ORIGINAL ARTICLE



Microarray Analysis of Gene Expression Changes in Neuroplastin 65-Knockout Mice: Implications for Abnormal Cognition and Emotional Disorders

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Abstract Neuroplastin 65 (Np65) is an immunoglobulin superfamily cell adhesion molecule involved in synaptic formation and plasticity. Our recent study showed that Np65-knockout (KO) mice exhibit abnormal cognition and emotional disorders. However, the underlying mechanisms remain unclear. In this study, we found 588 differentiallyexpressed genes in Np65-KO mice by microarray analysis. RT-PCR analysis also revealed the altered expression of genes associated with development and synaptic structure, such as Cdh1, Htr3a, and Kcnj9. In addition, the expression of Wnt-3, a Wnt protein involved in development, was decreased in Np65-KO mice as evidenced by western blotting. Surprisingly, MRI and DAPI staining showed a significant reduction in the lateral ventricular volume of Np65-KO mice. Together, these findings suggest that ablation of Np65 influences gene expression, which may contribute to abnormal brain development. These results provide clues to the mechanisms underlying the altered brain functions of Np65-deficient mice.

Keywords Neuroplastin $65 \cdot$ Microarray analysis \cdot Gene expression profile \cdot Htr3a \cdot Wnt

Huanhuan Li, Jiujiang Zeng and Liang Huang have contributed equally to this work.

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Introduction

Neuroplastin (Np) is a member of the immunoglobulin (Ig) superfamily of cell adhesion molecules and exists in two isoforms, Np65 and Np55 [1]. Np65 contains extracellular Ig1-2-3 modules, while Np55 only contains extracellular Ig2-3. Thus, Np65 can be differentiated from Np55 by its extracellular Ig1. Np55 is expressed in various organs and cell types, whereas the expression of Np65 is brain-specific and restricted to neurons.

Np65 undergoes trans- and cis-homophilic bindings as well as several heterophilic bindings with fibroblast growth factor receptors, the $\alpha 1$ or $\alpha 2$ subunit of GABA_A receptors, and the basigin-monocarboxylate transporter [2–4]. Np65 has been implicated in the regulation of synaptic plasticity and the maintenance of excitatory/inhibitory balance. Antibodies specific for Np65 or recombinant Np65 block long-term potentiation (LTP) in the hippocampal CA1. The induction of LTP also increases the expression of Np65 in postsynaptic densities [5]. In addition, *Nptn*-deficient neurons exhibit impaired inhibitory transmission [6].

Previous studies have suggested that Np65 is associated with cognition and emotional states. Polymorphisms in the human *NPTN* gene have been shown to correlate with cortical thickness and intellectual abilities in adolescents as well as in patients with schizophrenia [7, 8]. *Nptn*-deficient mice exhibit retrograde amnesia, depressive-like behaviors, and decreased social interactions [9]. In addition, mutation of the *Nptn* gene results in deafness in mice, suggesting that *NPTN* is a novel deafness gene [10, 11]. We have previously demonstrated that *Np65* knock-out (KO) mice exhibit enhanced hippocampal-dependent spatial memory in the Morris water maze and step-through passive avoidance tests [12], but the underlying mechanisms were unclear. In this study, we used custom-designed microarray

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analysis to profile differentially-expressed genes in Np65-KO mice, in order to explain the altered brain functions in Np65-deficient mice.

Materials and Methods

Animals

The homozygous *Np65*-KO mice were obtained from engineered mouse models; this caused Np65-Ig1 deficiency in single chromosome as previously described [12]. Wildtype (WT) littermates served as controls. Animals were housed in a temperature-controlled environment under a 12 h light/dark cycle (08:00–20:00) with food and water *ad libitum*. All protocols complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Ethics Committee of Tongji University School of Medicine, and conformed to Directive 2010/63/EU and NIH guidelines.

Microarray Experiments

Microarray analysis was performed as previously described [13]. Briefly, animals were sacrificed after deep anesthesia with intraperitoneal (i.p.) injection of 1% pentobarbital sodium (30 mg/kg). Hippocampi from adult *Np65*-KO mice (3 months old) and age-matched WT mice (n = 3/genotype) were dissected and immediately frozen in liquid nitrogen. The samples were stored at -80° C until use.

Total RNA was extracted from the hippocampal tissue using TRIzol (15596026, Thermo Fisher Scientific, Waltham, MA) and further purified with an RNeasy Mini Kit (74104, Qiagen, Hilden, Germany). RNA concentration and quality were evaluated by spectrophotometry (Nano-Drop ND-1000, Thermo Fisher Scientific, Waltham, MA). One microgram of total RNA was amplified and labeled with a One-Color Quick Amp Labeling Kit (5190-0442, Agilent Technologies, Santa Clara, CA). The fluorescencelabeled cRNA was hybridized onto the Whole Mouse Genome Oligo Microarray (4 × 44K, Agilent Technologies, Santa Clara, CA) using the Agilent Gene Expression Hybridization Kit (5188-5242, Agilent Technologies, Santa Clara, CA). Chips were washed and scanned by a microarray scanner (G2565BA, Agilent Technologies, Santa Clara, CA). Raw data were then normalized and analyzed using the GeneSpring GX Software Package (v11.5, Agilent Technologies). The microarray experiment was performed with 3 biological and experimental repeats. Normalized values were used to screen for differentiallyexpressed genes from biological and experimental repeats before all replicates were combined. Genes with a foldchange of > 2.0 and a *P* value < 0.05 were selected for Gene Ontology (GO) and pathway analysis.

Gene Ontology and Pathway Analysis

The fold-changes of differential expression were determined by the abundance ratio of *Np65*-KO and WT mice. Hierarchical clustering was used to analyze the differentially-expressed genes. GO analysis was applied to analyze the cellular components, biological functions, and biological processes of the differentially-expressed genes (www. geneontology.org). Pathway analysis was used to reveal significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the differentially-expressed genes.

Quantitative Real-Time Reverse-Transcription PCR

Quantitative real-time reverse-transcription PCR (RT-PCR) was performed as previously described [14]. Adult *Np65*-KO and WT mice (n = 4/genotype) were sacrificed after anesthesia with 1% pentobarbital sodium (30 mg/kg, i.p.) and the forebrain was harvested to extract total RNA using TRIzol (15596026, Thermo Fisher Scientific, Waltham, MA). RNA concentration and quality were determined by NanoDrop (ND-1000, Thermo Fisher Scientific, Waltham, MA). cDNA was generated using reverse transcriptase (PrimeScriptTM RT reagent Kit, RR0747Q, Takara Bio, Tokyo, Japan). The first-strand cDNA was used as a template for RT-PCR analysis. The primers for RT-PCR analysis (Table 1) were designed by the NCBI primer designing tool [15] and synthesized by Sangon (Shanghai, China). Each RT-PCR reaction was carried out in a 20 µL volume using SYBR Green Master Mix (RR820O, Takara Bio, Tokyo, Japan), started at 30 s at 95°C for initial denaturation, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C in the ABI 7500 Real-Time PCR System. A total of 3 independent samples per subject were run in duplicate for RT-PCR. β-actin was used as the reference gene. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression levels of genes.

