Critical signaling pathways during Wallerian degeneration of peripheral nerve
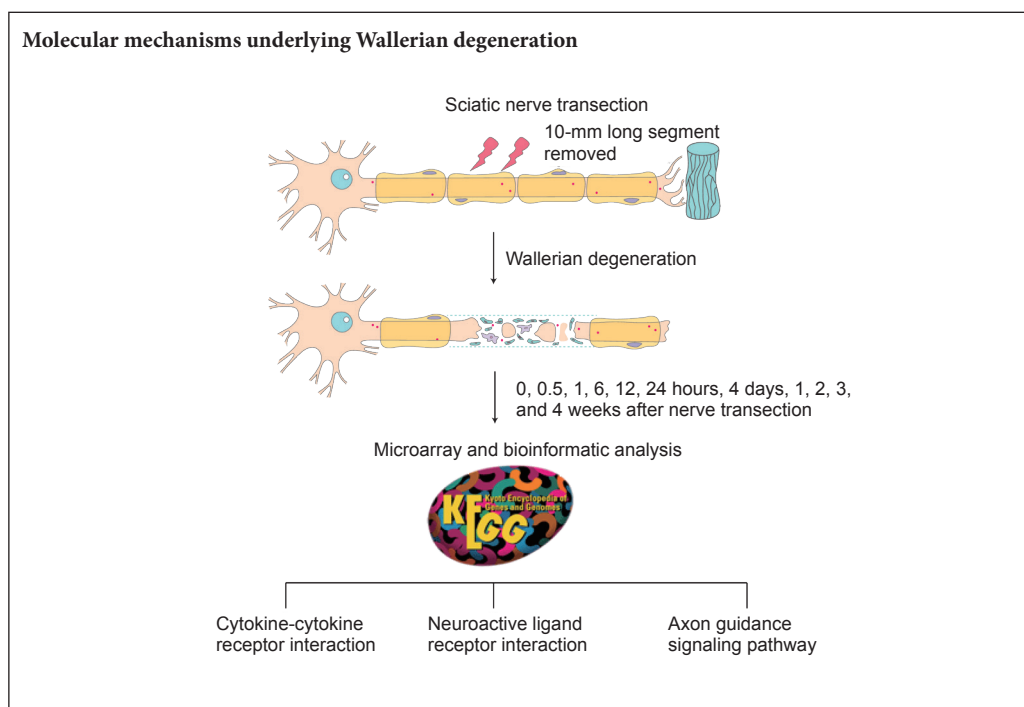
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Graphical Abstract

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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 Barres, 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
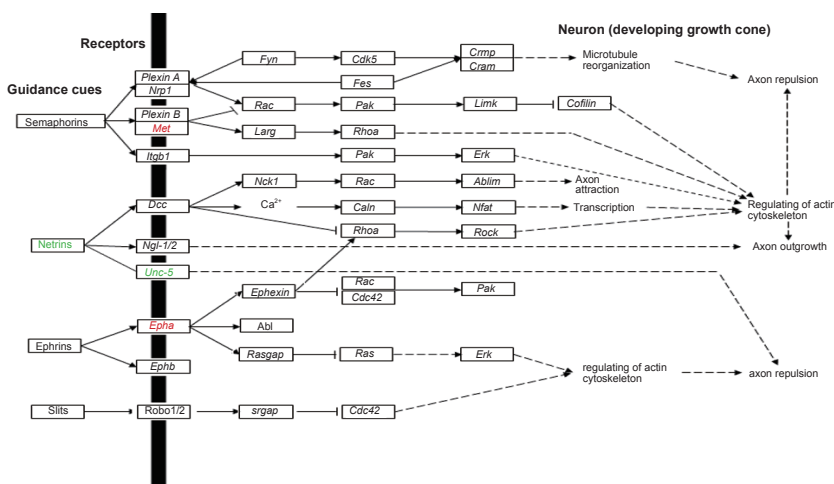

