Critical signaling pathways during Wallerian degeneration of peripheral nerve

Qiong Cheng, Ya-xian Wang, Jun Yu, Sheng Yi

Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education, Co-innovation Center of Neuroregeneration, Nantong University, Nantong, Jiangsu Province, China

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Graphical Abstract

Molecular mechanisms underlying Wallerian degeneration

Abstract

Wallerian degeneration is a critical biological process that occurs in distal nerve stumps after nerve injury. To systematically investigate molecular changes underlying Wallerian degeneration, we used a rat sciatic nerve transection model to examine microarray analysis outcomes and investigate significantly involved Kyoto Enrichment of Genes and Genomes (KEGG) pathways in injured distal nerve stumps at 0, 0.5, 1, 6, 12, and 24 hours, 4 days, 1, 2, 3, and 4 weeks after peripheral nerve injury. Bioinformatic analysis showed that only one KEGG pathway (cytokine-cytokine receptor interaction) was significantly enriched at an early time point (1 hour post-sciatic nerve transection). At later time points, the number of enriched KEGG pathways initially increased and then decreased. Three KEGG pathways were studied in further detail: cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction, and axon guidance. Moreover, temporal expression patterns of representative differentially expressed genes in these KEGG pathways were validated by real time-polymerase chain reaction. Taken together, the above three signaling pathways are important after sciatic nerve injury, and may increase our understanding of the molecular mechanisms underlying Wallerian degeneration.

Key Words: nerve regeneration; Wallerian degeneration; sciatic nerve transection; peripheral nerve regeneration; microarray; bioinformatic analysis; Kyoto Enrichment of Genes and Genomes; signaling pathway; cytokine-cytokine receptor interaction; neuroactive ligand-receptor interaction; axon guidance; neural regeneration
Introduction

Wallerian degeneration is an important degenerative process that occurs in distal nerve stumps in response to nerve fiber injury (Coleman, 2005; Coleman and Freeman, 2010). It occurs in both the central nervous system and peripheral nervous system, although it normally occurs within 1 to 2 weeks after injury in the peripheral nervous system but does not occur until a few months or even years after injury to the central nervous system (Griffin et al., 1992; George and Griffin, 1994). Timely occurrence of Wallerian degeneration in the peripheral nervous system may contribute to axonal regeneration due to clearance of myelin debris and growth inhibitors, and subsequent establishment of a regenerative microenvironment (Avellino et al., 1995; Vargas and Barnes, 2007). However, delayed Wallerian degeneration in the central nervous system may hinder axonal regeneration. Accordingly, it is believed that Wallerian degeneration plays a key role in nerve regeneration (Lunn et al., 1989; Brown et al., 1991, 1992).

Considering the importance of Wallerian degeneration, numerous studies have been performed to identify underlying biological changes. These studies show that macrophages, monocytes, and Schwann cells work together to remove axon and myelin debris, and clear a path for subsequent axonal regrowth and nerve regeneration (Geuna et al., 2009; Sta et al., 2014). These morphological and genetic studies have identified many central factors, including nicotinamide mononucleotide adenylyltransferase 2 (Coleman and Freeman, 2010; Gilley and Coleman, 2010; Gilley et al., 2013). Furthermore, emerging high-throughput studies have been performed to decipher global molecular changes. For example, in our previous study, we jointly re-annotated and re-analyzed microarray data (Yao et al., 2012, 2013) using bioinformatic tools including Euclidean distance calculation, hierarchical clustering, principle component analysis, gene ontology analysis, Kyoto Enrichment of Genes and Genomes (KEGG) analysis, and Ingenuity Pathway Analysis. Altogether, we obtained an integrated global view of genetic changes in injured distal nerve stumps (Yu et al., 2016; Yi et al., 2017). In particular, KEGG analysis outcomes identified pathways with P-values less than 0.05, indicating they may play critical roles in Wallerian degeneration (Yi et al., 2017).

Taking the importance of signaling pathways into account, in the current study, we examined in detail these significantly enriched pathways in distal nerve stumps at various time points following sciatic nerve transection. Our aim was to achieve greater insight into dynamic molecular changes underlying Wallerian degeneration and identify critical biological processes for treatment of peripheral nerve repair and regeneration.

