, KIF5A, UNC5C, MAPK8IP3, WEE1, VLDLR, FOXB1, FGFR1, VIM, FOXG1, PRKG1, STRN, LEF1, LHX8, CNTNAP1, VEGFA, JAG1, DPYSL2, PBX1
GO:0097485, neuron projection guidance	9	NEUROG2, CHL1, EPHA5, KIF5A, NTN1, UNC5C, MAPK8IP3, FOXG1, VEGFA
GO:0007420, brain development	16	LEF1, NUMBL, ULK1, EPHA5, FOXB1, FGFR1, SKI, FOXG1, LHX8, NEUROG2, E2F1, PRKG1, MAPK8IP3, ABR, IGF1R, UNC5C
GO:0007417, central nervous system development	19	ABR, FGFR1, IGF1R, UNC5C, VIM, LEF1, NUMBL, ULK1, EPHA5, FOXB1, SKI, FOXG1, LHX8, NEUROG2, E2F1, PRKG1, MAPK8IP3, MYCN, TNFRSF21
GO:0030900, forebrain development	12	LEF1, NUMBL, EPHA5, FOXB1, FGFR1, SKI, FOXG1, VIM, NTN1, DPYSL2, JAG1, PBX1
GO:0036445, neuronal stem cell division	3	FGFR1, NUMBL, LEF1
GO:0050767, regulation of neurogenesis	17	FOXG1, VEGFA, SKI, FGFR1, VIM, NTN1, DPYSL2, JAG1, PBX1, NEUROG2, MAPK8IP3, ULK1, MYCN, TNFRSF21, NUMBL, SIRT2, VLDLR
GO:0007405, neuroblast proliferation	5	FOXG1, VEGFA, FGFR1, NUMBL, LEF1
GO:0051960, regulation of nervous system development	18	FOXG1, VEGFA, SKI, FGFR1, VIM, NTN1, SIRT2, TNFRSF21, DPYSL2, JAG1, PBX1, NEUROG2, MAPK8IP3, ULK1, MYCN, NUMBL, EPHA5, VLDLR
GO:0045666, positive regulation of neuron differentiation	8	BNIP2, FGFR1, IL6, NCOA1, NEUROD1, PHOX2B, TGIF1, TGIF2
GO:0021872, forebrain generation of neurons	5	FGFR1, NUMBL, FOXG1, LEF1, LHX8
GO:0045664, regulation of neuron differentiation	13	FGFR1, VIM, NTN1, FOXG1, JAG1, PBX1, NEUROG2, MAPK8IP3, ULK1, VEGFA, NUMBL, VLDLR, DPYSL2
GO:0038179, neurotrophin signaling pathway	4	RAP1A, RAF1, DDIT4, SLC9A6
GO:0021954, central nervous system neuron development	4	FGFR1, FOXG1, LHX8, NEUROG2
GO:0097485, neuron projection guidance	9	B3GNT1, EFNA4, USP33, OTX2, SCN1B, PLA2G10, EPHA7, ISL1, PTPRM
GO:0021953, central nervous system neuron differentiation	6	ULK1, FGFR1, FOXG1, LEF1, LHX8, NEUROG2
GO:0001764, neuron migration	5	FOXG1, NEUROG2, CHL1, NTN1, PRKG1
GO:0050808, synapse organization	8	FGFR2, AFG3L2, LRRC4, C1QL3, THBS2, LRRTM2, SNCG, SLC9A6
GO:0002052, positive regulation of neuroblast proliferation	2	FOXG1, VEGFA
GO:0021885, forebrain cell migration	3	FGFR1, FOXG1, FOXB1
GO:0045665, negative regulation of neuron differentiation	3	FOXG1, JAG1, PBX1
GO:0050769, positive regulation of neurogenesis	5	FOXG1, VEGFA, NTN1, NUMBL, VIM
GO:0021846, cell proliferation in forebrain	2	FGFR1, NUMBL

GO, Gene Ontology.

Table IV. KEGG pathway annotations for miRNA targets.

