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Gene expression profiling of the rat sciatic nerve in early Wallerian degeneration after injury[☆]

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Abstract

Wallerian degeneration is an important area of research in modern neuroscience. A large number of genes are differentially regulated in the various stages of Wallerian degeneration, especially during the early response. In this study, we analyzed gene expression in early Wallerian degeneration of the distal nerve stump at 0, 0.5, 1, 6, 12 and 24 hours after rat sciatic nerve injury using gene chip microarrays. We screened for differentially-expressed genes and gene expression patterns. We examined the data for Gene Ontology, and explored the Kyoto Encyclopedia of Genes and Genomes Pathway. This allowed us to identify key regulatory factors and recurrent network motifs. We identified 1 546 differentially-expressed genes and 21 distinct patterns of gene expression in early Wallerian degeneration, and an enrichment of genes associated with the immune response, acute inflammation, apoptosis, cell adhesion, ion transport and the extracellular matrix. Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed components involved in the Jak-STAT, ErbB, transforming growth factor- β , T cell receptor and calcium signaling pathways. Key factors included interleukin-6, interleukin-1, integrin, c-sarcoma, carcinoembryonic antigen-related cell adhesion molecules, chemokine (C-C motif) ligand, matrix metalloproteinase, BH3 interacting domain death agonist, baculoviral IAP repeat-containing 3 and Rac. The data were validated with real-time quantitative PCR. This study provides a global view of gene expression profiles in early Wallerian degeneration of the rat sciatic nerve. Our findings provide insight into the molecular mechanisms underlying early Wallerian degeneration, and the regulation of nerve degeneration and regeneration.

Key Words

Wallerian degeneration; sciatic nerve; microarrays; expression profiling; rats; neural regeneration

Abbreviations

WD, Wallerian degeneration; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology

INTRODUCTION

Wallerian degeneration (WD) occurs both in the peripheral nervous system and the central nervous system. Peripheral nerve injuries are quite common in clinical practice, and many studies have been conducted on the underlying molecular mechanisms. WD in the peripheral nervous system occurs following various traumatic injuries, such as nerve fiber transection or crush, and is also

known as anterograde degeneration, orthograde degeneration and secondary degeneration. In WD, axonal injury induces a series of responses in the distal nerve segment^[1-2]. WD can also be caused by inflammatory or autoimmune responses, in addition to traumatic injuries.

A sequence of events unfolds during WD in the distal stump of an axotomized nerve. WD begins with the disintegration and degeneration of the axoplasm and axolemma, which is complete within 24

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hours. Axonal breakdown is mediated by calcium influx and involves the activation of axonal proteases. An important aspect of this response in peripheral nerves is the recruitment and activation of macrophages that leads to the phagocytosis and clearance of myelin and axonal debris. The rapid clearance of myelin, which contains axonal growth inhibitors, appears to contribute to axonal regeneration after peripheral nerve injury^[3-10].

WD is currently the focus of much research in neuroscience. Although the mechanisms underlying WD have been studied extensively, the molecular changes in the distal stump during early WD, such as patterns of differential gene expression and the molecular networks involved, are unclear^[11-18].

We report here on gene expression patterns in early WD of the rat distal nerve stump at 0, 0.5, 1, 6, 12 and 24 hours after sciatic nerve injury using microarray and bioinformatic analysis. The data were validated with real-time quantitative PCR. We analyzed the data using Series Test Cluster of Gene Ontology (STC-GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Our results will help us better understand gene expression profiles and patterns, as well as the signaling pathways and key regulatory networks involved in early WD.

RESULTS

Gene expression profiling and regulatory patterns during early WD after sciatic nerve injury

Gene Ontology (GO) analysis of differentially-expressed genes

To screen for differentially-expressed genes during WD

after sciatic nerve injury, we examined the expression of genes at different time points, compared with the 0-hour time point, thereby obtaining 1 546

differentially-expressed genes and 21 significant patterns of gene expression in early WD (Figure 1). The data summarizes all possible expression patterns and statistical judgments based on $P < 0.05/N$ as the standard.