Materials and Methods

Rat sciatic nerve transection

A total of 66 adult, 2-month-old, male Sprague-Dawley rats weighing 180 to 220 g were obtained from the Experimental Animal Center, Nantong University, China (animal license No. SCXK [Su] 2014-0001 and SYXK [Su] 2012-0031). All animal surgery procedures were performed in accordance with Institutional Animal Care guidelines of Nantong University, and ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (No. 20160229-007). The experiment followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and...
| Classification | Cytokine | Cytokine receptor | Classification | Cytokine | Cytokine receptor |
|----------------|----------|------------------|----------------|----------|------------------|
| Chemokines     | Cxcl1, Cxcl2, Cxcl3/5/6/7 | Il8rb | PDGF Family | Pdgfa, Pdgfb, Pdgfc | Pdgfra |
|                | Cxcl6, Il8 | Il8ra |                |                  |                  |
|                | Cxcl9/10/11/12 | Cxcr3 |            |                  |                  |
|                | Cxcl12 | Cxcr4 |            |                  |                  |
|                | Cxcl13 | Bl1 |            |                  |                  |
|                | Cxcl16 | Cxcr6 |            |                  |                  |
|                | Xcl1/2 | Xcr1 |            |                  |                  |
|                | Cxcl1 | Cx3cr1 |            |                  |                  |
|                | Cd1 | Ccr8 |            |                  |                  |
|                | Cd20 | Ccr6 |            |                  |                  |
|                | Cd25 | Ccr9 |            |                  |                  |
|                | Cd17/22 | Ccr4 |            |                  |                  |
|                | Cd19/21 | Ccr7 |            |                  |                  |
|                | Cd2/7/12/13 | Ccr2 |            |                  |                  |
|                | Cd3/4/5 | Ccr5 |            |                  |                  |
|                | Cd3/5/7/14/15/16/23 | Ccr1 |            |                  |                  |
|                | Cd3/7/8/11/13/15/24/26/28 | Ccr3 |            |                  |                  |
|                | Cd27/28 | Gpr2 |            |                  |                  |
| Hematopoietins | If6 | If6r, Il6st |            |                  |                  |
|                | Il1 | Il11r, Il6st |            |                  |                  |
|                | Osm | Osmtr, Il6st |            |                  |                  |
|                | Lf | Lfmr, Il6st |            |                  |                  |
|                | Ctnf, Bsf3 | Ctnfr, Lfmr, Il6st | |                  |                  |
|                | Ctf1 | Lfmr, Il6st |            |                  |                  |
|                | Cs3 | Csf3r |            |                  |                  |
|                | Lep | Lepr |            |                  |                  |
|                | Il4/13 | Il4r, Il13ra1 |            |                  |                  |
|                | Il12 | Il12rb1/2 |            |                  |                  |
|                | Il23a | Il23r, Il12rb1 |            |                  |                  |
|                | Il2 | Il2ra, Il2rb, Il2rg | |                  |                  |
|                | Il4 | Il4r, Il2rg |            |                  |                  |
|                | Il7 | Il7r, Il2rg |            |                  |                  |
|                | Il9 | Il9r, Il2rg |            |                  |                  |
|                | Il15 | Il15ra, Il2rb, Il2rg | |                  |                  |
|                | Il21 | Il21r, Il21rg |            |                  |                  |
|                | Tslp | Il7r, Tslpr |            |                  |                  |
|                | Epo | Epo |            |                  |                  |
|                | Ght/2 | Ghr |            |                  |                  |
|                | Prl | Prl |            |                  |                  |
|                | Tpo | Mpl |            |                  |                  |
| IL-17 family   | Il17a | Il17r |            |                  |                  |
|                | Il17b | Il17rb |            |                  |                  |

