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Research Paper

DLK mediates the neuronal intrinsic immune response and regulates glial reaction and neuropathic pain

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ABSTRACT

Inflammatory response triggered by nerve injury plays important roles in the development of neurological disorders, such as neuropathic pain. The signaling events leading to inflammation in the nervous system remain poorly understood. Here, by deleting *Dlk* in sensory neurons driven by *Wnt1a-Cre*, we show that dual leucine zipper kinase (DLK) is required for the neuronal intrinsic immune response to induce cytokines and chemokines such as *Ccl2*, *Ccl7*, and *Ccl12* upon nerve injury. The DLK-controlled injury response in sensory neurons could regulate CD11b$^+$ immune cell infiltration in the dorsal root ganglia, as well as microgliosis and astrogliosis in the spinal dorsal horn but not the ventral horn. Deficiency of *Dlk* drastically alleviates the neuropathic pain elicited by chronic constriction injury of the sciatic nerve. Thus, DLK is an essential component that mediates the neuronal intrinsic immune response to nerve injury in sensory neurons and regulates inflammation in the spinal cord.

1. Introduction

The length of axons causes unique challenges for neurons to maintain their structural integrity and exert proper function. Nerve injuries can trigger profound inflammatory reactions in the nervous system, contributing to neurological disorders such as neurodegeneration and neuropathic pain (Grace et al., 2014; Russo and McGavern, 2016; Witcher et al., 2015). In the somatosensory nervous system, injury to the sensory axons in the periphery could lead to inflammation in the somatosensory pathway, which promotes development of neuropathic pain, a chronic condition characterized by allodynia with decreased pain threshold and hyperalgesia with increased response to noxious stimuli. The underlying molecular mechanism mediating the inflammatory response has been investigated to determine potential therapeutic options.

Previous studies have provided evidence that neurons could elicit intrinsic immune responses under pathological or physiological conditions. For instance, it was reported that sensory neurons could express certain cytokines and chemokines, such as *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12*, after nerve injury (Guan et al., 2016; Murphy et al., 1995; Schreiber et al., 2001; Wang et al., 2018). In addition, neurons in the central nervous system could produce a collection of cytokines and chemokines against viral infection (Hou et al., 2013; Limatola and Ransohoff, 2014; Mukherjee et al., 2013; Old and Malcangio, 2012). We recently found that sensory neurons engage the SARM1-JNK-cJun signal axis, which responds to distal axonal damage and mediates an intrinsic neuronal immune response, resulting in the production of chemokines and cytokines, including *Ccl2*, *Ccl7,* and *Csf1*, and infiltration of CD11b$^+$ immune cells into the dorsal root ganglia (DRG) (Wang et al., 2018). In-depth studies of the neuronal intrinsic immune response would help us better understand the underlying mechanism of the frequently observed inflammation associated with neurological diseases (Heneka et al., 2014; Xanthos and Sandkuhler, 2014).

Dual leucine zipper kinase (DLK; *Map3k12*) plays pleiotropic roles in the neuron pathophysiology, including neuronal survival and death, axon degeneration and regeneration, and neuropathic pain (Ghosh et al., 2011; Hammarlund et al., 2009; Huntwork-Rodriguez et al., 2013; Larhammar et al., 2017; Le Pichon et al., 2017; Miller et al., 2009; Sheu et al., 2018; Shin et al., 2012; Simon et al., 2016; Watkins et al., 2013; Wlaschin et al., 2018; Yan et al., 2009). In the injured axons segregated from the neuronal cell bodies, DLK functions redundantly with three other MAP3K family members, MEKK4, MLK2, and MAP3K13 (also known as LZK), to activate JNK for axonal self-destruction, namely, Wallerian degeneration (Gerdts et al., 2016; Le

Abbreviations: DLK, Dual leucine zipper kinase; STAT3, signal transducer and activator of transcription 3; DRG, dorsal root ganglia; SNI, sciatic nerve injury; CCI, chronic constriction injury; PWT, paw withdrawal threshold; PWL, paw withdrawal latency

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Pichon et al., 2017; Miller et al., 2009; Summers et al., 2018; Welsbie et al., 2017; Yang et al., 2015). Interestingly, genetic ablation or pharmacological inhibition of DLK has recently been shown to drastically mitigate neuropathic pain triggered by spared nerve injury to branches of the sciatic nerve, suggesting its prominent role in promoting pathological pain (Wlaschin et al., 2018). As a chronic condition afflicting above 7% of the general population (Bouhassira et al., 2008; Colloca et al., 2017), treatment of neuropathic pain stands as an unmet medical need. Understanding the signaling events mediated by DLK will therefore provide key molecular insights into precision intervention of the pain conditions.

In this study, through genetic deletion of *Dlk* in sensory neurons driven by *Wnt1a-Cre* (Danielian et al., 1998; Lewis et al., 2013), we show that DLK plays critical roles in regulating neuronal intrinsic immune response *in vitro* and in response to acute and chronic sciatic nerve injury *in vivo*. The DLK-controlled injury response mediated the inflammation in the DRG and spinal dorsal horn in response to nerve injury. DLK was important for production of cytokines, chemokines, and other immune-related genes leading to infiltration of the immune cells into the DRG, and for the glial reaction in the spinal dorsal horn. In addition, *Dlk* deficiency alleviated neuropathic pain elicited by chronic constriction injury of the sciatic nerve. The revealed function of DLK in neuronal immune signaling may indicate new therapeutic opportunities for neuropathic pain targeting the inflammatory response.