Pathway ID	Definition	P-value	Count	Genes
4014	Ras signaling pathway	1.03134×10^{-6}	20	EFNA4, FASL, FGF15, FGFR1, FGFR2, GNG5, IGF1R, INSR, MAP2K1, PAK4, PAK6, PLA2G10, PLA2G3, RAF1, RALA, RAP1A, RASSF1, SHOC2, SOS2, TBK1
4010	MAPK signaling pathway	0.00018	17	CASP3, FASL, FGF15, FGFR1, FGFR2, FLNC, FOS, GNA12, IL1A, MAP2K1, MAP3K1, MAP4K3, MKNK1, RAF1, RAP1A, SOS2, TGFBR1
4110	Cell cycle	0.00055	8	CDKN1A, E2F1, E2F5, MCM3, RBL1, RBL2, WEE1, YWHAQ
4068	FoxO signaling pathway	0.00087	8	CCNG2, CDKN1A, FOXG1, IGF1R, PIK3CA, PLK3, RBL2, SLC2A4
4722	Neurotrophin signaling pathway	0.00337	9	ARHGDIA, FASL, MAP2K1, MAP3K1, NFKBIA, PRKCD, RAF1, RAP1A, SOS2
4151	PI3K-Akt signaling pathway	0.01463	11	CDKN1A, CRTC2, FGFR1, GNB5, IGF1R, LAMB3, OSM, PIK3CA, RBL2, VEGFA, YWHAQ
4360	Axon guidance	0.01524	8	EFNA4, EPHA7, LIMK2, LRRC4, PAK4, PAK6, SEMA4C, SEMA6D
5214	Glioma	0.01612	4	CDKN1A, E2F1, IGF1R, PIK3CA
4730	Long-term depression	0.01771	5	GNA12, IGF1R, MAP2K1, PPP2R1A, RAF1
4066	HIF-1 signaling pathway	0.02363	5	CDKN1A, IGF1R, LDHA, PIK3CA, VEGFA
4520	Adherens junction	0.02465	4	FGFR1, IGF1R, LEF1, SSX2IP
4024	cAMP signaling pathway	0.02635	10	ADRB2 FOS HCN2 LIPE MAP2K1 NFKBIA PDE3A PPARA RAF1 RAP1A

KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen activated protein kinase; PI3K, phosphoinositide-3-kinase; HIF, hypoxia inducible factor; cAMP, cyclic adenosine monophosphate.

may be important in the mechanism of glyphosate-induced neurotoxicity.

It is widely acknowledged that miRNAs can regulate target mRNAs and affect the activities of cells and tissues, implicating the importance of target gene prediction (46). For example, among miR-34b-5p target genes, *Chl1*, *Fgfr1*, *Foxg1* and *Nrn1* were reported to be involved in certain neurological disorders, including autism, schizophrenia and bipolar disorder (47-49).

A large number of the target genes identified in the present study are known to be involved in multiple pathways, including the Wnt and Notch signaling pathways. Certain target genes, including *Numbl* and *Adam10*, were detected by bioinformatics methods. It is well established that the expression of *Numbl* and *Adam10* in the brain could regulate neural development, synaptogenesis and neural stem cells via the Notch and Wnt/β-catenin signaling pathways (50-52). These two pathways are important for maintaining and protecting neural connections in the developing brain (53,54).

In the current study, a PCR array was used to validate the effects of the Wnt and Notch signaling pathways in this study and to help elucidate the mechanism of glyphosate-induced neurotoxicity. A number of targets were identified within the Notch and Wnt signaling pathways that are known to be closely associated with neurogenesis and behavioral deficits in mice. The results of the PCR array indicated that several genes were affected by glyphosate exposure. *Nr4a2* is a transcription

factor that is highly expressed in the brain and is crucial for the formation or maintenance of dopaminergic neurons in the central nervous system (CNS) (55). *Wnt7b*, known to be involved in Wnt signaling, may affect early neural progenitor differentiation by regulating the expression of pro-neural transcription factors, including the T-domain transcription factors Tbr1 and Tbr2 (56). *Wnt7b* is also involved in Celsr3-Fzd3 signaling, in which it regulates the timing of neural progenitor cell fate via Notch activation (57). In the present study, the miRNA expression levels of *Nr4a2* and *Wnt7b* were decreased in the PFC of mouse offspring following glyphosate exposure. These gene expression changes may be associated with abnormal neural differentiation.