Western Blotting

Adult *Np65*-KO and WT mice (4 months old, n = 3/genotype) were used for western blotting. Briefly, animals were decapitated after deep anesthesia with 1% pentobarbital sodium (30 mg/kg i.p.). Forebrains were collected and frozen in nitrogen and then stored at -80° C until use. The total proteins were extracted using RIPA lysis buffer (P0013B, Beyotime) with 1 mmol/L PMSF (ST506, Beyotime). Protein concentrations were measured using the BCA Protein Assay Kit (P0010, Beyotime,

Table 1 Primers used in RT-PCR.	Gene	NCBI Accession	Forward Primer	Reverse Primer
	Cdh1	NM_009864	CAGCCGGTCTTTGAGGGATT	TGACGATGGTGTAGGCGATG
	Cdh4	NM_009867	ACAACCGTCCCGAGTTCATC	TCATCTGCATCGTTGGCTGT
	Htr3a	NM_013561	CAGACCACCTCCTGGCTAAC	GATGCTGTCTGTGGGGGATGG
	Htr4	NM_008313	ACGTCCTCATGCCCATTTCC	ACCACTGCAAGGAACGTGAG
	Kcnj9	NM_008429	TCTTCTTCGTGCTCGCCTAC	CGAAGCCGTTGAGGTTGTTG
	Pla2g4e	NM_177845	CTCCAACTGCCTACACCCAG	CCTCTGGGTTGAGTGGGAAC
	Xafl	NM_001037713	AGAGCCCATCCCAGAGTCAA	CAGATTGCTAAGCTGCACGG
	Lactb	NM_030717	GGCTATGCAGACGTGGAGAA	CAGTTTAGCCAGAGCCACCA
	Actb	NM_007393	GCTGTATTCCCCTCCATCGTG	AGTCCTTCTGACCCATTCCCA

Jiangsu, China), then 10 ng of total protein was separated by SDS-PAGE and transferred to the PVDF membrane. After blocking with 5% bovine serum albumin (BSA), the membranes were incubated overnight at 4°C with primary antibody against Wnt-3 (1:1,000, Santa Cruz Biotechnology, Dallas, TX) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1,000, Santa Cruz Biotechnology, Dallas, TX). Subsequently, the membranes were incubated with HRP-conjugated goat anti-mouse secondary antibody (1:1,000, Beyotime) for 2 h at room temperature. The labeled proteins were detected by using the ImageQuant LAS 4000mini system (GE Healthcare Life Sciences, Chicago, IL). The protein levels were normalized to that of GAPDH from three independent experiments.

Magnetic Resonance Imaging (MRI)

A separate cohort of mice (4 months old, n = 5/genotype) was used in MRI analysis. MRI data were acquired with a 7.0 T animal MRI scanner (PharmaScan, Bruker Biospin GmbH, Germany) with 4-channel phased array coil. T2-weighed (T2-wt) MRI was performed using a rapid acquisition with relaxation enhancement (RARE) sequence with TR/TE = 4200/36 ms, RARE factor = 8, and averaging number = 3. The geometric parameters for the scan were: slice number = 18, slice thickness = 0.5 mm, matrix = 256 × 256, and FOV = 21 × 21 mm².

4',6-Diamidino-2-Phenylindole (DAPI) Staining

Adult *Np65*-KO and WT mice (n = 4, 2 months old) were anesthetized with 1% pentobarbital sodium intraperitoneally and perfused with 4% paraformaldehyde. The brain was removed, postfixed for 10 h–16 h, and cryoprotected in 20% sucrose. Coronal sections (10 µm, at the level of the lateral ventricle, 0 mm–2 mm from bregma) were prepared for DAPI staining. In brief, the sections were blocked in 5% BSA (B2064-100G, Sigma-Aldrich, St. Louis, MO) with 0.3% Triton X-100 (ST795, Beyotime), then incubated with DAPI (1:300, Beyotime) diluted in 1% BSA with 0.3% Triton X-100 for 10 min at room temperature. The sections were then rinsed with PBS and covered with Permount for fluorescent microscopy (Eclipse 80i, Nikon Corp., Tokyo, Japan).

Statistics

Statistics were calculated using SPSS Statistics software (v22.0, IBM). All data are presented as mean \pm SEM. Independent samples were tested by the unpaired Student's *t*-test (two-tailed). The Mann-Whitney *U* test was used to determine the significance of data with an abnormal distribution or unequal variance. Statistical significance was set at *P* < 0.05.

Results

Microarray Analysis of Differentially-Expressed Genes in the Hippocampus of *Np65*-KO Mice

All genes are shown in a scatter plot with normalized intensity in Fig. 1A (details in Table S1). Of the 34397 targeted genes by the Mouse 4×44 K Gene Chip, 481 (1.4%) were up-regulated and 418 (1.2%) were down-regulated by 2-fold in the *Np65*-KO mice (Fig. 1B). Using P < 0.05 as the criterion, 367 genes were significantly higher and 221 genes were significantly lower in *Np65*-KO mice as compared to age-matched WT mice (Fig. 1C and D, Table S2). These differentially-expressed genes were primarily located on chromosomes 7, 9 and 11. Notably, the *NPTN* gene resides on chromosome 9 (Fig. 1E).

Gene Ontology and Pathway Analysis of Differentially-Expressed Genes

Using the criterion of P < 0.001, GO analysis showed that the upregulated genes were involved in several cellular components, including extracellular region, plasma



Fig. 1 Differentially-expressed genes in the hippocampus of *Np65*-KO mice. A Scatter plot of normalized intensity derived from microarray chips with WT and *Np65*-KO mice. Dots above the red line denote upregulated genes, and dots below the green line denote downregulated genes. B Venn diagram showing the percentages of differentially-expressed genes categorized by fold-change. C Hierarchical clustering of differentially-expressed genes. N1–N3, *Np65*-KO mice; W1–W3, WT mice. D Fold changes of differentially-expressed genes in *Np65*-KO mice. E Chromosome distributions of differentially-expressed genes in *Np65*-KO mice. Red bars, numbers of upregulated genes; green bars, numbers of downregulated genes.

membrane, and desmosome (Fig. 2A). The molecular functions of the upregulated genes were mainly associated with co-receptor activity. The down-regulated genes were involved in various binding actions, such as ankyrin binding, lipoprotein binding, and Wnt-protein binding (Fig. 2B). The main biological processes were the cellular response to interferon-beta, embryonic hindlimb morphogenesis, negative regulation of neuron differentiation, cellular process, positive regulation of epidermis development, and negative regulation of transmembrane receptor protein serine/threonine kinase signaling pathway. The downregulated genes were associated with several biological processes, including post-transcriptional regulation of gene expression, developmental growth involved in morphogenesis, regulation of translation, cell adhesion, developmental growth, and response to stimuli (Fig. 2C).

According to the pathway analysis, 8 pathways were significantly up-regulated in *Np65*-KO mice, including glycerophospholipid metabolism, pancreatic secretion, and regulation of actin cytoskeleton. Among the 9 down-regulated pathways, the most prominent was cytokine-cytokine receptor interaction. The other downregulated pathways were involved in bladder cancer, chemical carcinogenesis, and drug metabolism (Fig. 2D).

Functional Analysis of Differentially-Expressed Genes in *Np65*-KO Mice

The differentially-expressed genes were divided into four categories: cell adhesion, development, neurotransmission and ion channel, and signal transduction. Previous studies have suggested that Np65 may interact with other cell



Fig. 2 GO and pathway analysis of differentially-expressed genes in Np65-KO mice. A–C Cellular components (A), molecular functions (B), and biological processes (C) of differentially-expressed genes. D Significantly changed pathways in Np65-KO mice.

adhesion molecules like fibroblast growth factor receptor (FGFR) to activate intracellular signaling [2]. Interestingly, the expression of several cell adhesion molecules was altered in Np65-KO mice. Fgfr4, immunoglobulin superfamily member 1 (Igsf1), interleukin 7 receptor (Il7r), and members of the integrin superfamily such as integrin alpha 1 (Itgal) and integrin alpha 9 (Itga9) were upregulated, while interleukin 1 receptor, type II (Il1r2) and protein tyrosine phosphatase receptor type D (Ptprd) were down-regulated in Np65-KO mice. In Np65-KO mice, the expression levels of genes associated with Ca²⁺ binding, including Ca²⁺-binding protein 5 (Cabp5), calbindin 1 (Calb1), and calmodulin-like 4 (Calml4) were significantly increased. However, several cadherins, including cadherin 1 (Cdh1), cadherin 4 (Cdh4), cadherin 6 (Cdh6), protocadherin 7 (Pcdh7), protocadherin 12 (Pcdh12), and protocadherin 17 (Pchd17) were downregulated in Np65-KO mice (Table 2).