Figure 1 Significant differentially expressed genes in axon guidance signaling.
Up-regulated genes are labeled in red, while down-regulated genes are labeled in green. The figure was modified based on Kyoto Enrichment of Genes and Genomes Orthology Database (rno04360). *Fyn*: fyn proto-oncogene; *Cdk5*: cyclin dependent kinase 5; *Crmp*: collapsing response mediator protein; *Cram*: dihydropyrimidinase like 5; *Nrp1*: neuropilin 1; *Fes*: feline sarcoma oncogene; *Rac*: Rac protein; *Pak*: p21-activated kinase; *Limk*: LIM domain kinase; *Met*: met proto-oncogene; *Larg*: rho guanine nucleotide exchange factor 12; *Rhoa*: ras homolog family member A; *Itgb1*: integrin subunit beta 1; *Erk*: extracellular regulated MAP kinase; *Dcc*: deleted in colorectal carcinoma netrin 1 receptor; *Nck1*: non-catalytic region of tyrosine kinase adaptor protein 1; *Ablim*: actin binding LIM protein; *Caln*: protein phosphatase 3 catalytic subunit alpha; *Nfat*: nuclear factor of activated T-cells; *Rock*: rho kinase; *Ngl-1/2*: netrin-G1 ligand 1/2; *Unc-5*: netrin receptor unc-5; *Epha*: Eph receptor A; *Cdc42*: cell division cycle 42; *Abl*: Abelson murine leukemia viral oncogene homolog; *Rasgap*: RAS p21 protein activator 1; *Ephb*: Eph receptor B; *Robo1/2*: roundabout homolog 1/2; *srgap*: SLIT-ROBO rho GTPase activating protein.

Table 2 Significant differentially expressed genes in cytokine-cytokine receptor interaction

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		Pdgfa, Pdgfb, Pdgfd	Fdgfrb
	Cxcl9/10/11/12	Cxcr3		Vegfa, Vegfb	Flt1, *Kdr*
	Cxcl12	Cxcr4		Vegfa, Vegfe, Vegfod	*Kdr*
	Cxcl13	Blr1		Vegfod	Flt4
	Cxcl16	Cxcr6		Hgf	*Met*
	Xcl1/2	Xcr1		Egf	Egfr
	Cx3cl1	Cx3cr1		Csf1	Csf1r
	Ccl1	Ccr8		Kitlg	Kit
	Ccl20	Ccr6		Flt3lg	Flt3
	Ccl25	Ccr9	Interferon family	*Ifna*, Ifnb1, Ifnw1, Ifnk, Ifnt1	Ifnar1/2
	Ccl17/22	Ccr4		Ifng	Ifngr1/2
	Ccl19/21	Ccr7	IL-10 family	Il10	Il10ra, Il10rb
	Ccl2/7/12/13	Ccr2		Il19, Il20	Il20ra, Il20rb
	Ccl3/4/5	Ccr5		Il20, Il24	Il22ra1, Il20rb
	Ccl3/5/7/14/15/16/23	Ccr1		Il22	Il22ra1, Il10rb,
	Ccl5/7/8/11/13/15/24/26/28	Ccr3			Il22ra2
	Ccl27/28	Gpr2		Il28A/B, Il29	Il28ra, Il10rb
Hematopoietins	*Il6*	Il6r, Il6st	TNF family	Tnfsf10	Sf10a/b/c/d, Sf11b
	Il11	Il11ra, Il6st		Tnfsf11	Sf11a, Sf11b
	Osm	Osmr, Il6st		Tnfsf12	Sf12a
	Lif	Lifr, Il6st		Tnf	Sf1a, Sf1b
	Cntf, Bsf3	Cntfr, Lifr, Il6st		Lta	Sf1a, Sf1b, Ltbr, Sf14
	Ctf1	Lifr, Il6st		Ltb	Ltbr
	Csf3	*Csf3r*		Tnfsf14	Ltbr, Sf14, Sf6b
	Lep	Lepr		Faslg	Sf6b, Fas
	Il4/13	Il4r, IL13ra1		Cd40lg	Cd40
	Il12	Il12rb1/2		Tnfsf7	Sf7
	Il23A	Il23R, Il12rb1		Tnfsf8	Sf8
	Csf2	Csf2ra/b		Tnfsf9	Sf9
	Il3	Il3ra, Csf2rb		Tnfsf4	Sf4
	Il5	Il5ra, Csf2rb		Tnfsf18	Sf18
	Il2	Il2ra, Il2rb, Il2rg		Tnfsf13	Sf17
	Il4	Il4r, Il2rg		Tnfsf13b	Sf17, Sf13b, Sf13c
	Il7	Il7r, Il2rg		Eda	Edar, Xedar
	Il9	Il9r, Il2rg	TGF-β family	Tgfb1, Tgfb2, Tgfb3	Tgfbr1, Tgfbr2
	Il15	Il15ra, Il2rb, Ir2rg		INHBA, INHBB, INHBC, INHBE	Acvr1, Acvr2
	Il21	Il21r, Il21rg		Amh	Amhr2, Acvr1
	Tslp	Il7r, Tslpr		Bmp2	Bmpr2, Acvr1,
	Epo	Epor			Bmpr1a/b
	Gh1/2	Ghr		Bmp7	Acvr2, Bmpr1b
	Prl	Prlr		Gdf5	Acvr2, Bmpr1a
	Tpo	Mpl	IL-1 family	Il17b, Il17e	Il17rb
IL-17 family	Il17a	Il17r		*Il1A*, Il1b	Il1r1, *Il1r2*, Il1rap
	Il17b, Il17e	Il17rb		Il18	Il18r1, Il18rap