Dkk1, a secreted inhibitor of Wnt/β-catenin signaling, is required for proper neural development and induces the rapid disassembly of synapses (58). Its dysfunction contributes to synaptic degeneration during early stages of neurodegenerative diseases (58), including Alzheimer's disease, Parkinson's disease (PD) and epilepsy (59), as well as impaired motor behavior (60). However, the role of *Dkk1* in glyphosate-induced neurotoxicity in mouse offspring is completely unknown. *Dixdc1*, known as a positive regulator of the Wnt signaling pathway, was recently reported to play a role in neurogenesis (61) and the development of cortical dendrites and synapses (62). It is also essential for neural progenitor proliferation and migration during embryonic
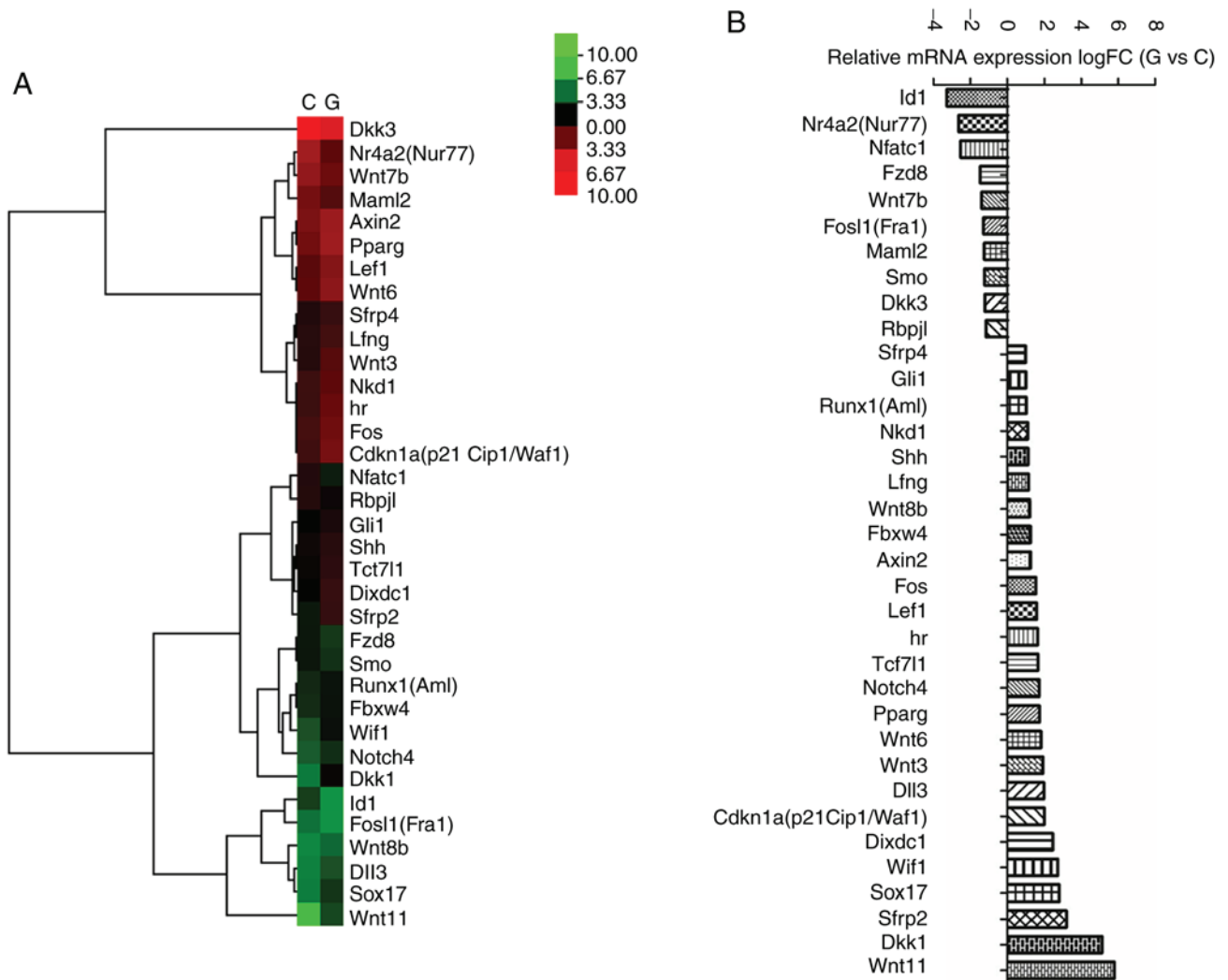
Figure 3. Differentially expressed genes involved in the Wnt and Notch pathways. The mouse Wnt and Notch signaling pathway RT2 profiler™ PCR array plates were used, which contained 84 key genes involved in the Wnt pathway and 26 key genes involved in the Notch pathway. (A) Each column represents a sample; each row represents a gene. Red color indicates upregulated genes; green color indicates downregulated genes in the glyphosate group. Genes were considered differentially expressed at fold change ≤0.5 and ≥2. (B) Relative mRNA expression in the two groups. PCR, polymerase chain reaction; C, control group; G, glyphosate-treated group.