In addition, a subset of genes associated with neuronal development, such as chemokine (C-C motif) receptor 5 (*Ccr5*), forkhead box O3 (*Foxo3*), myelin basic protein (*Mbp*), myocyte enhance factor 2C (*Mef2c*), and Wnt inhibitory factor 1 (*Wif1*), were significantly downregulated in *Np65*-KO mice, while several genes involved in glial cell development, including neurotrophin 3 (*Ntf3*), glial cell missing homolog 1 (*Gcm1*), and transformation related protein 73 (*Trp73*), were upregulated. More

interestingly, the expression of eye development-related genes was also altered in *Np65*-KO mice. The expression of aldehyde dehydrogenase family 1 subfamily A3 (*Ald-h1a3*), retinitis pigmentosa GTPase regulator interacting protein 1 (*Rpgrip1*), crumbs homolog 1 (*Crb1*), visual system homeobox 1 homolog (*Vsx1*), keratin 12 (*Krt12*), and secreted frizzled-related sequence protein 5 (*Sfrp5*) were down-regulated. Myosin VIIA (*Myo7a*) and collagen triple helix repeat containing 1 (*Cthrc1*), which are associated with inner ear receptor cell development, were also decreased in *Np65*-KO mice (Table 2).

The expressions of genes related to the structure and function of synapses were also altered in *Np65*-KO mice. Notably, expression of the serotonin receptor 4 (*Htr4*) gene was significantly increased, whereas the expression of serotonin receptor 3A (*Htr3a*) was significantly decreased in *Np65*-KO mice. Moreover, two ion channel-related genes displayed significant downregulations in *Np65*-KO mice: Cl⁻ channel Ca²⁺ activated 5 (*Clca5*) and K⁺ inwardly-rectifying channel subfamily J member 9 (*Kcnj9*) (Table 2).

MAPK signaling is essential for various physiological and pathological processes, such as neural plasticity and memory. We found that the expression of mitogenactivated protein kinase kinase 7 (Map2k7) was significantly decreased, while phospholipase A2, group IVE (Pla2g4e), serine/threonine kinase 38 like (Stk38l), and

Table 2 Selected differentially-expressed genes in the hippocampus of Np65-KO mice.