Up-regulated genes are labeled in red, while down-regulated genes are labeled in green. The table was modified based on Kyoto Enrichment of Genes and Genomes Orthology Database (rno04060). Cxcl: Chemokine (C-X-C motif) ligand; Il8rb/b: (Cxcr) interleukin 8 receptor A/B; Cxcr: C-X-C motif chemokine receptor; Blr1: C-X-C motif chemokine receptor type 5; Xcr1: X-C motif chemokine receptor 1; Xcl: Chemokine (C motif) ligand; Cxcl1: C-X-C motif chemokine ligand 1; CX3CR1: chemokine (C-X3-C motif) receptor 1; Ccl: C-C motif chemokine ligand; Csf: colony stimulating factor receptor; Gpr: G-protein regulator; If: interleukin; Ifnar: interleukin 6 receptor; Il6st: interleukin 6 signal transducer; Osm: oncostatin M; Lif: leukemia inhibitory factor; Ctnf: ciliary neurotrophic factor; Bsf: bicoid stability factor; Ctf: cardiotrophin; Csf: colony stimulating factor; Epo: erythropoietin; Gh: growth hormone; Prl: prolactin; Tpo: thyroid peroxidase; Mpl: MPL proto-oncogene; thrombopoietin; Pdgf: platelet derived growth factor; Vegf: vascular endothelial growth factor; Fli: fms related tyrosine kinase; Kdr: kinase insert domain receptor; Hgf: hepatocyte growth factor; Met: met proto-oncogene; Egf: epidermal growth factor; Kif1g: KIT ligand; Ifn: interferon; Tnf: tumor necrosis factor superfamily; Lt: lymphotoxin; Faslg: fas ligand; Cd40lg: CD40 ligand; Acn: ectodysplasin-A; Xdar: ectodysplasin A2 receptor; Tgfb: transforming growth factor beta; Inhb: inhibin beta; Acvr: activin A receptor; Amh: anti-Mullerian hormone; Bmp: bone morphogenetic protein; Gdf: growth differentiation factor.
Rat sciatic nerve transection was performed, as previously described (Yu et al., 2016; Yi et al., 2017). Briefly, Sprague-Dawley rats were equally and randomly divided into eleven groups with 6 rats in each group. Following anesthetization, rat hair was shaved and the surgical area cleansed with 75% ethanol. An incision was made on the lateral aspect of the mid-thigh of the rat left hind limb, the sciatic nerve was lifted, and a 10-mm long segment was removed from the middle of the femur. At 0.5, 1, 6, 12, and 24 hours, 4 days, and 1, 2, 3, and 4 weeks post-sciatic nerve transection, rats were sacrificed by decapitation and distal nerve stumps were collected. Sham-operated rats (rats with sciatic nerves exposed but uninjured) were used as controls and designated as 0 hour post-sciatic nerve transection.

**RNA extraction and microarray analysis**

RNA samples were extracted from distal nerve stumps using Trizol reagent (Life Technology, Carlsbad, CA, USA). Remaining DNA was removed using RNeasy spin columns (Qiagen, Valencia, CA, USA). Purified RNA samples were then quantified using a NanoDrop ND-1000 spectrophotometer (Infinigen Biotechnology Inc., City of Industry, CA, USA).

Microarray analysis was performed in accordance with previous studies (Yao et al., 2012, 2013). Briefly, an Affymetrix GeneChip Oven 640 and Gene Array Scanner 3000 (Affymetrix, Santa Clara, CA, USA) were used to obtain microarray outcomes. These outcomes were then analyzed by the R software platform (v.2.13.0) and limma (linear regression model) package (Ritchie et al., 2015).