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; *Il8ra/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; *Cx3cl1*: C-X3-C motif chemokine ligand 1; *CX3CR1*: chemokine (C-X3-C motif) receptor 1; *Ccl*: C-C motif chemokine ligand; *Ccr*: chemokine (C-C motif) receptor; *Gpr*: G-protein regulator; *Il*: interleukin; *Il6r*: interleukin 6 receptor; *Il6st*: interleukin 6 signal transducer; *Osm*: oncostatin M; *Lif*: leukemia inhibitory factor; *Cntf*: ciliary neurotrophic factor; *Bsf*: bicoid stability factor; *Ctf*: cardiotrophin; *Csf*: colony stimulating factor; *Lep*: leptin; *Tslp*: thymic stromal lymphopoietin; *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; *Flt*: fms related tyrosine kinase; *Kdr*: kinase insert domain receptor; *Hgf*: hepatocyte growth factor; *Met*: met proto-oncogene; *Egf*: epidermal growth factor; *Kitlg*: KIT ligand; *Ifn*: interferon; *Tnfsf*: tumor necrosis factor superfamily; *Lt*: lymphotoxin; *Faslg*: fas ligand; *Cd40lg*: CD40 ligand; *Eda*: ectodysplasin-A; *Xedar*: 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.

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
6 hours	rno04640	Hematopoietic cell lineage	1.63E-03
	rno04080	Neuroactive ligand-receptor interaction	6.83E-03
	rno04060	Cytokine-cytokine receptor interaction	9.00E-03
	rno04630	Jak-STAT signaling pathway	1.28E-02
	rno04010	MAPK signaling pathway	2.94E-02
12 hours	rno04080	Neuroactive ligand-receptor interaction	7.70E-06
	rno04060	Cytokine-cytokine receptor interaction	1.26E-04
	rno04630	Jak-STAT signaling pathway	4.23E-04
	rno04640	Hematopoietic cell lineage	1.02E-03
	rno05200	Pathways in cancer	3.86E-03
	rno04020	Calcium signaling pathway	9.22E-03
	rno04360	Axon guidance	3.76E-02
	rno04910	Insulin signaling pathway	4.24E-02
	rno05020	Prion diseases	4.75E-02
24 hours	rno04080	Neuroactive ligand-receptor interaction	1.31E-04
	rno04640	Hematopoietic cell lineage	1.15E-02
	rno04020	Calcium signaling pathway	2.35E-02
4 days	rno04080	Neuroactive ligand-receptor interaction	1.35E-02
	rno05219	Bladder cancer	4.84E-02
1 week	rno04080	Neuroactive ligand-receptor interaction	3.74E-03
	rno05200	Pathways in cancer	1.26E-02
	rno05410	HCM	1.78E-02
	rno05218	Melanoma	4.92E-02
2 weeks	rno05410	HCM	3.60E-03
	rno05414	Dilated cardiomyopathy	2.45E-02
	rno04080	Neuroactive ligand-receptor interaction	4.16E-02
3 weeks	rno04514	Cell adhesion molecules	8.23E-03
	rno04670	Leukocyte transendothelial migration	1.18E-02
	rno05410	HCM	1.78E-02
	rno04530	Tight junction	1.93E-02
	rno05414	Dilated cardiomyopathy	2.23E-02
	rno04080	Neuroactive ligand-receptor interaction	3.62E-02
4 weeks	rno04080	Neuroactive ligand-receptor interaction	1.72E-03
	rno00910	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).