Cell adhesion Fifth NM_008011 Hibrohlast growth factor receptor 4 2.0 0.0001 $Fgf1$ NM_008372 Interleukin 7 receptor 2.1 0.0002 Ilr NM_008372 Interleukin 7 receptor, type II -2.1 0.0002 Ilr NM_008372 Interleukin 1 receptor, type II -2.3 0.015 Chh NM_009864 Cadherin 1 -2.4 0.010 Chh NM_009864 Cadherin 1 -2.4 0.010 Chh NM_009864 Cadherin 1 -2.4 0.010 Chh NM_009864 Cadherin 7 -5.8 0.020 $Pehl$ NM_0113758 Protocalherin 17 -2.2 0.040 $Pehl$ NM_001013753 Protocalherin 17 -2.2 0.040 $Pehl$ NM_001013753 Protocalherin 17 -2.2 0.040 $Pehl$ NM_001013753 Protocalherin 17 -2.2 0.041 $Pehl$ NM_001013753 Protocalherin 17 -2.1 0.040 Development	Category	NCBI accession	Full name	Fold change	P value
$F_{gf}f$ NM_00811 Fibroblast growth factor receptor 4 2.0 0.000 lpf NM_00877 Intractakin 7 receptor 2.1 0.000 lln NM_00875 Intractakin 7 receptor, type II -2.1 0.000 pnd XR_107615 Protein tyrosine phosphatase, receptor type D -2.3 0.001 Chd NM_009867 Catherin 1 -2.4 0.001 Chd NM_009867 Catherin 6 -2.2 0.003 Chd NM_00122758 Protocatherin 7 -5.8 0.020 $Pch17$ NM_0013225 Integrin alpha 1 3.0 0.047 $lpad$ NM_00135228 Integrin alpha 9 0.0 0.004 $lpad$ NM_00103228 Integrin alpha 9 -2.0 0.004 $lpad$ NM_00103228 Integrin alpha 9 -2.0 0.004 $lpad$ NM_0103228 Integrin alpha 9 -2.0 0.004 Mfp NM_010777 Myelin basic protein 73 -2.1 0.004 $Mfg1$ <t< td=""><td>Cell adhesion</td><td>1</td><td></td><td></td><td></td></t<>	Cell adhesion	1			
Instrume NML (39372) Immunoglobulin superfamily, member 1 2.3 0.002 ID7 NML (008372) Interleukin 7 receptor, type II -2.1 0.0002 Pinrd XRL (17615) Protein tyrosine phosphatse, receptor type D -2.3 0.013 Cdh1 NML 009867 Cadherin 1 -2.1 0.004 Cdh4 NML 009867 Cadherin 4 -2.1 0.004 Cdh5 NML 009867 Cadherin 6 -2.2 0.043 Pedh7 NML 00137738 Protocadherin 17 -2.3 0.045 Pedh17 NML 00103753 Protocadherin 17 -2.2 0.000 Igal NML 00103753 Protocadherin 17 -2.1 0.009 Perdor NML 00103753 Protocadherin 17 -2.1 0.009 Proxa NML 00103753 Protocadherin 40x0 3 -2.0 0.004 Igad NML 001013753 Protocadherin 5 -2.1 0.009 Proxa NML 001013753 Protocadherin 6 -2.2 0.001 Verof <	Fgfr4	NM_008011	Fibroblast growth factor receptor 4	2.0	0.004
$II7$ NM_008372 Inerleakin 1 receptor, type II 2.1 0.006 $II1/2$ NM_010355 Interleakin 1 receptor, type II -2.1 0.002 $Cdhi$ NM_009864 Cadherin 1 -2.4 0.010 $Cdhi$ NM_009867 Cadherin 6 -2.2 0.004 $Cdhi$ NM_009867 Cadherin 6 -2.2 0.004 $Cdhi$ NM_00112758 Protocalherin 17 -5.8 0.020 $Pcdh1$ NM_001013753 Protocalherin 12 -2.3 0.0045 $Pcdh2$ NM_00103228 Integrin alpha 1 .3.0 0.041 $Dga9$ NM_00103228 Integrin alpha 1 .3.0 0.0041 $Dcvelopment$ -2.1 0.004 0.000 0.004 $Mcf2a$ NM_00177 Myein basic protein -2.1 0.004 $Mf1$ NM_00116034 Neurotrophin 3 .3.2 0.044 $Mf2a$ NM_0016402 Transformation related protein 73 .2.3 0.005 $Mf1$ NM_0016403 <td< td=""><td>Igsfl</td><td>NM_177591</td><td>Immunoglobulin superfamily, member 1</td><td>2.3</td><td>0.002</td></td<>	Igsfl	NM_177591	Immunoglobulin superfamily, member 1	2.3	0.002
III12NM_010555Interleukin 1 receptor, type II-2.10.002PprudXR_010761Protein tyrosine phosphatase, receptor type D-2.30.015Cdh1NM_009867Cadherin 1-2.40.010Cdh5NM_007666Cadherin 6-2.10.0043Pcdh7NM_0012758Protocadherin 7-5.80.020Pcdh12NM_0013753Protocadherin 12-2.30.0045Pcdh17NM_00103753Protocadherin 12-2.30.0047IgalNM_00103753Protocadherin 19-0.00.000Pcdh17NM_00103753Integrin alpha 910.40.0000Development-2.10.00910.0001Cc5NM_009917Chemokine (C-C motif) receptor 5-2.10.0091Foxa3AK143198Forkhaal box 03-2.00.014MphNM_010171Mycine basic protein-2.20.014Mg2cNM_012582Mycoyte enhancer factor 2C-2.10.003Mg1NM_010144Neurotophin 33.20.044Gm1NM_008103Gital cells missing homolog 12.90.019Tp73NM_011642Tanaformation related protein 73-2.10.000Rgrip1NM_02879Retinitis pigmentosa GTPase regulator interacting protein 1-2.50.020Ch1NM_02865Visai System homolog 1-2.40.000Kuh12NM_01868Visai System homolog 1-2.40.000Kuh12NM_01877Ca ²⁺¹ binding protein	Il7r	NM_008372	Interleukin 7 receptor	2.1	0.006
PhysicXR_107615Protein tyrosine phosphatase, receptor type D-2.30.015Cdh1NM_009864Cadherin 1-2.40.0010Cdh5NM_007660Cadherin 4-2.10.004Cdh6NM_007660Cadherin 7-2.30.0020Pcdh7NM_00112758Protocadherin 7-2.30.0045Pcdh7NM_00103753Protocadherin 17-2.20.0040Iga0NM_01013753Protocadherin 17-2.20.0040Iga0NM_00103728Integrin alpha 910.40.0000Development0.004Cer5NM_00917Chenokine (C-C motif) receptor 5-2.10.0041Fox3AK143198Forkhead box 03-2.00.0044Mfp2NM_00177Myclin basic protein-2.10.0041Mfp2NM_00116403Nuce rehareer factor 2C-2.10.0041Mfp3NM_00116403Nuce rehareer factor 2C-2.10.0041Mfp4NM_00116403Nucerotophi 3-2.30.0042Mfp3NM_00116403Nucerotophi 3-2.10.0032Mfp4NM_001870Calacells missing homolog 1-2.50.020Thr2NM_01803Alderlyde delydrogenase family 1, subfamily A3-2.10.0036Mgrif NM_018780Scretted frizzle-related sequence protein 5-3.40.015Karl 12NM_018780Calacell riside-related sequence protein 5-2.00.000GrbfNM_018780Calacell riside-related sequenc	Il1r2	NM_010555	Interleukin 1 receptor, type II	-2.1	0.002
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Cub4NM_0009867Cadherin 4-2.10.004Cdb6NM_0017566Cadherin 6-2.20.043Pcdh7NM_00112758Protocadherin 7-5.30.045Pcdh71NM_001013753Protocadherin 12-2.30.045Pcdh71NM_001013753Protocadherin 17-2.20.000Iga1NM_0010137232Integrin alpha 13.00.047Iga9NM_133721Integrin alpha 910.40.000Development0.004Cro5NM_00917Chemokine (C-C motif) receptor 5-2.10.004Mp7NM_01077Myelin basic protein-2.20.014Mp7NM_01077Myelin basic protein-2.10.001Foxo3NM_011915Wit inhibitory factor 1-2.10.001Mg7NM_00116403Fiactor factor 2C-2.10.001Mg7NM_00116403Riecells missing homolog 12.90.019Tp73NM_0011642Transformation related protein 732.30.040AldhidaNM_053080Aldehyde deplacegares family 1, subfamily A3-2.10.002Crb1NM_133239Crumbs homolog 1-2.50.039Yax1NM_01877Ca ³⁺ actin 12-2.90.000Myr5NM_01877Ca ³⁺ binding protein 5-2.00.000Myr5NM_01877Ca ³⁺ binding protein 5-2.00.000Myr5NM_01877Ca ³⁺ binding protein 5-2.10.003Myr5NM_	Cdh1	NM_009864	Cadherin 1	-2.4	0.010