2. Materials and methods

2.1. Antibodies

Primary antibodies used in this study were mouse anti-Flag (Sigma, F1804; RRID:AB_262044), rabbit anti-β-Tubulin (Cell Signaling Technology Cat# 2146, RRID:AB_2210545), chicken anti-GFP (Aves Labs Cat# GFP-1010, RRID:AB_2307313), rat anti-CD11b (AbD Serotec Cat# MCA74EL, RRID:AB_2129277), rat anti-CD68 (Thermo Fisher Scientific Cat# 12-0681-82, RRID:AB_2572569), rabbit anti-p-cJun (Cell Signaling Technology #3270, RRID:AB_2129575), rabbit anti-p-STAT3 (Tyr705) (Cell Signaling Technology Cat# 9145, RRID:AB_2491009), rabbit anti-Iba1 (Abcam Cat# ab178846, RRID:AB_2636859), mouse anti-NeuN (Millipore Cat# MAB377, RRID:AB_2298772), goat anti-GFAP (Abcam Cat# ab53554, RRID:AB_880202), rabbit anti-Tuj1 (Covance Cat# MRB-435P-100, RRID:AB_663339), goat anti-TrkA (R and D Systems Cat# AF1056, RRID:AB_2283049), goat anti-TrkB (R and D Systems Cat# AF1494, RRID:AB_2155264), goat anti-TrkC (R and D Systems Cat# AF1404, RRID:AB_2155412). Rabbit anti-SARM1 was produced by immunization with a peptide derived from mouse SARM1 protein (PSQDSSAGSDTSLEGATPMG) as described (Wang et al., 2018). Rabbit anti-p-DLK(S302) was produced by immunization with a peptide derived from mouse DLK protein (CDKSTKM-pS- FAGTVAW) and affinity-purified from the antisera using agarose resin conjugated with the corresponding peptide. In addition, Alexa dye-conjugated secondary antibodies were from Thermo Fisher Scientific.

2.2. Animals

Animals were maintained on the 12 h/12 h light/dark cycle with the chow diet and water available *ad libitum*. The surgical and experimental procedures in mice were performed in compliance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University. 8 to 12 weeks-old mice maintained in specific pathogen-free conditions were utilized in the experiments. Wildtype C57BL/6 mice were purchased from Charles River International. *Wnt1a-Cre* (JAX, 022501; RRID:IMSR_JAX:022501), *Stat3*$^{fl/fl}$ (JAX, 016923; RRID:IMSR_JAX: 016923), and *Ccl2*$^{fl/fl}$ (JAX, 016849; RRID:IMSR_JAX:016849) was from the Jackson Laboratory. *Dlk*$^{fl/fl}$ mice were generated with targeting vector containing the loxP sites inserted to flank the exons 3–6 of *Dlk* gene. The targeting donor DNA fragment was electroporated into the mouse ES cells of C57BL/6 background. The ES cells harboring the targeted allele were injected into the blastocysts. The offspring were genotyped and bred with *Rosa26-FLP* (JAX, 009086; RRID:IMSR_JAX:009086) mice to determine the germline transmission and simultaneously to remove the Neo-cassette to obtain *Dlk*$^{fl/+}$, which were further bred to produce *Dlk*$^{fl/fl}$ mice. The mice were in-house bred to produce the littermates for experiments. Age-matched littermates were subjected to surgery and tissues were harvested for RNA and protein level analysis. Sex- and age- matched littermates were subjected to the behavior test.

2.3. Surgical procedures

For the surgery of sciatic nerve injury (SNI), mice of the indicated genotypes were anesthetized with Avertin, and the skin on their left hindlimb was shaved and prepared with iodine and alcohol. An incision was made between the knee and the hip joint, and the gluteal muscles were separated with a pair of sterile forceps to expose the sciatic nerve. The nerve was completely transected with a pair of sterile surgical scissors. The gluteal muscles were then brought back into the original anatomical position, and the skin incision was closed by 5–0 sutures. Chronic constriction injury (CCI) was performed according to the method modified for mice (Bennett and Xie, 1988; Sommer and Schafers, 1998). Briefly, mice were anesthetized, and the skin on their left hindlimb was shaved and prepared with iodine and alcohol. The left sciatic nerve was exposed at the level of the mid-thigh. Three ligatures were loosely tied around the nerve with 1 mm interval using 4–0 sutures. The gluteal muscles were then brought back into the original anatomical position, and the skin incision was closed by 5–0 sutures.