| Table 1 List of significantly involved Kyoto Enrichment of Genes and Genomes at various time points during Wallerian degeneration identified by bioinformatic analysis |
|---------------------------------------------------------------|
| Time point | Pathway entry | Pathway name | P value |
| 1 hour | rno04060 | Cytokine-cytokine receptor interaction | 4.62E-02 |
| 1 hour | rno04660 | Hematopoietic cell lineage | 1.63E-03 |
| 1 hour | rno04080 | Neuroactive ligand-receptor interaction | 6.83E-03 |
| 1 hour | rno04660 | Cytokine-cytokine receptor interaction | 9.00E-03 |
| 1 hour | rno04630 | Jak-STAT signaling pathway | 1.28E-02 |
| 1 hour | rno04010 | MAPK signaling pathway | 2.94E-02 |
| 6 hours | rno04080 | Neuroactive ligand-receptor interaction | 7.70E-06 |
| 6 hours | rno04060 | Cytokine-cytokine receptor interaction | 1.26E-04 |
| 6 hours | rno04630 | Jak-STAT signaling pathway | 4.23E-04 |
| 6 hours | rno04640 | Hematopoietic cell lineage | 1.02E-03 |
| 6 hours | rno05200 | Pathways in cancer | 3.86E-03 |
| 6 hours | rno04020 | Calcium signaling pathway | 9.22E-03 |
| 6 hours | rno04360 | Axon guidance | 3.76E-02 |
| 6 hours | rno04910 | Insulin signaling pathway | 4.24E-02 |
| 6 hours | rno05020 | Prion diseases | 4.75E-02 |
| 12 hours | rno04080 | Neuroactive ligand-receptor interaction | 1.35E-02 |
| 12 hours | rno04640 | Hematopoietic cell lineage | 1.15E-02 |
| 12 hours | rno04020 | Calcium signaling pathway | 2.35E-02 |
| 12 hours | rno05219 | Bladder cancer | 4.84E-02 |
| 24 hours | rno04080 | Neuroactive ligand-receptor interaction | 1.31E-04 |
| 24 hours | rno04640 | Hematopoietic cell lineage | 1.15E-02 |
| 24 hours | rno04020 | Calcium signaling pathway | 2.35E-02 |
| 4 days | rno04080 | Neuroactive ligand-receptor interaction | 1.35E-02 |
| 4 days | rno05219 | Bladder cancer | 4.84E-02 |
| 4 days | rno05219 | HCM | 4.92E-02 |
| 1 week | rno04080 | Neuroactive ligand-receptor interaction | 3.74E-03 |
| 1 week | rno05200 | Pathways in cancer | 1.28E-02 |
| 1 week | rno05410 | HCM | 1.78E-02 |
| 1 week | rno05218 | Melanoma | 4.92E-02 |
| 2 weeks | rno05410 | HCM | 3.60E-03 |
| 2 weeks | rno05414 | Dilated cardiomyopathy | 2.45E-02 |
| 2 weeks | rno04080 | Neuroactive ligand-receptor interaction | 4.16E-02 |
| 3 weeks | rno04514 | Cell adhesion molecules | 8.23E-03 |
| 3 weeks | rno04670 | Leukocyte transendothelial migration | 1.18E-02 |
| 3 weeks | rno05410 | HCM | 1.78E-02 |
| 3 weeks | rno04530 | Tight junction | 1.93E-02 |
| 3 weeks | rno05414 | Dilated cardiomyopathy | 2.23E-02 |
| 4 weeks | rno04080 | Neuroactive ligand-receptor interaction | 3.62E-02 |
| 4 weeks | rno04080 | Neuroactive ligand-receptor interaction | 1.72E-03 |
| 4 weeks | rno080910 | Nitrogen metabolism | 4.60E-02 |

MAPK: Mitogen-activated protein kinases; HCM: hypertrophic cardiomyopathy.

"Consensus Author Guidelines on Animal Ethics and Welfare" by the International Association for Veterinary Editors (IAVE).
Bioinformatic analysis
Expression levels of mRNAs at 0.5, 1, 6, 12, and 24 hours, 4 days, and 1, 2, 3, and 4 weeks post-sciatic nerve transection were compared with those at 0 hour post-nerve transection. Genes with fold changes > 2 or < −2 (absolute value of log2 fold change > 1) and adjusted P-values < 0.05 were considered to be differentially expressed. Differentially expressed genes were then systematically analyzed using Database for Annotation, Visualization, and Integrated Discovery to enrich significantly involved KEGG pathways.