Functional classification by GO is an internationally standardized classification system for gene function offering a dynamic, updated and controlled vocabulary employing strictly defined concepts to comprehensively describe the properties of genes and their products in any organism. GO encompasses three domains: molecular function, cellular component and biological process^[19-21]. GO analysis was conducted using gene expression patterns in a series of experiments, followed by significant and separate analyses of different gene expression trends in early WD. Quantitative changes in selected enriched GO biological processes were present and found to alter the expression of genes involved in these processes. Based on the GO database, the regulated genes were distributed into functional categories; these categories included genes with putative functions in the innate immune response, activation of the acute inflammatory response, promotion of chemokine production, Ras signal transduction, ion transport, nerve growth factor processing, regulation of gene-specific transcription, regulation of gene expression, promotion of axonogenesis, cytokine production, cytokinesis, neurological processing, neural tube development, regulation of cell differentiation and apoptosis (Figure 2).

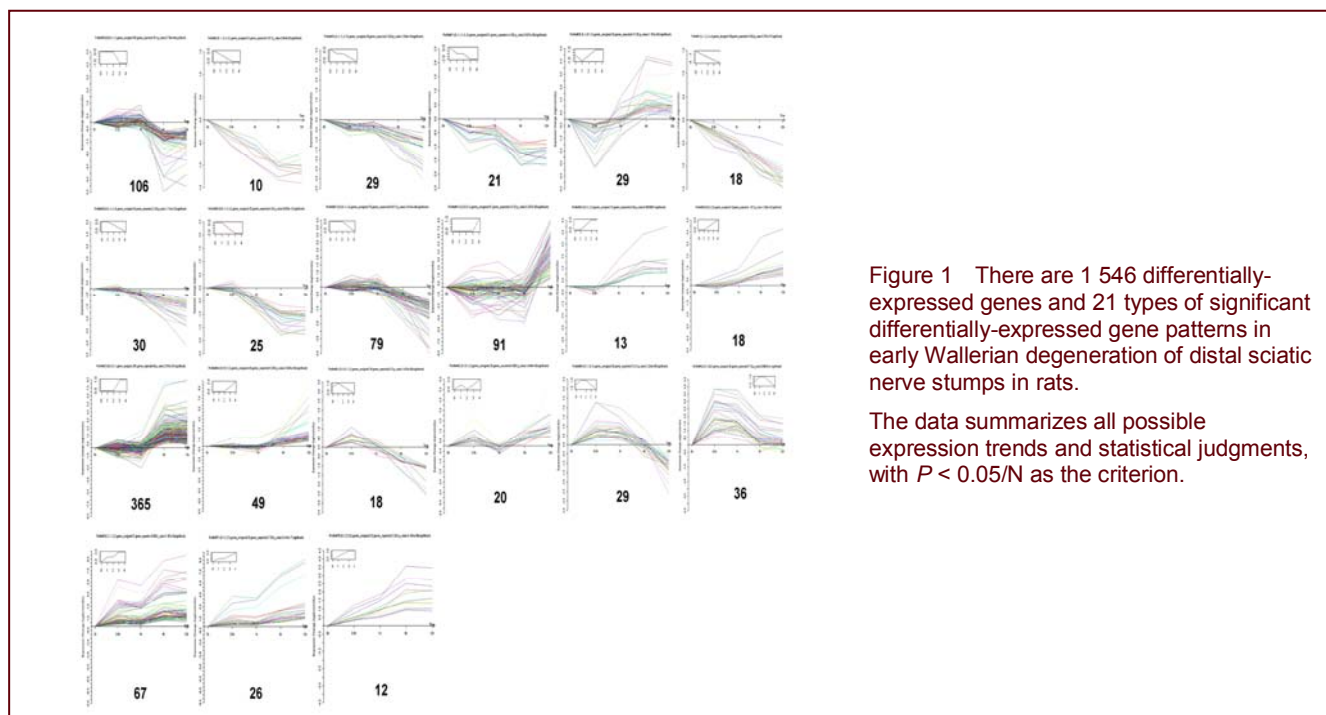


Figure 1 There are 1 546 differentially-expressed genes and 21 types of significant differentially-expressed gene patterns in early Wallerian degeneration of distal sciatic nerve stumps in rats.

The data summarizes all possible expression trends and statistical judgments, with $P < 0.05/N$ as the criterion.