2.4. Behavioral tests

Behavioral tests were conducted by experimenters blind to conditions. To assess mechanical allodynia, mechanical paw withdrawal threshold (PWT) was measured by von Frey hair test (range 0.008–2 g). Mice were placed in a transparent plastic box on a metal mesh and acclimatized for 30 min prior to testing. Each mouse was tested more than five times at a specific force manually, and the mechanical PWT was calculated using the up–down paradigm (Chaplan et al., 1994). To assess thermo hyperalgesia, paw withdrawal latency (PWL) was measured by hot plate test. Mice were placed on 52.5 °C metal plate. The latency was determined by the onset of hind paw lifts and/or licking, flinching or jumping. Sex- and age- matched littermates were subjected to the behavior test. Both male and female mice were performed on the behavior test, and there was no sex-dependent phenotypes detected.

2.5. Tissue processing

Female or male mice were euthanized at indicated time points after SNI or CCI, and indicated tissues were harvested as following. (1) For the qPCR analysis, L4 DRG were acutely dissected out, and the total RNA was extracted by RNeasy Mini Kit (Qiagen) followed by SYBR Green (Thermo Fisher Scientific) qPCR analysis on Step One Plus Real Time PCR System (Applied Biosystems) with two technical replicates using primers listed in Table S1. And the analyzed genes were selected from RNA-Seq data. *Ppib* (Cyclophilin-B) was selected as the reference gene based on its stability (Yue et al., 2014). The fold change of mRNA level was normalized to the basal level of WT mice. (2) For the RNA-Seq analysis, the total RNA from eight DRG was pooled followed by high-throughput sequencing. The RNA-Seq data were deposited at The Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/docs/) with accession numbers SRR9056046, SRR9056047, SRR9056048, SRR9056049, SRR9056050, SRR9056051. (3) For the fluorescent immunohistochemistry, L4 DRG and L1/L2 spinal cords were acutely dissected out, and fixed in PBS/1% PFA at 4 °C overnight, and then

dehydrated in 30% sucrose. The tissues were then processed for 10 μm cryosections. The sections were immunolabeled with indicated primary antibodies and corresponding Alexa dye-conjugated secondary antibodies, and imaged by fluorescence microscopy. For quantification of cells stained positive for CD11b, CD68, p-STAT3 (p-STAT3$^+$) and p-cJun (p-cJun$^+$) in DRG, the number of CD11b$^+$, CD68$^+$, p-STAT3$^+$ or p-cJun$^+$ cells was normalized to the total areas of the representative sections. For quantification of cells stained positive for TrkA, TrkB, TrkC, and Tuj1 in DRG, the cells were quantified in the indicated total area of the representative sections. For quantification of cells stained positive for Iba1 and GFAP in L1/L2 spinal cords, the indicated cells were quantified in an area of 0.2 mm^2 in the dorsal horn and 0.1 mm^2 in the ventral horn of the representative sections.

2.6. Neuronal cultures

For the *in vitro* neuronal culture, DRG were dissected from E13.5 mouse embryos of the indicated genotypes, and dissociated in 0.05% Trypsin/EDTA (Life Technologies) at 37 °C for 10 min. After washing once with Neurobasal medium containing with 2% B-27 (Thermo Fisher Scientific), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 30 ng/ml mouse NGF (Sino Biological Inc.), neurons were re-suspended in the Neurobasal/B27 medium supplemented with 0.25% methyl-cellulose. On the 24-well plates coated with poly-L-ornithine and 5% heat inactivated FBS (Corning) in PBS, 1.5×10^5 cells in 4 μl Neurobasal/B27 medium were seeded in the center of coated 24-well plates. For lentiviral shRNA (TRC, The RNAi Consortium) knockdown of *Sarm1* (TRCN0000193858) and lentiviral gene overexpression of *Dlk*, the neurons were transduced at 24 h after the culture setup at MOI (multiplicity of infection) of 10. 3 days after the culture setup, neurons were changed to fresh Neurobasal/B27 medium supplemented with the mitotic inhibitor (5 μM 5-fluoro-2′-deoxyuridine and 5 μM uridine) to eliminate the non-neuronal cells. For the *in vitro* injuries, distal axons of the cultured neurons were completely transected by a sterile scalpel (Fine Science Tools) and removed under a dissecting microscope. (1) To determine expression levels of the genes, the culture media were removed and 500 μl Trizol was added to each well to lyse the neurons. The total RNAs were extracted by RNeasy Mini Kit (Qiagen) and processed for SYBR Green (Thermo Fisher Scientific) qPCR analysis. (2) To detect the activation of cJun or the expression of GFP, the neurons were immunolabeled with indicated primary antibodies and corresponding Alexa dye-conjugated secondary antibodies, and imaged by fluorescence microscopy. (3) To detect the activation of DLK, the culture media were removed and 200 μl RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1.0% NP-40) with protease and phosphatase inhibitors was added to each well to lyse the neurons. The cell lysate was prepared for immunoblotting assay.

2.7. RNA-Seq analysis

The RNA sequencing data were mapped by HISAT2. StringTie was used to obtain FPKM values. The genes upregulated or downregulated by 2-fold or higher were analyzed further. Enrichment of biological process and signaling pathways were analyzed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (Huang Da et al., 2009a, 2009b).

2.8. Statistical methods

Cells in fluorescent images were quantified by Nikon NIS-Elements AR Analyser. Fluorescent images were analyzed by ImageJ (1.48 V). