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

HUA JI1, LINHAO XU1, ZHENG WANG1, XINLI FAN1 and LIHUI WU2

Departments of 1Basic Medicine and 2Clinical Medicine, Hangzhou Medical College, Hangzhou, Zhejiang 310053, P.R. China

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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: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, Runxl, 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.
They were then provided with normal drinking water. A total pregnancy and lactation and weaning occurred on PND 21, normal drinking water. The offspring received it indirectly via embryonic day (E) 14 to PND 7 and were then provided with 1/20th of the glyphosate no-observed-adverse-effect level, as described previously (4). The mothers received treatment from 1% Roundup (Monsanto Company, St. Louis, MO, USA), containing 48 g glyphosate isopropylamine salt per 100 cm² (Mirasan Company, St. Louis, MO, USA) and purified with an RNase Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. 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 miRNAs 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-34c-5p, mmu-miR-484, mmu-miR-19b-3p, mmu-miR-21a-5p, mmu-miR-142a-5p, mmu-miR-540-5p, mmu-miR-183-3p, mmu-miR-181a-5p, mmu-miR-470-5p, mmu-miR-19b-3p, mmu-miR-476a-5p, mmu-miR-3475-3p, mmu-miR-34b-5p, mmu-miR-320-3p, 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 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 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 cm² 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 <6±2 dRm 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 RNase Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. 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.

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through a poly (A) polymerase reaction using the MystiCq® microRNA cDNA Synthesis mix (Sigma-Aldrich; Merck, KgA, Darmstadt, Germany) and then reverse transcribed into cDNA by ReadyScript™ reverse transcriptase and oligo-dT adapter primers (Sigma-Aldrich; Merck KgA). Individual miRNAs were quantified using SYBR Green qPCR ReadyMix™. Reverse primers were MystiCq® Universal PCR Primer (Sigma-Aldrich; Merck KgA) and the forward primers were the specific MystiCq miRNA qPCR assay primers, mmu-miR-34b-5p, 5'-AGGCCAG TGAATTAGCTGAT TTG-3' ; mmu-miR-322-5p, 5'-CAG CAGAA TAT CAGGTT GTGTTGGA-3' ; mmu-miR-376b-5p, 5'-GTGGATATCC TCTCTACTGGTAA-3' purchased from Sigma-Aldrich (Merck KgA) and Wegenie Biotechnology Corporation (Shanghai, China) synthetic primers as follows: mmu-let-7a-5p, 5'-TGAAGTAGTGTAGG TTATAGTT-3'; mmu-miR-19b-3p, 5'-TGTGCA AACATCCATGCAAA ACTGA-3'; mmu-miR-34c-5p, 5'-AGGCCAGGTGAT TACGGT-3'; mmu-miR-93-5p, 5'-CAAATGTCGTTGTC GTGCAGTAG-3' ; mmu-miR-142a-5p, 5'-CATAAAGTGAAG ACGACTACT-3'; mmu-miR-181a-5p, 5'-AACATTCAACGCGT GTCGCGTAGT-3'; mmu-miR-183-3p, 5'-TGTGAATTACA CGAAGGCCCTA-3'; mmu-miR-320-3p, 5'-AAAGGC TGGTGTACGAGGGCC-3' ; mmu-miR-375-5p, 5'-GCG ACGAGCCCTCGCAACAAAC-3' ; mmu-miR-376a-5p, 5'-GTTGAGTCTTCTCTCTATGAGT-3'; mmu-miR-381-3p, 5'-TATACA AGGCAAGCCTCT CGAGT-3'; mmu-miR-470-5p, 5'-TCTCTTGGACTGGCCACTGTTGAGT-3'; mmu-miR-484, 5'-TCCAGGCTCAGTCCCCCTCCCGGAT-3'; mmu-miR-540-5p, 5'-CAAGGGTACCCTCTGACCTCT GT-3'; mmu-miR-592-5p, 5'-ATTGTGCTCAATAGCCGAT GATGTG-3'; mmu-miR-3098-3p, 5'-TTCTTGCTGCTCGCTT TAGGA-3'; mmu-miR-3475-3p, 5'-TCTGGGAGGCAACT GGTGTTGAA-3'; U1, 5'-CTTACCTGCAGGCAGGAGGA TATA-3'. The protocol of miRNA RT-qPCR array analysis was as previously described (23) and as specified on the Wcgene website (wgene.com). The mouse U1 small nuclear rna gene were used to normalize expression. The 2-ΔΔCq 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).