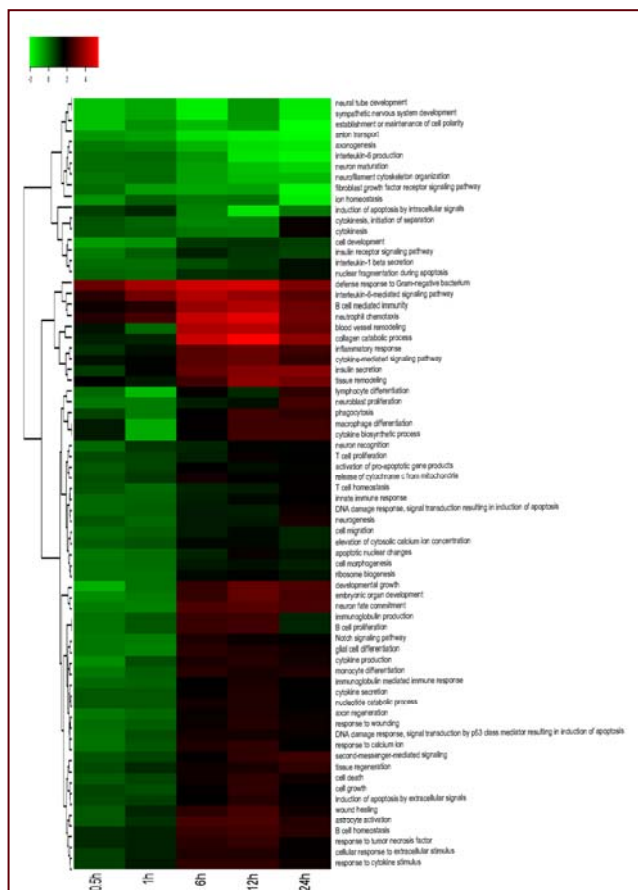


Figure 2 Hierarchical cluster analysis showing partition clustering of genes most highly expressed in the distal nerve stumps after sciatic nerve injury.

Gene Ontology analysis of distal sciatic nerve stumps of rats at 0.5, 1, 6, 12 and 24 hours post-surgery.

Fisher exact test and chi-square test were applied to the differentially-expressed genes, and significance analysis was performed with the target genes by screening for $P < 0.05$. The heat map shows the intensity of expression as a function of color, with higher expression in red and lower expression in green or black.

KEGG Pathway analysis of differentially-expressed genes during WD

Based on the GO database, Fisher's Exact Test and Chi Square tests were applied to the differentially-expressed genes, significance analysis was performed with the pathways involving target genes, and significant pathways were obtained by screening for $P < 0.05$. The KEGG Pathway database comprises information on networks of molecular interactions for numerous organisms, permitting functional classification. Pathway-based analysis provides insight into biological functions and interactions of genes. Based on a comparison against the GO database using BLAST with an E value cutoff of 10^{-5} , 1 546 genes had significant matches in the database and were assigned to 70 KEGG pathways in early WD. KEGG pathway analysis identified numerous pathways, including those relating to B-cell

receptor signaling, janus kinase and signal transducer and activator of transcription (Jak-STAT) signaling, apoptosis, cytokine-cytokine receptor interactions, toll-like receptor signaling, tight junctions, neuroactive ligand-receptor interactions, axon guidance, Wnt signaling, p53 signaling, T-cell receptor signaling, leukocyte transendothelial migration, vascular endothelial growth factor signaling, adherens junctions, cell adhesion molecules, ErbB signaling, gap junctions, transforming growth factor- β signaling, mitogen-activated protein kinase signaling, the extracellular matrix-receptor interactions, actin cytoskeleton regulation, calcium signaling and the cell cycle (Figure 3).

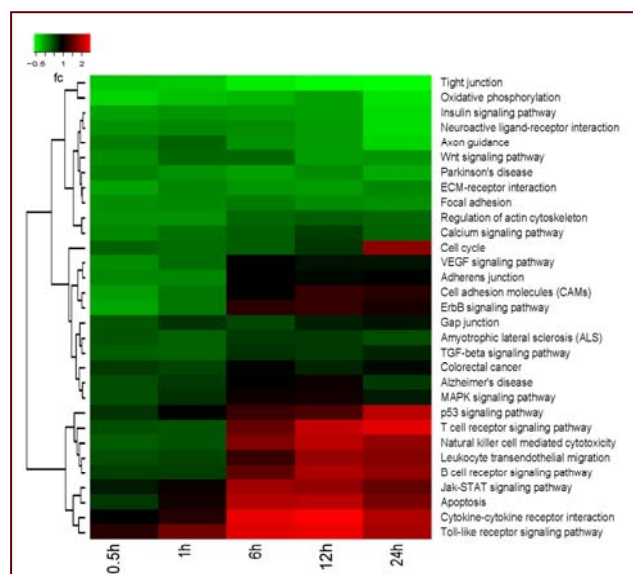


Figure 3 Hierarchical cluster analysis showing partition clustering of genes most highly expressed in the distal nerve stumps after sciatic nerve injury. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of distal sciatic nerve stumps of rats at 0.5, 1, 6, 12 and 24 hours post-surgery.