Cdb6NM_007666Cadherin 6-2.20.043Pcdh7NM_007786Protocadherin 17-5.80.020Pcdh17NM_001013738Protocadherin 12-2.30.045Pcdh17NM_00103328Integrin alpha 13.00.047Itga9NM_00103328Integrin alpha 910.40.000Development0.000Evelopment0.000Pcva0AK143198Forkhead box 03-2.00.004Mp/0NM_009917Myein basic potein-2.20.014Mg2cNM_025282Myocyte enhancer facto 2C-2.10.034Mg7aNM_00116403Neurotrophin 33.20.044Gen1NM_001164034Neurotrophin 32.30.040AlfalaNM_053080Aldehyde dpotein 732.30.040AlfalaNM_053080Aldehyde gpotease family 1, subfamily A3-2.10.008Rprj0NM_033879Retinitis pigmentosa GTPase regulator interacting protein 1-2.50.029Vx1NM_0054068Visual system homeobox 1 homeolog-3.40.015Kr12NM_016161Keratin 12-2.90.000Gyrb5NM_01877Ca ²⁴ binding protein 5-2.00.006Myo274NM_00563Myo371Laimodulin-like 43.00.007Gyrb5NM_018376Ca ²⁴ binding protein 5-2.90.000Gyrb5NM_018376Ca ²⁴ binding protein 5-2.10.006G	Cdh4	NM_009867	Cadherin 4	-2.1	0.004
Pcdh7NM_00112278Protocadherin 7-5.80.020Pcdh12NM_00103753Protocadherin 12-2.30.045Pcdh17NM_00103753Integrin alpha 13.00.0471IggdNM_00103228Integrin alpha 90.00.0091IggdNM_009917Chemokine (C-C motif) receptor 5-2.10.009Fcxx3NM_011277Myelin basic protein-2.00.0041Mef2cNM_0013753Myocyte enhancer factor 2C-2.10.0034Myf1NM_011915Wat inhibitory factor 1-2.10.0014Mef2cNM_00116434Neurotrophin 33.20.0441Myf1NM_0116434Neurotrophin 33.20.0401Myf3NM_0116434Neurotrophin 32.30.040Aldh1a3NM_028103Gilal cells missing homolog 1-2.50.020Crb1NM_028104Crumshomolog 1-2.50.020Crb1NM_028080Nethydrogenase family 1, subfamily A3-2.10.000Sprgir1NM_01876Screted fitzled-related sequence protein 1-2.50.020Crb1NM_028068Nyels wits homolog 1-2.50.020Syr3NM_01876Screted fitzled-related sequence protein 5-2.00.000Syr3NM_018778Collagen triple helix repeat containing 1-2.10.000Syr3NM_01877Ca ²⁺ binding protein 5-2.00.000Syr3NM_01877Calmodulin-like 43.00.007Crb1N	Cdh6	NM_007666	Cadherin 6	-2.2	0.043
Pcdh12 NM_017378 Protocadherin 12 -2.3 0.045 Pcdh17 NM_001033228 Protocadherin 17 -2.2 0.000 Igal NM_001033228 Integrin alpha 9 10.4 0.000 Development - -2.1 0.009 Cr5 NM_009917 Chemokine (C- motif) receptor 5 -2.1 0.009 Fca03 AK143198 Forkhead box 03 -2.0 0.004 Mp/ NM_001777 Myelin basic protein -2.1 0.001 Mg2c NM_001164034 Neurotrophin 3 3.2 0.044 Gcn1 NM_00116403 Glial cells missing homolog 1 2.9 0.019 Nrg73 NM_011642 Transformation related protein 73 2.3 0.040 Aldh1a3 NM_053080 Aldehyde dehydrogenase family 1, subfamily A3 -2.1 0.008 Rpgrip1 NM_023879 Retrinit is pigmentosa GTPase regulator interacting protein 1 -2.5 0.029 Vx1 NM_00866 Kisual system homeobox 1 homolog -3.4 0.015	Pcdh7	NM_001122758	Protocadherin 7	-5.8	0.020
Pcdh17NL_001013753Protocadherin 17 -2.2 0.000Irga1NM_00103328Integrin alpha 13.00.047Irga9NM_133721Integrin alpha 910.40.000Development0.00910.0010.001Ecr5NL_009917Chemokine (C-C motif) receptor 5 -2.1 0.004MbpNM_010777Myelin basic protein -2.2 0.014Mef2cNM_025282Myocyte enhancer factor 2C -2.1 0.001Myf1NM_0116403Neurotopin 3 3.2 0.004Gem1NM_00116403Reurotopin 3 2.2 0.014Gem1NM_00116403Cilial cells missing homolog 1 2.9 0.019Trp73NM_0116403Cilial cells missing homolog 1 2.5 0.020Grb1NM_023879Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020Grb1NM_0133239Crumbs homolop 1 -2.5 0.030Stprjp1NM_01661Keratin 12 -2.9 0.000Stprjp3NM_01876Scerted frizted-related sequence protein 5 -2.0 0.006Gubp5NM_01887Callenge tripte helix repeat containing 1 -2.1 0.000Stprjp3NM_01887Callenge tripte helix repeat containing 1 -2.1 0.000Stprjp3NM_01887Callenge tripte helix repeat containing 1 -2.1 0.000Stprjp3NM_01887Callenge tripte helix repeat containing 1 -2.1 0.000ChrlaNM_00813 <td>Pcdh12</td> <td>NM_017378</td> <td>Protocadherin 12</td> <td>-2.3</td> <td>0.045</td>	Pcdh12	NM_017378	Protocadherin 12	-2.3	0.045
IrgalNM_001033228Integrin alpha 13.00.047Irga9NM_113721Integrin alpha 910.40.000Development0.0190.009Ccr5NM_009917Chemokine (C-C motif) receptor 5-2.10.009Foxo3AK143198Forkhead box 03-2.00.014Mdp2NM_010777Myecite basic protein-2.20.014Mdp2NM_0010775Myecite anancer factor 2C-2.10.0031Wif1NM_00116034Neurotrophin 33.20.0404Gcm1NM_00164034Neurotrophin 32.90.019Trp73NM_00164034Neurotrophin 732.30.0404Aldhla3NM_005080Aldehyde dehydrogenase family 1, subfamily A3-2.10.008Rpgrip1NM_0053809Aldehyde dehydrogenase family 1, subfamily A3-2.50.020Vcx1NM_0054068Visual system homeobox 1 homolog-3.40.015Kr12NM_01661Keratin 12-2.90.000S/pr5NM_013770Ca ²⁺ binding protein 5-2.00.006My07aNM_00863Myosin VIIA-2.80.001Cubrl<	Pcdh17	NM_001013753	Protocadherin 17	-2.2	0.000
Inga9NM_133721Integrin alpha 910.40.000DevelopmentCar5NM_00917Chemokine (C-C motif) receptor 5-2.10.009Faxa3AK143198Forkhead box 03-2.00.004MbpNM_010777Myein basic protein-2.20.014Mef2cNM_025282Myocyte enhancer factor 2C-2.10.001Mf3NM_001164034Neurotrophin 33.20.044Gcm1NM_001164034Neurotrophin 32.30.040Adhla3NM_053080Aldehyde dehydrogenase family 1, subfamily A3-2.10.003Tp733NM_011642Transformation related protein 732.30.040Adhla3NM_053080Aldehyde dehydrogenase family 1, subfamily A3-2.10.008Rpgrip1NM_023879Retinitis pigmentosa GTPase regulator interacting protein 1-2.50.020Crb1NM_133239Crumbs homolog 1-2.90.000Str12NM_00661Keratin 12-2.00.006MyoraNM_00863Myosin VIIA-2.80.013AdurbChalgen triple helix repeat containing 1-2.10.000NurotarsmisutarCollagen triple helix repeat containing 12.10.003Cubr2NM_00863Gabindin 12.10.001Cubr2NM_00863Calbindin 12.10.003Cubr2NM_00863Gabindin 12.10.001Cubr2NM_00863Gabindin 12.10.001Cubr2NM_00863<	Itgal	NM_001033228	Integrin alpha 1	3.0	0.047
Development V $CerS$ NM_009917 Chenokine (C-C motif) receptor 5 -2.1 0.0091 $Roads$ AK14319 Forkhead box 03 -2.0 0.0041 $Meplo$ NM_010777 Myelin basic protein -2.2 0.014 $Meflo$ NM_025282 Myocyte enhancer factor 2C -2.1 0.034 $Meflo$ NM_0101915 Went inhibitory factor 1 -2.1 0.001 $Mflo$ NM_00116403 Neurotrophin 3 3.2 0.040 $frar NM_008103 Glial cells missing homolog 1 2.9 0.019 Trp73 NM_011642 Transformation related protein 73 2.3 0.040 Aldh1a3 NM_033080 Aldehyde dehydrogenase family I, subfamily A3 -2.1 0.002 Crb1 NM_04068 Visual system homeobox 1 homolog -2.5 0.030 Vxr1 NM_05605 Reratin 12 -2.0 0.000 Strp5 NM_01877 Caleert frizzled-related sequence protein 5 -2.0 0.000 Strp5 NM_00863$	Itga9	NM_133721	Integrin alpha 9	10.4	0.000