Wnt and Notch signaling pathway PCR array. On the basis of the KEGG functional annotation analysis, the mouse Wnt and Notch signaling pathway RT² profile™ PCR array plates (Wgene 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 Wegenie 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 demonstrated 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 (GO:0030182) and brain development (GO:0042007), 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).

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 (pic tar.org/) software was used to predict target miRNAs. 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.
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. In addition, it provided useful insights for further investigation of the role of targeted miRNAs in the neurotoxic effects of glyphosate on the developing brain.

### 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             | 2.47263     |
| 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             | 2.12466     |
| 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      |

#### 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     |

**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
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
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 reported to 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, FOXG1, VEGFA, VIM, STRN, LHX8, CNTNAP1, VLDLR |
| GO:0031175, neuron projection development                                | 22    | IGF1R, NTN1, NUMBL, ULK1, SLITRK4, NRN1, NEUROG2, CHL1, EPHA5, KIF5A, UNC5C, MAPK8IP3, FOXB1, VIM, STRN, LHX8, CNTNAP1, VLDLR |
| GO:0030182, neuron differentiation                                       | 27    | IGF1R, NTN1, NUMBL, ULK1, SLITRK4, NRN1, NEUROG2, CHL1, EPHA5, KIF5A, UNC5C, MAPK8IP3, FOXB1, VIM, STRN, 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, VEGFA, 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, VEGFA, E2F1, PRKG1, MAPK8IP3, MYCN, TNFRSF21 |
| GO:0030900, forebrain development                                        | 12    | LEF1, NUMBL, EPHA5, FOXB1, FGFR1, SKI, FOXG1, LHX8, VEGFA, E2F1, PRKG1, MAPK8IP3 |
| 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, VLDLR |
| GO:0045666, positive regulation of neuron differentiation               | 8     | BNIP2, FGFR1, IL6, NCOA1, NEUROD1, PHOX2B, TGF1, TGF2                         |
| 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, DDT4, SLCA9A                                                    |
| 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, SNC, SLCA9A                      |
| GO:002052, positive regulation of neuroblast proliferation              | 2     | FOXG1, VEGFA                                                                 |
| GO:0021885, forebrain cell migration                                      | 3     | FOXG1, FGFR1, 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.
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
cortical development (63). Rare missense variants in \textit{Dixdc1} have been identified in ASD patient cohorts via genetic sequencing (63), indicating that \textit{Dixdc1} may be associated with morphological defects associated with NDDs. \textit{Runx1} and \textit{Shh}, which are involved in the Notch signaling pathway, have been reported to serve critical functions in the developing brain (64-66). For example, \textit{Runx1} may be upregulated after injury to promote neuronal differentiation, in order to facilitate repair of the CNS. Therefore, upregulated \textit{Runx} expression is associated with brain injury and disease (64). \textit{Shh} influences neurogenesis and neural patterning during development of the CNS (67). Dysregulated \textit{Shh} signaling may lead to neurological disorders such as ASD, depression and PD, as well as locomotor deficits (68). \textit{Lef}-1, a direct \textit{Wnt}/\textit{β}-catenin signaling target (69), is a crucial determinant of neurogenesis and of neural progenitor fates in the brain (70,71). It also regulates \textit{β}-catenin-dependent transcription of neural progenitor genes in the neocortex (72). \textit{Axin2}, a classic \textit{Wnt} target gene and \textit{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 \textit{Axin} expression in neuronal progenitors leads to an enlarged neocortex and autistic-like behaviors (74). In the present study, the mRNA expression levels of \textit{Dkk1}, \textit{Dixdc1}, \textit{Runx1}, \textit{Shh}, \textit{Lef}-1 and \textit{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

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.
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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