Based on the KEGG database, Fisher exact test and Chi square test were applied to the differential genes, significance analysis was performed with the pathways involving target genes, and the significant pathways were obtained by screening for $P < 0.05$.

The heat map shows the intensity of expression as a function of color, with higher expression in red and lower expression in green or black.

Key network analysis of differentially-expressed genes during WD

External stimuli affect cellular behavior, as reflected in protein interactions and gene expression kinetics, and we infer from this the presence of dynamic gene regulatory networks. These networks are computed based on fold-changes in gene expression and gene interactions in pathways. The relationships among the gene expression data were inferred using a Continuous Time Recurrent Neural Network (CTRNN) as an abstract, dynamic model for gene regulatory networks mediating

the cellular decision to migrate upon an external stimulus. The model describes the mutual influence of genes and their stimulus responses as dynamic elements, regardless of how such interactions or stimuli are realized in concrete biological terms. Using a genetic algorithm, we estimated the model parameters. A heat map and dendrogram showed partition clustering of genes most highly expressed in the distal nerve stumps after sciatic nerve injury.

Key networks were constructed from active experiments. The gene expression data was used as the input for the generation of biological networks, using the *analyze network* algorithm with default settings. This is a variant of the *shortest paths* algorithm with main parameters of relative enrichment and relative saturation of networks with canonical pathways. Genes encoding signal transducers or factors involved in processes such as cell death, the immune response, transport and transcriptional regulation showed injury-specific gene expression. The expression of numerous genes is required for regeneration^[22-24]. In this study, we used CTRNN to dynamically model GO processes for gene regulatory networks. A number of key networks were identified, including those comprising interleukin-6, interleukin-1, integrin (ITG), c-sarcoma, carcinoembryonic antigen-related cell adhesion molecules, chemokine (C-C motif) ligand, matrix metalloproteinase, BH3 interacting domain death agonist (BID), baculoviral IAP repeat-containing 3 (BIRC3) and Rac (a subfamily of the Rho family of GTPases) (Figure 4). These key networks cover the most important pathways and play important roles in regulating gene expression profiles through mainly biological GO processes during WD of the sciatic nerve. The data obtained here are consistent with

published data and support the conclusion that genetic programs of regenerating and developing peripheral nerves exhibit relevant differences. The data revealed that signaling molecules significantly regulated in the lesioned distal stump after sciatic nerve injury were steadily up and down-regulated. All of these molecules were significantly regulated after sciatic nerve injury and are known to modulate signaling pathways composing the key networks^[15, 25-28]. The data also suggest that the functional group of transcriptional regulators identified by the gene expressional changes may be an essential prerequisite for nerve degeneration and/or regeneration/development^[29-31].

Real-time quantitative PCR assay

Although the reliability of our array data is demonstrated by a number of genes which have been previously described in response to peripheral nerve injury [such as c-jun (Herdegen and Zimmerman 1994), cyclin D1 (Kim *et al.* 2000), GADD45 (Befort *et al.* 2003), phospholipase A2 (Edström *et al.* 1996), PMP22 (Spreyer *et al.* 1991) and the p75NTR receptor (Heumann *et al.* 1987)], we have performed additional validation experiments for a number of genes, the expression or regulation of which has not been described in this context. We carried out real-time quantitative PCR for the following genes: BID, BIRC3, ITGA11, and ACVR1C. Real-time quantitative PCR reactions were run in triplicate. Levels of mRNAs were normalized against GAPDH. Expression levels for each time point were compared to the 0-hour time point and statistically analyzed (Figure 5). The real-time quantitative PCR results are consistent with the gene expression patterns identified by microarray analysis.

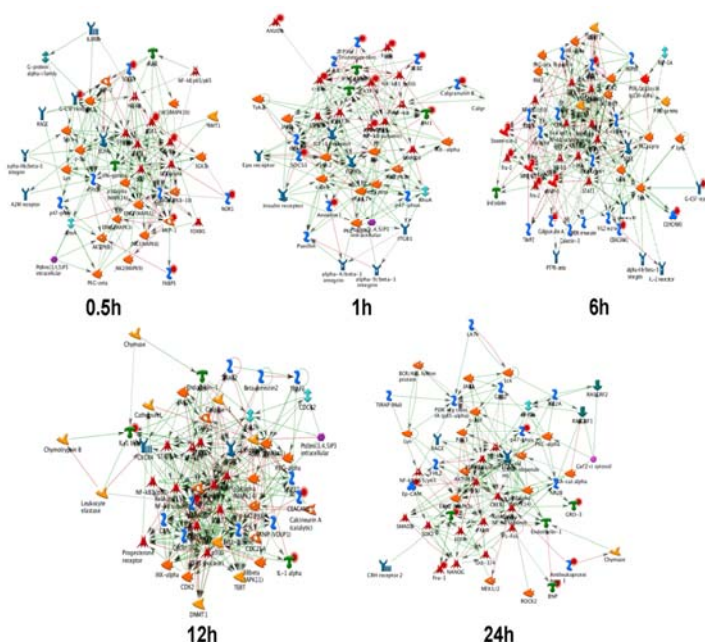
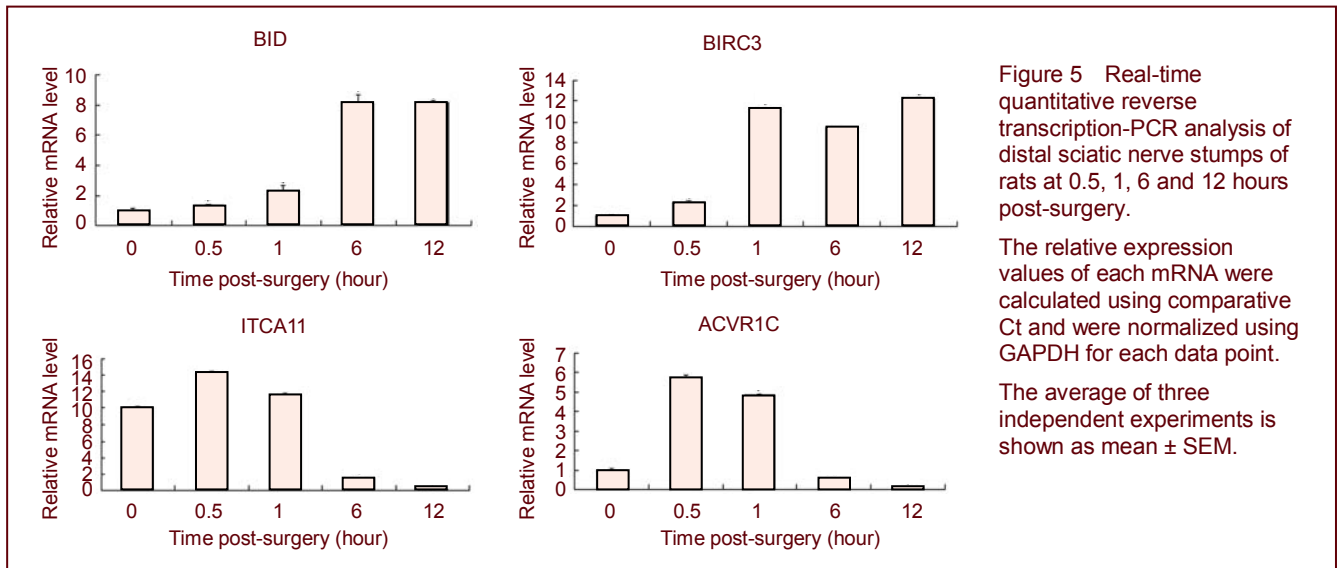


Figure 4 Key network analysis of distal sciatic nerve stumps of rats at 0.5, 1, 6, 12 and 24 hours post-surgery.

The gene expression data were used as the input file for the generation of biological networks using the *analyze network* algorithm with default settings.

The networks are prioritized based on the number of fragments of canonical pathways. Thick cyan lines indicate the fragments of canonical pathways.