Ccr5 NM_009917 Chemokine (C-C motif) receptor 5 -2.1 0.009 $Faxa3$ AK143198 Forkbad box 03 -2.0 0.004 Mbp NM_010777 Myelin basic protein -2.2 0.014 $Mg/2c$ NM_02582 Myocyte enhancer factor 2C -2.1 0.001 $Nf/3$ NM_0116403 Neutorophin 3 3.2 0.044 $Gcn1$ NM_008103 Gilal cells missing homolog 1 2.9 0.019 $Tp73$ NM_011642 Transformation related protein 73 2.3 0.040 $Aldh1a3$ NM_025879 Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020 $Crb1$ NM_025870 Visual system homoolog 1 -2.5 0.020 $Vx1/L$ NM_010661 Keratin 12 -2.9 0.000 $Strp5$ NM_010863 Secreted frizzled-related sequence protein 5 -2.0 0.000 $Vx1/L$ NM_026878 Collagen triple helix repeat containing 1 -2.1 0.004 $Vx1/L$ NM_0108763 Secreted frizzled-related s	Development				
$Foxo3$ AK143198 Forkhead box 03 -2.0 0.004 Mpp NM_010777 Myelin basic protein -2.2 0.014 $Mef2c$ NM_012528 Myocyte enhancer factor 2C -2.1 0.001 $Mf3$ NM_01150 Wit inhibitory factor 1 -2.1 0.001 $Mf3$ NM_001164034 Neurotrophin 3 3.2 0.044 $Gcml$ NM_008103 Glial cells missing homolog 1 2.9 0.019 $Trp73$ NM_011642 Transformation related protein 73 2.3 0.040 $Aldh1a3$ NM_023879 Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.039 $Vxi1$ NM_054068 Visual system homeobox 1 homolog -3.4 0.015 $Kr12$ NM_016610 Keratin 12 -2.9 0.000 $Syp5$ NM_018780 Secreted firzized-related sequence protein 5 -2.0 0.006 $Myo7a$ NM_026778 Collagen triple helix repeat containing 1 -2.1 0.000 $Cahp5$ NM_013871 Ca	Ccr5	NM_009917	Chemokine (C-C motif) receptor 5	-2.1	0.009
MbpNM_010777Myelin basic protein-2.20.014Mef2cNM_025282Myocyte enhancer factor 2C-2.10.034Wif1NM_011915Wnt inhibitory factor 1-2.10.004Mof3NM_001164034Neurotrophin 33.20.044Gcm1NM_008103Glial cells missing homolog 12.90.019Trp73NM_011642Transformation related protein 732.30.040Aldh1a3NM_053080Aldehyde dehydrogenase family 1, subfamily A3-2.10.002Crb1NM_13239Crumbs homolog 1-2.50.020Crb1NM_054068Visual system homolog 1-2.50.039Vsx1NM_064068Visual system homolog-3.40.015Kr12NM_010661Keratin 12-2.00.000Sfp5NM_01780Scereted frizzled-related sequence protein 5-2.00.000Vsv1NM_026778Collagen triple helix repeat containing 1-2.10.000Neurotamsmisorialto channel-2.10.000CablnAK038856Calbindin 12.10.033Cabln4NM_013801S-hydroxytryptamine (serotonin) receptor 3A-2.50.001Clca5NM_01351S-hydroxytryptamine (serotonin) receptor 42.40.003Krif9NM_008429K ⁺ inwardly-rectifying channel, subfamily J, member 94.20.000Kap24eNM_01351S-hydroxytryptamine (serotonin) receptor 3A-2.50.001Clca5NM_01042557Mitog	Foxo3	AK143198	Forkhead box O3	-2.0	0.004
Mef2c NM_025282 Myocyte enhancer factor 2C -2.1 0.034 Wif1 NM_011915 Wt inhibitory factor 1 -2.1 0.001 N/f3 NM_001164034 Neurorophin 3 3.2 0.044 Granl NM_008103 Glial cells missing homolog 1 2.9 0.019 Trp73 NM_011642 Transformation related protein 73 2.3 0.040 Aldh1a3 NM_053080 Aldehyde dehydrogenase family 1, subfamily A3 -2.1 0.008 Rpgrip1 NM_023879 Retnitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020 Crb1 NM_010661 Keratin 12 -2.9 0.000 Sfrp5 NM_018780 Scereted frizzled-related sequence protein 5 -2.0 0.006 Myo24 NM_008663 Myosin VIIA -2.8 0.0401 Cuhrc1 NM_026778 Calgen triph ehix repeat containing 1 -2.1 0.004 Calp5 NM_013877 Ca ²⁺ binding protein 5 3.5 0.031 Calp4 NM_028456 Calmoluin-like 4 3.0 0.007 Cpk2 NM_0098463 S-hydroxytryp	Mbp	NM_010777	Myelin basic protein	-2.2	0.014
Wift NM_011915 Wnt inhibitory factor 1 -2.1 0.001 Ntf3 NM_001164034 Neurotrophin 3 3.2 0.044 Gcm1 NM_008103 Glial cells missing homolog 1 2.9 0.019 Trp73 NM_011642 Transformation related protein 73 2.3 0.040 Aldh1a3 NM_023879 Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020 Crb1 NM_03239 Crumbs homolog 1 -2.5 0.039 Vsx1 NM_010661 Keratin 12 -2.9 0.000 Strp5 NM_018780 Secreted frizzled-related sequence protein 5 -2.0 0.006 Myo7a NM_008663 Myosin VIA -2.8 0.000 Cthrc1 NM_008663 Myosin VIA -2.8 0.000 Cthrc1 NM_01877 Ca ²⁺ binding protein 5 -2.0 0.000 Cubp5 NM_013871 Calbroduin-like 4 3.0 0.007 Cubp2 NM_008313 S-hydroxytryptamine (serotonin) receptor 4 2.4 0.043	Mef2c	NM_025282	Myocyte enhancer factor 2C	-2.1	0.034
$Nf3$ $NL_001164034$ Neurotrophin 3 3.2 0.044 $Gcml$ NL_008103 Glial cells missing homolog 1 2.9 0.019 $Trp73$ NL_011642 Transformation related protein 73 2.3 0.040 $Aldh1a3$ $NL_0053080$ Aldehyde dehydrogenase family 1, subfamily A3 -2.1 0.008 $Rpgrip1$ NL_023879 Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020 $Crb1$ NL_023879 Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.039 $Vxil$ $NL_0054068$ Visual system homeobox 1 homolog -3.4 0.015 $Krt12$ NL_010661 Kertain 12 -2.9 0.000 $Sfrp5$ NL_010863 Myosin VIIA -2.8 0.040 $Chrc1$ NL_026778 Collagen triple helix repeat containing 1 -2.1 0.000 Neurotransmissionand channel 2.1 0.001 $Calp5$ NL_013877 Ca^{2+} binding protein 5 3.5 0.031 $Calhof$ NL_008613 S-hydroxytryptamine (serotonin) receptor 4 2.4 0.043 $Chrl3$ NL_009813 S-hydroxytryptamine (serotonin) receptor 3A -2.5 0.001 $Clrd3$ $NL_0108271$ Grahoxytryptamine (serotonin) receptor 3A -2.5 0.001 $Clrd3$ $NL_01042557$ Mitogen-activated fortein kinase 7 -2.67 0.000 $Rap2k7$ $NL_001042557$ Mitogen-activated protein kinase 7 -2.67 0.000 $Rap2k7$ $NL_$	Wif1	NM_011915	Wnt inhibitory factor 1	-2.1	0.001
Gcm1NM_008103Glia cells missing homolog 12.90.019Trp73NM_011642Transformation related protein 732.30.040Aldl1a3NM_053080Aldehyde dehydrogenase family 1, subfamily A3-2.10.008Rpgrip1NM_023879Retinitis pigmentosa GTPase regulator interacting protein 1-2.50.020Crb1NM_013329Crumbs homolog 1-2.50.039Vsr1NM_054068Visual system homeobox 1 homolog-3.40.015Krt12NM_010661Keratin 12-2.90.000Sfrp5NM_018780Secreted frizzled-related sequence protein 5-2.00.006Myo7aNM_008663Myosin VIIA-2.80.040Cthrc1NM_026778Callegn triple heix repeat containing 1-2.10.006Nup07aNM_013877Ca ²⁺ binding protein 53.50.011Calb1AK038856Calbindin 12.10.004Calm14NM_138304Calmodulin-like 43.00.007Cpk2NM_009946Complexin 22.10.033Htr3aNM_0083135-hydroxytryptamine (serotonin) receptor 4A2.40.043ClacaNM_178697C1 ⁻ channel Ca ²⁺ activated 52.90.000Krif9NM_008429K ⁺ inwardly-recitiying channel, subfamily J, member 94.20.000Signal transducturV-2.670.0000.013Signal transducturSerier/threonine kinase 38 like4.800.013Pip6r1NM_17234 <td>Ntf3</td> <td>NM_001164034</td> <td>Neurotrophin 3</td> <td>3.2</td> <td>0.044</td>	Ntf3	NM_001164034	Neurotrophin 3	3.2	0.044