Quantitative real time-polymerase chain reaction
RNA samples (0.5 μg) were reverse transcribed to cDNA using the Prime-Script Reagent Kit (TaKaRa, Dalian, China) for subsequent amplification. Quantitative real time-polymerase chain reaction (RT-PCR) was then performed using SYBR Green Premix Ex Taq (TaKaRa) with specific primer pairs on an Applied Biosystems Stepone real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer pair sequences were: Eph receptor A4 (EphA4), (forward) 5′-CGC CGT AGT AGT GGG TG-3′ and (reverse) 5′-GTC TGT TGA CTG GCT CA-3′; met proto-oncogene (Met), (forward) 5′-CGC TGC AGG CTG ATT TA-3′ and (reverse) 5′-GGT GAA ATG TGC TGT GCG AG-3′; interleukin 11 (Il11), (forward) 5′-CGG ACT GGA ACG GCT ACT TC-3′ and (reverse) 5′-GAG GAT TGC GAT GGT GGC TT-3′; prostaglandin E receptor 2 (Ptger2), (forward) 5′-TTC TAT GGC GGA GAC GG-3′ and (reverse) 5′-GGT CCC ACT TTT CCT TCC GGG-3′; and glyceraldehyde 3-phosphate dehydrogenase (Gapdh), (forward) 5′-CCT TCA TGG ACC TCA ACT ACA TG-3′ and (reverse) 5′-CCT CTC CAT GGT GAA GAC-3′. Ct values were obtained for mRNA quantification using the ΔΔCt method and Gapdh as the reference gene.

Statistical analysis
Data were presented as the mean ± SEM. Differences between groups were tested by one-way analysis of variance using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). P-values < 0.05 were considered statistically significant.

Results
Overview of significantly involved KEGG pathways during Wallerian degeneration
Our previous study showed that many KEGG pathways are significantly involved in Wallerian degeneration (Yi et al., 2017). To further study these activated KEGG pathways, we analyzed enriched KEGG pathways at various time points post-sciatic nerve transection. KEGG pathways with P-values less than 0.05 are listed in Table 1.

At 0.5 hour post-sciatic nerve transection, no KEGG pathway was significantly involved. At 1 hour post-nerve transection, only one KEGG pathway, cytokine-cytokine receptor interaction was significantly activated. At 6 hours post-nerve transection, hematopoietic cell lineage, neuroactive ligand-receptor interaction, Jak-STAT signaling pathway, and mitogen-activated protein kinases signaling pathway were also activated. At 12 hours post-nerve transection, more KEGG pathways were involved including those involved in cancer, calcium signaling pathway, axon guidance, insulin signaling pathway, and Prion diseases. At later time points, the number of significantly involved KEGG pathways decreased. Some of the above-mentioned pathways (e.g., neuroactive ligand-receptor interaction) remained highly activated. Some novel KEGG pathways emerged at longer time points post-nerve transection, such as cell adhesion molecules (CAMs) and tight junctions.

Cytokine-cytokine receptor interaction
Cytokine-cytokine receptor interaction (rno04060) was the only KEGG pathway activated immediately post-sciatic nerve transection, and strongly activated thereafter at early stages (1, 6, and 12 hours) following nerve transection. A schematic network of the cytokine-cytokine receptor interaction was built based on the KEGG Orthology Database (http://www.genome.jp/kegg/ko.html), with differentially expressed genes labeled (Table 2). Many chemokines, cytokines, and their corresponding receptors, including C-X-C motif chemokine ligand 2 (Cxcl2), interleukin 6 (Il6), interleukin-11 (Il11), colony stimulating factor 3 receptor (Csf3r), colony stimulating factor 2 (Csf2), MET proto-oncogene, receptor tyrosine kinase (Met), interferon A (Ifna), interleukin 1α (Il1a), and interleukin 1R2 (Il1r2) were significantly up-regulated in injured distal nerve stumps. Only one gene, kinase insert domain receptor (Kdr), a gene that encodes vascular endothelial growth factor receptor, was down-regulated post-scientific nerve transection.

Neuroactive ligand-receptor interaction
Neuroactive ligand-receptor interaction (rno04080) was another KEGG pathway activated at a relatively early time point (6 hours post-sciatic nerve transection). Further, this KEGG pathway was activated at all time points from 6 hours to 4 weeks post-nerve transection, suggesting that it is critical for Wallerian degeneration and subsequent nerve regeneration. Receptors of numerous neurotransmitters and mediators, such as histamine receptor (Htr), prostaglandin E2 receptor 1 (Ptger1), adenosine receptor (Adora), and leukotriene B4 receptor (Ltbr4) were up-regulated. In contrast, dopamine receptor (Drd), neurotensin receptor (Ntsr), MAS proto-oncogene or mas-related G-protein coupled receptor A (Mas1), cysteinyl-leukotriene receptor (Cysltr), corticotropin-releasing hormone receptor (Crhr), metabotropic glutamate receptor (Grm), purinergic receptor P2X (P2rx), and leptin receptor (Lepr) were down-regulated (Table 3).
Table 3 Significant differentially expressed genes in neuroactive ligand-receptor interaction