Up-regulated genes are marked with red circles; down-regulated genes with blue circles. The checker board pattern indicates mixed expression for the gene between files or between multiple tags for the same gene.



DISCUSSION

WD is a process that occurs before nerve regeneration and can be described as a cleaning or clearing process that prepares the distal stump for reinnervation. The macrophage response required to clear myelin from the distal portion of damaged peripheral nerves is well underway in the first week^[32-34], and myelin and axonal debris are cleared within 7–14 days. Macrophages in degenerating peripheral nerves are mainly of hematogenous origin^[35-36]. Understanding the factors that regulate the rapid macrophage responses in the peripheral nervous system during WD and comparing the differences in the expression of these molecules in the lesioned peripheral nervous system may provide insight into the reasons for slow WD in the peripheral nervous system.

In this study, we performed validation experiments for a number of genes; the expression and regulation of a number of these has not been described previously. The BID gene is a pro-apoptotic member of the Bcl-2 protein family responsible for apoptotic signaling. The expression of BID is up-regulated by the tumor suppressor p53, a protein transcription factor, and participates in p53-mediated apoptosis, as part of the cell response to stress. BIRC3 is a member of the Inhibitor of Apoptosis family, and inhibits apoptosis by interfering with the activation of caspases. It inhibits apoptosis induced by serum deprivation, but does not significantly affect apoptosis mediated by reactive oxygen species. ITGA11 plays a role in cell signaling and cellular shape regulation, motility, and the cell cycle. Integrins work alongside other proteins, such as cadherins, immunoglobulin superfamily cell adhesion molecules, selectins and syndecans to mediate cell-cell and cell-matrix interactions and signaling. ACVR1C is a

dimeric growth and differentiation factor that belongs to the transforming growth factor beta superfamily. ACVR1C is a structurally related receptor that mediates BMP signaling.

In this study, large-scale microarray analysis of WD in the lesioned peripheral nervous system was conducted, which can be used to discover novel genes associated with nerve degeneration and regeneration. WD is a complex process that involves a large number of diverse and overlapping signals, requiring the participation of numerous endogenous signaling molecules. Distinct up- and down-regulation of gene expression after nerve crush indicates that these genes are probably of functional relevance for WD and/or nerve repair. GO and functional analysis demonstrated that genes with similar functions often display similar regulation profiles^[37-38]. Of the thousands of genes analyzed using microarrays, numerous differentially-expressed transcripts were identified in early WD. Preferentially up-regulated genes comprised growth factors and cytokines and their receptors, cell adhesion and extracellular matrix molecules, and those involved in axonal growth and plasticity. We compared gene expression patterns in the sciatic nerve during developmental myelination and during remyelination after injury. Pro-inflammatory chemokines and cytokines play an important role in WD after peripheral nerve injury. These pro-inflammatory signals are down-regulated in a timely manner to ensure that the inflammatory response in the injured nerve is limited. The factors that suppress the pro-inflammatory state are not fully understood.

In our study, we applied microarray technology using a genome-wide, systems-based approach to explore transcriptional responses that provide evidence of biological processes/pathways impacting WD after peripheral nerve injury. Future studies, for example, by using microarray analysis to compare gene expression

patterns in peripheral nerves during WD in a congenital hypomyelinating rat model, will help us understand how the peripheral nervous and immune systems interact to modulate nerve repair, and identify the molecules that drive these responses, such as in the injured peripheral nerve during early WD.

Due to the complexity of the peripheral nervous system, especially in the context of injury, which involves the proliferation, recruitment and differentiation of various cell types, it is difficult to precisely determine the roles of the hundreds of genes that are regulated, or their relative importance in the regenerative repair process. For these reasons, we decided to focus our attention on genes that are differentially regulated in the sciatic nerve distal stump, as opposed to where cell bodies of axotomized neurons are located. Furthermore, analyzing gene expression profiles in the distal nerve stump offers the possibility to study how genes are regulated after sciatic nerve injury in WD^[12, 39-42]. Our results revealed distinct patterns of up and down-regulation of genes in the injured distal nerve, which suggests that these genes play a role in nerve degeneration and/or regeneration. Further studies are necessary to determine whether these differentially-expressed genes are also expressed by Schwann cells *in vivo*, and to clarify their functions during WD after sciatic nerve injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at Jiangsu Key Laboratory of Neuroregeneration, Nantong University, China, from October 2009 to March 2011.