Trp73NM_011642Transformation related protein 732.30.040Aldh1a3NM_053080Aldehyde dehydrogenase family 1, subfamily A3 -2.1 0.008Rpgrip1NM_023879Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020Crb1NM_033239Crumbs homolog 1 -2.5 0.039VsrlNM_054068Visual system homeobox 1 homolog -3.4 0.015Kr12NM_010661Keratin 12 -2.9 0.000Sfrp5NM_018780Secreted frizzled-related sequence protein 5 -2.0 0.006Myo7aNM_00863Myosin VIIA -2.8 0.040Cthrc1NM_008673Myosin VIIA -2.8 0.040Neurotransmissichange protein 5 -2.1 0.006Nucoff78Calbindin 1 2.1 0.004Calb1AK088856Calbindin 1 2.1 0.004Calm14NM_013877Ca ²⁺ binding protein 5 3.5 0.031Calm14NM_008313S-hydroxytryptamine (serotonin) receptor 4 2.4 0.043Htr3aNM_00946Complexin 2 2.1 0.033Htr3aNM_013561S-hydroxytryptamine (serotonin) receptor 3A -2.5 0.001Clca5NM_178697C1 channel Ca ²⁺ activated 5 2.9 0.000Signal transducturV 2.4 0.433 2.4 0.433Htr3aNM_00142557Mitogen-activated protein kinase kinase 7 -2.67 0.000Signal transducturV 4.64 <	Gcm1	NM_008103	Glial cells missing homolog 1	2.9	0.019
Aldh1a3NM_053080Aldehyde dehydrogenase family 1, subfamily A3 -2.1 0.008Rpgrip1NM_023879Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020Crb1NM_133239Crumbs homolog 1 -2.5 0.039Vsx1NM_054068Visual system homeobox 1 homolog -3.4 0.015Krt12NM_010661Keratin 12 -2.9 0.000Sfrp5NM_01870Secreted frizzled-related sequence protein 5 -2.0 0.006Myo7aNM_00863Myosin VIIA -2.8 0.040Cthrc1NM_026778Collagen triple helix repeat containing 1 -2.1 0.000Neurotransmissiand on channel2.10.004Cabl5NM_013877Ca ²⁺ binding protein 5 3.5 0.031Cabl4AK038856Calbindin 1 2.1 0.004Cahl4NM_0083135-hydroxytryptamine (serotonin) receptor 4 2.4 0.043Hrr34NM_0083135-hydroxytryptamine (serotonin) receptor 3A -2.5 0.001Clca5NM_178697CI channel Ca ²⁺ activated 5 2.9 0.000Kcrij9NM_008429K ⁺ inwardly-rectifying channel, subfamily J, member 9 4.2 0.000Signal transductM_178457Mitogen-activated protein kinase kinase 7 -2.67 0.000Signal transductM_178457Phospholipase A2, group IVE 4.64 0.000Signal transductSerine/threonine kinase 38 like 4.64 0.003Sid381NM_17234S	Trp73	NM_011642	Transformation related protein 73	2.3	0.040
Rpgrip1NM_023879Retinitis pigmentosa GTPase regulator interacting protein 1 -2.5 0.020 Crb1NM_133239Crumbs homolog 1 -2.5 0.039 VsrlNM_054068Visual system homeobox 1 homolog -3.4 0.015 Krt12NM_010661Keratin 12 -2.9 0.000 Sfrp5NM_018780Secreted frizzled-related sequence protein 5 -2.0 0.006 Myo7aNM_008663Myosin VIIA -2.8 0.040 Chrrc1NM_026778Collagen triple helix repeat containing 1 -2.1 0.000 Neurotransmissi-and ion channel 2.1 0.004 Cabp5NM_013877Ca ²⁺ binding protein 5 3.5 0.031 Calb1AK038856Calbindin 1 2.1 0.004 Cylx2NM_009946Complexin 2 2.1 0.033 Htr4NM_0083135-hydroxytryptamine (serotonin) receptor 4 2.4 0.043 Htr3aNM_0135615-hydroxytryptamine (serotonin) receptor 3A -2.5 0.001 Clca5NM_178697CI ⁻ channel Ca ²⁺ activated 5 2.9 0.000 Kerj9NM_008429K ⁺ inwardly-rectifying channel, subfamily J, member 9 4.2 0.000 Signal transcluct	Aldh1a3	NM_053080	Aldehyde dehydrogenase family 1, subfamily A3	-2.1	0.008
$Crb1$ NM_133239Crumbs homolog 1 -2.5 0.039 $Vsrl$ NM_054068Visual system homeobox 1 homolog -3.4 0.015 $Krl2$ NM_010661Keratin 12 -2.9 0.000 $Sfrp5$ NM_018780Secreted frizzled-related sequence protein 5 -2.0 0.006 $Myo7a$ NM_008663Myosin VIIA -2.8 0.040 $Cthrc1$ NM_026778Collagen triple helix repeat containing 1 -2.1 0.004Neurotransmissi	Rpgrip1	NM_023879	Retinitis pigmentosa GTPase regulator interacting protein 1	-2.5	0.020
Vsx1NM_054068Visual system homeobox 1 homolog-3.40.015Krt12NM_010661Keratin 12-2.90.000Sfrp5NM_018780Secreted frizzled-related sequence protein 5-2.00.006Myo7aNM_008663Myosin VIIA-2.80.040Cthrc1NM_026778Collagen triple helix repeat containing 1-2.10.000Neurotransmissi	Crb1	NM_133239	Crumbs homolog 1	-2.5	0.039
Kr12NM_010661Keratin 12 -2.9 0.000Sfrp5NM_018780Secreted frizzled-related sequence protein 5 -2.0 0.006Myo7aNM_008663Myosin VIIA -2.8 0.040Cthrc1NM_026778Collagen triple helix repeat containing 1 -2.1 0.000Neurotransmissi-mu -2.1 0.000Neurotransmissi-mu -2.1 0.000Cabp5NM_013877Ca ²⁺ binding protein 5 3.5 0.031Calb1AK038856Calbindin 1 2.1 0.004Calml4NM_138304Calmodulin-like 4 3.0 0.007Cplx2NM_009946Complexin 2 2.1 0.033Htr3aNM_0083135-hydroxytryptamine (serotonin) receptor 4 2.4 0.043Kraj9NM_0135615-hydroxytryptamine (serotonin) receptor 3A -2.5 0.001Clca5NM_178697Cl ⁻ channel Ca ²⁺ activated 5 2.9 0.000Kraj9NM_008429K ⁺ inwardly-rectifying channel, subfamily J, member 9 4.2 0.000Signal transduct	Vsx1	NM_054068	Visual system homeobox 1 homolog	-3.4	0.015
$Shpp5$ NM_018780Secreted frizzled-related sequence protein 5 -2.0 0.0061 $Myo7a$ NM_008663Myosin VIIA -2.8 0.0401 $Cthrc1$ NM_026778Collagen triple helix repeat containing 1 -2.1 0.0001 Neurotransmissi- -2.1 0.0001 Neurotransmissi-CalabaCalaba 3.5 0.0311 $Cabp5$ NM_013877 Ca^{2+} binding protein 5 3.5 0.0311 $Calb1$ AK038856Calbindin 1 2.1 0.0007 $Cplx2$ NM_009946Camodulin-like 4 3.0 0.0071 $Cplx2$ NM_009313 5 -hydroxytryptamine (serotonin) receptor 4 2.4 0.0431 $Hr3a$ NM_013561 5 -hydroxytryptamine (serotonin) receptor 3A -2.5 0.001 $Clca5$ NM_178697 CI^- channel Ca^{2+} activated 5 2.9 0.000 $Signal transductrVV0.01042557Mitogen-activated protein kinase kinase 7-2.670.0001Pla2g4eNM_177845Phospholipase A2, group IVE4.640.0013Sit381NM_172734Serine/threonine kinase 38 like4.800.0131Ppfor1NM_001037713XIAP associated factor 15.990.0001LactomNI_030717Lactamase, beta24.300.0001$	Krt12	NM_010661	Keratin 12	-2.9	0.000
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<i>Lactb</i> NM_030717 Lactamase, beta 24.30 0.000	Xafl	NM_001037713	XIAP associated factor 1	5.99	0.000
	Lactb	NM_030717	Lactamase, beta	24.30	0.000