| Classification | Neuroactive ligand | Receptor |
|----------------|--------------------|----------|
| GPCRs Class A: | Acetylcholine       | Chrm     |
| Rhodopsin like receptors | Dopamine            | Adr      |
|                  | Histamine           | Hrh      |
|                  | 5-Hydroxytryptamine | Htr      |
|                  | Trace amine         | Tar      |
|                  | Angiotensin II, III | Agtr     |
|                  | Apelin              | AgtrI    |
|                  | Bombesin            | Gpr      |
|                  | Bradykinin          | Bdkrb    |
|                  | Anaphylatoxin       | C3ar, C5r|
|                  | Lipoxin A4          | Fpr1     |
|                  | Cholecystokinin     | Cckr     |
|                  | Endothelin          | Edhr     |
|                  | Galanin             | Galr     |
|                  | Ghrelin             | Ghsr     |
|                  | Kiss1 peptide       | Kiss1r   |
|                  | Melanocortin        | Mc1/3/5/4/5c, Mc2r |
|                  | Motilin             | Mhr      |
|                  | Neurexinulin U      | Nnr      |
|                  | Neuropeptide FF     | Ngfrr    |
|                  | Neuropeptide Y      | Npyr     |
|                  | Neuropeptide W/B    | Gpr7/8   |
|                  | Neurotensin         | Ntr      |
|                  | Opioids             | Opr      |
|                  | Orexin              | Octr     |
|                  | Oxytocin            | Oxtr     |
|                  | Somatostatin        | Sitr     |
|                  | Tachykinin          | Tac1/1/2/3 |
|                  | Urotensin II        | Uts2r    |
|                  | Vasopressin         | Avpr     |
|                  | Proteinase-activated like | Prar |
|                  | Prolactin-releasing peptide | Prlr |
|                  | Melanin-concentrating hormone | Mchrr |
|                  | Fish                | Fshr     |
|                  | Lh                  | Lhger    |
|                  | Tsh                 | Tshr     |
|                  | Prostaglandin PGD2  | Prgrr    |
|                  | Pg2                 | Piger    |
|                  | Pg2/alpha           | Pgfr     |

| Classification | Neuroactive ligand | Receptor |
|----------------|--------------------|----------|
| GPCRs Class B: Secretin like | Corticotropin releasing hormone | Ccr |
| Metabotropic glutamate Channels | N-Acetylaspartyl-gluatmate | Grn |
| Other receptors | Glutamate, L-Aspartate, L-Cysteic acid | Gri |
|                  | L-Homocysteic acid | Ntr |
|                  | Glycine, β-Alanine, Taurine | Ghr |
|                  | N-Arachidonoyl-dopamine, N-Oleoyl-dopamine | Trpv1 |
|                  | Acetylcholine      | Ghnm     |
|                  | Naucleotides       | P2x4 |
|                  | Glutamate, L-Aspartate, L-Cysteic acid | Ghr |
|                  | Acetylcholine      | Ghrn     |