Materials

Male Sprague-Dawley rats, weighing 180–200 g, were provided by the Experimental Animal Center of Nantong University. All animal tests were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

Methods

Establishment of sciatic nerve injury models

All rats were anesthetized using an injection of complex narcotics (10 mg/kg xylazine, 95 mg/kg ketamine, acepromazine 0.7 mg/kg) and the sciatic nerve was identified and lifted through an incision on the lateral aspect of the mid-thigh of the right hind limb. A 1-cm segment of the right sciatic nerve was excised, and the distal and proximal nerve stumps were separately

inserted into nearby inter-muscular spaces to prevent nerve regeneration. After the surgical incisions were closed, the animals were housed in temperature and humidity-controlled cages, maintained under a 12 hour light-dark cycle, and were allowed free access to water and food. All rats randomly divided into six groups, with six animals in each group, to undergo sciatic neurectomy. One group of rats was killed immediately and the other groups were killed at 0.5, 1, 6, 12 or 24 hours post surgery. Distal nerve stumps were obtained from these rats after euthanization.

RNA isolation and Affymetrix microarray scanning

Total RNA was isolated from the distal nerve stumps using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. An Affymetrix Genechip Hybridization Oven 640 and Gene Array Scanner 3000 were used for genechip analysis. The labeling and hybridization were performed at Shanghai Biochip Company, Ltd., China according to the protocols in the Affymetrix GeneChip System. Affymetrix GeneChip scan control software was used for scanning the microarray slides, and an Affymetrix Fluidics Station 450 was used for image analysis.

Bioinformatic analysis

Gene screening and bioinformatic analyses were performed by the Bioinformatics Center at the Key Laboratory of Systems Biology, Shanghai Institute for Biological Sciences. We selected differentially-expressed genes in a logical sequence according to Random Variance Model corrective ANOVA.

Differentially-expressed genes were identified by Affymetrix scan at each time point with ratios set for different genes. Using a strategy for clustering the early WD gene expression data, we defined some unique profiles. The expression model profiles were related to the actual or expected number of genes assigned to each model profile. Significant profiles demonstrated a higher probability than expected by Fisher's Exact Test and multiple comparison tests^[43-44]. Stc GO analysis was applied to determine the main functions of the differentially-expressed genes according to GO, the key functional classification of NCBI. Pathway analysis was used to determine the significant pathways of the differentially-expressed genes according to KEGG^[19-20, 45]. Gene regulatory networks were analyzed using a CTRNN. Key regulatory factors and networks were calculated according to gene fold-change expression and gene interaction in pathways^[46]. The networks were constructed extempore and unique for the uploaded data, using an analytical network algorithm with default settings for biological networks.

Real-time quantitative PCR assay

The distal nerve stumps of rats killed at 0, 0.5, 1, 6 or 12 hours following injury were dissected and then suspended in RNA Stabilization Reagent (Qiagen Tokyo, Japan) and stored at -80°C . The total RNA fraction was prepared from stored tissue specimens using an RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. The cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA), and real-time quantitative PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems), both according to the manufacturers' protocols. All reactions were run in triplicate. The cycle threshold (Ct) values were analyzed using the comparative Ct ($\Delta\Delta\text{Ct}$) method. Levels of mRNAs were normalized using GAPDH as a reference. The primers used in the real-time quantitative PCR assays are provided in supplementary information online. The relative expression values of each mRNA were calculated using comparative Ct and were normalized using GAPDH mature mRNA for each data point.

Statistical analysis

Microarray data were analyzed using GeneSpring GCOS1.2 software. The data were analyzed statistically using the two-sample (independent group) *t*-test, and differences were considered statistically significant at $P < 0.05$. All data were expressed as mean \pm SEM and analyzed by one-way analysis of variance and Scheffe's *post-hoc* test with the SPSS software package. All values of $P < 0.05$ were considered statistically significant.

Author contribution: Dengbing Yao, Meiyuan Li and Dingding Shen had full access to experimental design, data collection, data analysis and wrote the manuscript. Fei Ding, Shibi Lu and Qin Zhao provided experimental guidance, technical support and article validation. Xiaosong Gu was in charge of funding and interpretation.

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Conflicts of Interest: None declared

Ethical approval: This study was approved by the Animal Ethics Committee of Nantong University, China.

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