cortical development (63). Rare missense variants in *Dixdc1* have been identified in ASD patient cohorts via genetic sequencing (63), indicating that *Dixdc1* may be associated with morphological defects associated with NDDs. *Runx1* and *Shh*, which are involved in the Notch signaling pathway, have been reported to serve critical functions in the developing brain (64-66). For example, *Runx1* may be upregulated after injury to promote neuronal differentiation, in order to facilitate repair of the CNS. Therefore, upregulated *Runx* expression is associated with brain injury and disease (64). *Shh* influences neurogenesis and neural patterning during development of the CNS (67). Dysregulated *Shh* signaling may lead to neurological disorders such as ASD, depression and PD, as well as locomotor deficits (68). *Lef-1*, a direct Wnt/β-catenin signaling target (69), is a crucial determinant of neurogenesis and of neural progenitor fates in the brain (70,71). It also regulates β-catenin-dependent transcription of neural progenitor genes in the neocortex (72). *Axin2*, a classic Wnt target gene and β-catenin destruction complex scaffolding protein, has been reported to control the switch of intermediate progenitors

from a proliferative to a differentiated status in the developing cerebral cortex (73). Enhancing *Axin* expression in neuronal progenitors leads to an enlarged neocortex and autistic-like behaviors (74). In the present study, the mRNA expression levels of *Dkk1*, *Dixdc1*, *Runx1*, *Shh*, *Lef-1* and *Axin2* were elevated in the PFC of mouse offspring following glyphosate exposure. These gene expression changes may be associated with neural behavior abnormalities (including anxiety- and depression-like behaviors and decreased social interaction behaviors; data not shown) observed in the mouse offspring. Therefore, these genes maybe good targets for the prevention of glyphosate-induced neurotoxicity in the developing brain.

The present study was limited by utilizing a single treatment dose to examine miRNA expression changes in mouse offspring caused by glyphosate exposure during pregnancy and lactation, and the simple miRNA prediction using bioinformatics was not sufficient. Future studies will utilize multiple treatment doses to better illustrate the results and verify the authenticity and accuracy of the predicted target genes by luciferase gene assay. These additional measures will increase

our understanding of the mechanism of glyphosate-induced neurotoxicity and will help clarify the association between glyphosate and NDDs.

In conclusion, the current study focused on the changes in miRNA expression in the PFC of mouse offspring that were exposed to glyphosate during pregnancy and lactation. An miRNA microarray and PCR array were performed to examine the effects of glyphosate on the brain. The current findings provide a basis for identifying the mechanism of action of glyphosate-induced neurotoxicity in the developing brain, and for clarifying the association between glyphosate and NDDs.

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