Up-regulated genes are labeled in red, while down-regulated genes are labeled in green. The table was modified based on Kyoto Enriched Database of Genes and Genomes Orthology Database (rno04080). Gpcs: G-protein-coupled receptors; Chrm: cholinergic receptor; Adr: adrenaline receptor; Dred: dopamine receptor; Hrh: histamine receptor; Htr: 5-hydroxytryptamine receptor; Tar: trace amine receptor; Agtr: angiotensin II receptor; Agtr1: apelin receptor; Gpr: gastrin releasing peptide receptor; Bdkrb: bradykinin receptor; beta; C3ar/c5r: anaphylatoxin receptor; Fpr1: formyl peptide receptor; Ghr: corticotropin releasing hormone receptor; Edhr: endothelin receptor; Galr: galanin receptor; Ghsr: growth hormone secretagogue receptor; Kiss1: kiss receptor; Mhr: melanocortin receptor; Nnr: neurexinulin U receptor; Ngfrr: neuropeptide FF receptor; Npyr: neuropeptide Y receptor; Gpr: neuropeptides B and W receptor; Ntr: neuropeptide receptor; Opr: opioid receptors; Octr: octocin receptor; Oxytr: oxytocin receptor; Sitr: somatostatin receptor; Tacr: tachykinin receptor; Ut2r: urotensin 2 receptor; Avpr: arginine vasopressin receptor; Par: proteinase-activated like receptor; Prlr: prolactin releasing hormone receptor; Mchrr: melanin-concentrating hormone receptor; Fshr: follicle stimulating hormone receptor; Lhger: luteinizing hormone; Lhger: luteinizing hormone/chioriogonadotropin receptor; Prgrr: prostaglandin D receptor; Piger: prostatinergen hormone receptor; Pfrr: prostatinergen FF receptor; P2x2: prostacyclin receptor; Pger2: phosphorus receptor; Adora: adenosine A receptor; P2y: purinoceptor; Cnrt: cannabinoid receptor type 1; P2af: platelet-activating factor receptor; Gnrhr: gonadotropin releasing hormone receptor; Trh: thyrotropin releasing hormone receptor; Mhr: melanotin receptor; Egr: ed5; lysophosphatidic acid; St1p: sphingosine-1-phosphate; Edgs: endothelial differentiation genes; Lhrh: luteinising hormone B4 receptor; Mas1: MAS1 proto-oncogene G-protein-coupled receptor; Rsfr: relaxin/insulin like family peptide receptor; Ccylr: cysteinyl-leukotriene receptor; Calcr: calciitonin receptor; Ccr: corticotropin releasing hormone receptor; Gpr: gastrin releasing peptide receptor FTaylor: glucagon receptor; Glcgr: glucagon-like peptide receptor; Ghrhsr: growth hormone releasing hormone receptor; Pfrr: parathyroid hormone receptor; Pacapr: pituitary adenylate cyclase activating polypeptide type 1 receptor; Scrtr: secretin receptor; Vipr: vasoactive intestinal peptide receptor; Gnmr: glutamate receptor metabotropic; Gabr: gamma-aminobutyric acid; Gabbr: GABA B receptor; Glnr: glutamate ionotropic receptor NMDA type subunits; Gbr: GABA receptor; Chmr: cholinergic receptor; P2x2: purineric receptor P2X2; Gji: glutamate receptor; Glr: glycine receptor; Trpv1: transient receptor potential cation channel subfamily V member 1; Bzfr: tracer protein; Nsr31: nuclear receptor subfamily 3 group C member 1; Ghr: growth hormone receptor; Thr: thyroid hormone receptor; Lepr: leptin receptor; Prlr: prolactin receptor.
successful nerve regeneration and functional reconstruction. Accordingly, *Met*, a previously identified activated gene in the cytokine-cytokine receptor interaction pathway, was also involved in the axon guidance pathway. In addition, the ephrin receptor, *Epha*, was also up-regulated while the netrin receptor, *Unc-5*, was down-regulated (Figure 1).

**RT-PCR verification of microarray results**

RT-PCR experiments were performed to validate temporal expression patterns of representative differentially expressed genes involved in the KEGG pathways: cytokine-cytokine receptor interaction (*Il11*), neuroactive ligand-receptor interaction (*Ptger*), and axon guidance (*Epha* and *Met*). Consistent with our microarray analysis outcomes, RT-PCR confirmed that *Epha*, *Met*, *Il11*, and *Ptger* expression levels were robustly increased, especially at longer time points post-sciatic nerve transection (Figure 2).

**Discussion**

Wallerian degeneration helps form a penetrable pathway for axon regrowth, and consequently, is very important for subsequent nerve repair and regeneration (Bittner et al., 2016). Until now, many critical factors for Wallerian degeneration have been identified. However, Wallerian degeneration is a complex biological process mediated by a group of molecules instead of a single gene or protein. Thus, obtaining a global view of molecular changes during Wallerian degeneration is of utmost importance. Accordingly here, by combined use of microarray and bioinformatic analysis, we synthetically analyzed highly activated KEGG signaling pathways (cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction, and axon guidance signaling pathway) following sciatic nerve transection.