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).

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 significant 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 ATC AGT GGG TG-3′ and (reverse) 5′-GTC TGT TCG GTA CTG GCT CA-3′; met proto-oncogene (*Met*), (forward) 5′-CGC TGC AGG CTG TGG ATT TA-3′ and (reverse) 5′-GGT GAA ATG TGC TGT GCG AG-3′; interleukin 11 (*Il11*), (forward) 5′-CCG ACT GGA ACG GCT ACT TC-3′ and (reverse) 5′-GAC GAT GTC 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 TTC GGG-3′; and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), (forward) 5′-CCT TCA TTG ACC TCA ACT ACA TG-3′ and (reverse) 5′-CTT 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 1A (*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-sciatic 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 (*Ptger*), adenosine receptor (*Adora*), and leukotriene B4 receptor (*Ltb4r*) 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**).

Axon guidance
Besides cytokine-cytokine receptor interaction and neuroactive ligand-receptor interaction, two KEGG pathways significantly enriched at various time points during Wallerian degeneration, axon guidance signaling pathway (rno04360) was a KEGG pathway activated at 12 hours post-nerve transection, and also studied in detail based on its importance for

Table 3 Significant differentially expressed genes in neuroactive ligand-receptor interaction

Classification	Neuroactive ligand	Receptor	Classification	Neuroactive ligand	Receptor
GPCRs Class A: Rhodopsin like receptors	Acetylcholine	*Chrm*	GPCRs Class A: Rhodopsin like receptors	Pgi2	*Ptgir*
	Epinephrine, norephinephrine	*Adr*		Thromoxane A2	*Tbxa2r*
	Dopamine	*Drd*		Adenosine	*Adora*
	Histamine	*Hrh*		Nucleotides	*P2ry*
	5-Hydroxytrypatamine	*Htr*		Anandamide	*Cnr1*
	Trace amine	*Tar*		Platelet-activating factor	*Ptafr*
	Angiotensin II, III	*Agtr*		Gonadotropin-releasing hormone	*Gnrhr*
	Apelin	*Agtrl1*		Thyrotropin-releasing hormone	*Trhr*
	Bombesin	*Grpr*		Melatonin	*Mtnr*
	Bradykinin	*Bdkrb*		Lysophosphatidic acid	*Edgl*
	Anaphylatoxin	*C3ar, C5r*		S1P, dihydro-S1P	*Edgs*
	Lipoxin A4	*Fprl*		Leukotriene B4	*Ltb4r*
	Cholecystokinin	*Cckr*		Masproto-oncogene	*Mas1*
	Endothelin	*Ednr*		Relaxin	*Rxfp*
	Galanin	*Galr*		Cysteinyl-leukotriene	*Cysltr*
	Ghrelin	*Ghsr*	GPCRs Class B: Secretin like	Calcitonin	*Calcr*
	Kiss1 peptide	*Kiss1r*		Corticotropin releasing hormone	*Crhr*
	Melanocortin	*Mc1/3/4/5r, Mc2r*		Gastric inhibitory peptide	*Gipr*
	Motilin	*Mlnr*		Glucagon	*Gcgr*
	Neuromedin U	*Nmur*		Glucagon-like peptide	*Glpr*
	Neuropeptide FF	*Npffr*		Growth hormone-releasing hormone	*Ghrhr*
	Neuropeptide Y	*Npyr*		Parathyroid hormone	*Pthr*
	Neuropeptide W/B	*Gpr7/8*		*Pacap*	*Pacapr*
	Neurotensin	*Ntsr*		Secretin	*Sctr*
	Opioids	*Opr*	GPCRs Class C: Metabotropic glutamate	Metabotropic glutamate	*Grm*
	Orexin	*Hcrtr*		*Gaba*	*Gabbr*
	Oxytocin	*Oxtr*	Channels Other receptors	N-Acetylaspartyl-glutamate	*Grin*
	Somatostatin	*Sstr*		*Gaba*	*Gabr*
	Tachykinin	*Tacr1/2/3*		Acetylcholine	*Ghrn*
	Urotensin II	*Uts2r*		Nuclotides	*P2rx*
	Vasopressin	*Avpr*		Glutamate, L-Aspartate, L-Cysteic acid, L-Homocysteic acid	*Gri*
	Proteinase-activated like	*Par*			
	Prolactin-releasing peptide	*Prlhr*		Glycine, β-Alanine, Taurine	*Glr*
	Melanin-concentrating hormone	*Mchr*		N-Arachidonoyl-dopamine, N-Oleoyl-dopamine, Anandamide, Palmitoyl-ethanolamide	*Trpv1*
	Fsh	*Fshr*		Porphyrins	*Bzrp*
	Lh	*Lhcgr*		Cortisol	*Nr3c1*
	Tsh	*Tshr*		Growth hormone	*Ghr*
	Prostaglandin PGD2	*Ptgdr*		Triiodothyronine thyroxine	*Thr*
	Pge2	*Ptger*		Leptin	*Lepr*
	Pgf2-alpha	*Ptgfr*		Prolactin	*Prlr*