Fig. 3 Relative expression levels of selected genes in *Np65*-KO and WT mice. A Results of quantitative real-time PCR (RT-PCR) (n = 4 mice). B RT-PCR and microarray experimental results for relative gene expression in *Np65*-KO and WT mice. The relative expression levels were calculated as the ratio of the target gene expression level to the β -actin expression level in the same sample. Fold changes are shown as mean \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001.





protein phosphatase 6, regulatory subunit 1 (*Ppp6r1*) were significantly increased in *Np65*-KO mice. In addition, the expression level of X-linked inhibitor of apoptosis protein associated factor 1 (*Xaf1*), an apoptosis-promoting factor, was decreased. Most notably, β -lactamase (*Lactb*), which is involved in mitochondrial metabolism, was also significantly down-regulated in *Np65*-KO mice (24.30-fold, *P* < 0.001) (Table 2).

RT-PCR Analysis of Differentially-Expressed Genes

Np65 is highly expressed in the hippocampus and other brain regions, such as cortex and striatum [1]. Therefore, 8 genes related to the functions of Np65 were further selected for RT-PCR analysis. The results showed that 6 of these were also significantly changed in the forebrain of *Np65*-KO mice (Fig. 3A), including the downregulated *Cdh1* (fold change, 45.34, *P* < 0.001), *Htr3a* (fold change, 1.94, *P* < 0.01), *Xaf1* and *Lactb*, and the increased *Kcnj9* (fold change, 1.26, *P* < 0.05) and *Pla2g4e* (fold change, 1.17, *P* < 0.05) (Fig. 3B).

Decreased Expression of Wnt-3 in Np65-KO Mice

Microarray and RT-PCR analysis showed that the expression levels of several genes associated with development were altered in *Np65*-KO mice. Some differentiallyexpressed genes, such as *Wif1* and *Cdh1*, are involved in Wnt signaling. Wnt signaling is a crucial regulator of many developmental processes, such as cell proliferation, maintenance of stem cells, and cell fate determination [16]. Therefore, we examined the protein level of Wnt-3 in *Np65*-KO mice by western blotting. The results showed that the protein level of Wnt-3 was significantly lower in the forebrain of *Np65*-KO mice than in WT mice (Fig. 4).

Reduced Lateral Ventricles in Np65-KO Mice

Given that the dysfunction of Wnt signaling may influence brain development, we then assessed whether ablation of Np65 affects the brain morphology of mice. T2-wt images were used to assess region-specific volume changes. The gross brain architecture was not affected in *Np65*-KO mice.



Fig. 5 Reduction in lateral ventricles in *Np65*-KO mice. A T2-wt MRI showing a significant reduction in the lateral ventricles (LV) compared to WT mice. B DAPI staining showing a significant

reduction in the lateral ventricles in coronal sections from adult *Np65*-KO mice compared to WT mice. Scale bar, 200 μ m. ****P* < 0.001.

MRI morphometry also showed normal anatomy of the cerebral cortex, hippocampus, thalamus, hypothalamus, basal ganglia, and caudatoputamen of *Np65*-KO mice. However, the lateral ventricular volume was significantly reduced compared to WT mice (Fig. 5A), and this was further confirmed by DAPI staining (Fig. 5B). Thus, these results suggested that the absence of Np65 leads to altered architecture of the mouse brain.

Discussion

Np65 is specifically expressed in the brain and has been reported to mediate several cellular processes including cellcell adhesion, neurite outgrowth, and synaptic plasticity [2, 5, 17, 18]. Our previous studies have shown that *Np65*-KO mice exhibit abnormal cognitive and emotional behaviors [12]. To investigate the underlying mechanisms, we further analyzed the gene expression profiles in *Np65*-KO mice in this study. Our microarray analysis demonstrated a large number of differentially-expressed genes in *Np65*-KO mice; these genes are crucially involved in development, ion channels, neurotransmission, and signal transduction.

Our study identified many differentially-expressed genes involved in neuronal development, such as the decreased expressions of *Cdh1*, *Ccr5*, *Foxo3*, *Mbp*, *Wif1*, and *Mef2c*, as well as upregulation of *Ntf3*, *Gcm1*, and *Trp73*, implying that Np65 deletion affects the configuration of the brain. Coincidently, T2-wt MRI morphometry and brain slices stained with DAPI showed a significant reduction in lateral ventricular volume in *Np65*-KO mice compared to WT mice. The expression of Wnt-3 was significantly decreased in *Np65*-KO mice. Wnt signaling is a crucial regulator of developmental processes like cell proliferation and cell fate determination [16]. Dysregulation of Wnt signaling may contribute to neuropsychiatric disorders, such as depression and schizophrenia [19]. In this study, our findings suggested that Np65 deletion affects the Wnt signaling pathway by decreasing Wnt expression. Together, these differentially-expressed genes associated with development may contribute, at least in part, to changes in the ventricles and abnormal behaviors in *Np65*-KO mice.

Recent studies have reported that mutation of the NPTN gene results in deafness in mice [10]. It has been reported that Np65 may regulate the properties of synapses connecting the inner hair cells with spiral ganglion neurons [10]. Intriguingly, Zeng et al. reported that Np55 is expressed in stereocilia of outer but not inner hair cells and affects interactions of stereocilia with the tectorial membrane and cochlear amplification in mice with NPTN mutation [11]. Together, these recent findings clearly confirm NPTN as a novel deafness gene. Consistent with their reports, our microarray analysis showed that the genes associated with inner ear receptor cell development, myosin VIIA (Myo7a) and collagen triple helix repeat containing 1 (Cthrc1) were significantly decreased in Np65-KO mice, supporting the hypothesis that Np65 is involved in hearing.

It has been shown that Np65 is linked with ribbon synapse formation in the plexiform layers of the rat retina [20]. Retinal function, as assessed using the electroretinogram, is unaffected by the absence of NPTN [10]. Surprisingly, the involvement of Np65 in vision was demonstrated using the pupillary light reflex and flash visual evoked potentials (our unpublished data). In agreement with our finding, our microarray analysis showed that the expression of eye development-related genes, including Aldh1a3, Rpgrip1, Crb1, Vsx1, Krt12, and Sfrp5, was significantly downregulated in Np65-KO mice. Although these alterations in eye-development genes need to be confirmed, the reduced amplitude of the pupil in the pupillary light reflex and reduced first negative and positive amplitude of flash visual evoked potentials (our unpublished data) suggest that Np65 plays roles in vision.

Our previous studies have shown that *Np65*-KO mice appear to show enhanced memory in the Morris water maze and increased anxiety [12]. Central 5-hydroxytryptamine (5-HT) activity is involved in emotional and cognitive activities [21, 22]. Generally, stimulation of central 5-HT activity impairs cognition, while its inhibition enhances cognition in rodent models. Tropisetron, a selective 5-HT₃ receptor antagonist, has been confirmed to reverse the cognitive deficit in rats injected with A β (1–42) [23]. In addition, central 5-HT activity is closely associated with anxiety [24–27]. Among the 5-HT receptors, 5-HT₃ is the only ligand-gated ion channel that increases intracellular cations such as Ca^{2+} , Na^+ , and K^+ . Stimulation of 5-HT₃ receptors induces the rapid and transient depolarization of neurons. 5-HT receptor 3A-null mice exhibit anxiolytic behaviors, indicating that this receptor influences anxiety-like behavior [28]. More surprisingly, microarray and RT-PCR analysis demonstrated that *Htr3a* mRNA was significantly reduced in *Np65*-KO mice. How deletion of Np65 affects the expression of *Htr3a* remains to be determined. To date, the decreased expression of *Htr3a* may explain, at least in part, the changed cognitive and anxiety behaviors in *Np65*-KO mice.

In conclusion, the present study demonstrates that a large number of genes are differentially expressed in *Np65*-KO mice. Notably, microarray analysis in *Np65*-KO mice revealed altered expression of *Htr3a* and genes associated with development, hearing, and vision, which may provide important insights for understanding the role of Np65 in brain development as well as brain functions like cognition and emotion.

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Compliance with Ethical Standards

Conflict of interest All authors claim that there are no conflicts of interest.

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