Consistent with previous observations (Li et al., 2013; Yao et al., 2013; Yi et al., 2017), the KEGG pathway, cytokine-cytokine receptor interaction, was significantly involved from an early stage post-sciatic nerve injury. Detailed study of the cytokine-cytokine receptor interaction KEGG pathway suggests that most differentially expressed genes are related to immunoregulatory, inflammatory, and host defense processes such as *Cxcl2*, *Il6*, *Il11*, *Csf3r*, *Csf2*, *Il1a*, and *Il1r2*. Indeed, Wallerian degeneration has been thought of as an innate-immune response to external injuries (Rotshenker, 2011). Our outcomes demonstrate that immune reactions and inflammatory responses are initiated at an acute phase post-nerve injury, and suggest that innate immune and inflammatory responses in Wallerian degeneration might be critical for successful nerve regeneration and functional reconstitution.

The KEGG pathway, neuroactive ligand-receptor interaction, was significantly enriched at later time points (6 hours to 4 weeks post-nerve injury). Differentially expressed genes in this KEGG pathway are mainly neurotransmitter receptors (e.g., *Drd*, *Htr*, *Ntsr*, *Ptger*, *Adora*, *Grm*, and *P2rx*). It has been demonstrated that certain neurotransmitters can affect Wallerian degeneration, namely adenosine, guanosine, adenosine triphosphate, and adenosine (Press and Milbrandt, 2009; Shin et al., 2014). From the genetic aspect, our outcomes show that neurotransmitters and their receptors might be involved in Wallerian degeneration.

The axon guidance signaling pathway was also investigated. We found that receptors of semaphorins (*Met*), ephrins (*Epha*), and netrins (*Unc-5*) are differentially expressed following sciatic nerve transection. Semaphorins, ephrins, and netrins are proteins highly related to axon guidance. They play chemotropic roles during axon growth and attract a growing axon to move towards or away from higher concentrated regions. Interestingly, *Met* and *Epha* show differential expression patterns of representative differentially expressed genes involved in the KEGG pathways: cytokine-cytokine receptor interaction (*Il11*), neuroactive ligand-receptor interaction (*Ptger*), and axon guidance (*Epha* and *Met*). Consistent with our microarray analysis outcomes, RT-PCR confirmed that *Epha*, *Met*, *Il11*, and *Ptger* expression levels were robustly increased, especially at longer time points post-sciatic nerve transection (Figure 2).
temporal expression patterns compared with Unc-5, suggesting their roles in axon guidance and nerve regeneration might be different. Further studies will be performed to clarify their specific effects.

However, it is worth noting that although we obtained some knowledge of dynamic molecular changes and essential signaling pathways during Wallerian degeneration, it remains unclear which cell types mediate these dynamic changes. Schwann cells and macrophages are important cells in distal nerve stumps, and consequently these dynamic changes may occur in these cell types. In our future studies, single cell sequencing will be performed to further decipher temporal expression patterns of genes in each cell type, and the specific role of each cell type during Wallerian degeneration.

Taken together, we systematically investigated significantly enriched KEGG pathways in distal nerve stumps following sciatic nerve transection, and identified critical KEGG pathways for peripheral nerve repair and regeneration. Our results may help elucidate critical genes, signaling pathways, and biological processes during Wallerian degeneration, and might contribute to identification of potential treatments for peripheral nerve repair and regeneration.

Author contributions: SY conceived and designed the experiments, and wrote the paper. QC, YYW, JY, and SY performed the experiments and analyzed the data. QC and SY provided reagents/materials/analysis tools. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Research ethics: The study protocol was approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (No. 20160229-007). The experiment followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and “Consensus Author Guidelines on Animal Ethics and Welfare” produced by the International Association for Veterinary Editors (IAVE). The article was prepared in accordance with the “Animal Research: Reporting of In Vivo Experiments Guidelines” (ARRIVE Guidelines).

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