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 (rno04080). *Gpcrs*: G-protein-coupled receptors; *Chrm*: cholinergic receptor; *Adr*: adrenaline receptor; *Drd*: dopamine receptor; *Hrh*: histamine receptor; *Htr*: 5-hydroxytrypatamine receptor; *Tar*: trace amine receptor; *Agtr*: angiotensin II receptor; *Agtrl1*: apelin receptor; *Grpr*: gastrin releasing peptide receptor; *Bdkrb*: bradykinin receptor: beta; *C3ar/c5r*: anaphylatoxin receptor; *Fprl*: formyl peptide receptor; *Cckr*: cholecystokinin receptor; *Ednr*: endothelin receptor; *Galr*: galanin receptor; *Ghsr*: growth hormone secretagogue receptor; *Kiss1r*: kiss1 receptor; *Mcr*: melanocortin receptor; *Mlnr*: motilin receptor; *Nmur*: neuromedin U receptor; *Npffr*: neuropeptide FF receptor; *Npyr*: neuropeptide Y receptor; *Gpr*: neuropeptides B and W receptor; *Ntsr*: neurotensin receptor; *Opr*: opioids receptor; *Hcrtr*: hypocretin (orexin) receptor; *Oxtr*: oxytocin receptor; *Sstr*: somatostatin receptor; *Tacr*: tachykinin receptor; *Uts2r*: urotensin 2 receptor; *Avpr*: arginine vasopressin receptor; *Par*: proteinase-activated like receptor; *Prlhr*: prolactin releasing hormone receptor; *Mchr*: melanin-concentrating hormone receptor; *Fshr*: follicle stimulating hormone receptor; *Lh*: luteinizing hormone; *Lhcgr*: luteinizing hormone/choriogonadotropin receptor; *Ptgdr*: prostaglandin D receptor; *Ptger*: prostaglandin E receptor; *Ptgfr*: prostaglandin F receptor; *Pgi2*: prostacyclin; *Ptgir*: prostacyclin receptor; *Tbxa2r*: thromboxane A2 receptor; *Adora*: adenosine A receptor; *P2ry*: P2Y purinoceptor; *Cnr1*: cannabinoid receptor type 1; *Ptafr*: platelet-activating factor receptor; *Gnrhr*: gonadotropin releasing hormone receptor; *Trhr*: thyrotropin releasing hormone receptor; *Mtnr*: melatonin receptor; *Edgl*: lysophosphatidic acid receptor; *S1p*: sphingosine-1-phosphate; *Edgs*: endothelial differentiation genes; *Ltb4r*: leukotriene B4 receptor; *Mas1*: MAS1 proto-oncogene: G-protein-coupled receptor; *Rxfp*: relaxin/insulin like family peptide receptor; *Cysltr*: cysteinyl-leukotriene receptor; *Calcr*: calcitonin receptor; *Crhr*: corticotropin-releasing hormone receptor; *Gipr*: gastric inhibitory polypeptide receptor; *Gcgr*: glucagon receptor; *Glpr*: glucagon-like peptide receptor; *Ghrhr*: growth hormone releasing hormone receptor; *Pthr*: parathyroid hormone receptor; *Pacapr*: pituitary adenylate cyclase-activating polypeptide type I receptor; *Sctr*: secretin receptor; *Vipr*: vasoactive intestinal peptide receptor; *Grm*: glutamate receptor metabotropic; *Gaba*: gamma-aminobutyric acid; *Gabbr*: GABA B receptor; *Grin*: gutamate ionotropic receptor NMDA type subunits; *Gabr*: GABA receptor; *Chrn*: cholinergic receptor; *P2rx*: purinergic receptor P2X; *Gri*: glutamate receptor; *Glr*: glycine receptor; *Trpv1*: transient receptor potential cation channel subfamily V member 1; *Bzrp*: translocator protein; *Nr3c1*: nuclear receptor subfamily 3 group C member 1; *Ghr*: growth hormone receptor; *Thr*: thyroid hormone receptor; *Lepr*: leptin receptor; *Prlr*: prolactin receptor.
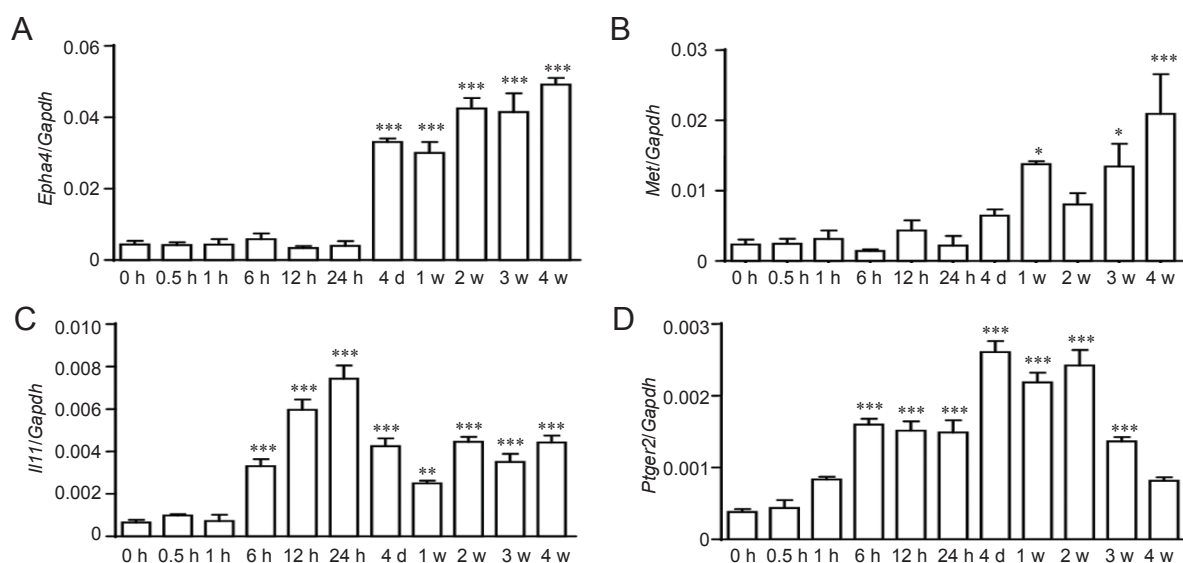
Figure 2 Quantitative real time-polymerase chain reaction for mRNA expression of *Epha4* (A), *Met* (B), *Il11* (C), and *Ptger2* (D).
Relative levels were normalized to *Gapdh*. Summarized data are from three independent experiments. Values are shown as the mean ± SEM. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, *vs.* 0 hour (one-way analysis of variance). *Epha4*: Eph receptor A4; *Met*: met proto-oncogene; *Il11*: interleukin 11; *Ptger2*: prostaglandin E receptor 2; *Gapdh*: glyceraldehyde 3-phosphate dehydrogenase; h: hour(s); d: days; w: week(s).

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 *Epha4*, *Met*, *Il11*, and *Ptger2* 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,

2014; 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*, *Ifna*, *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

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.

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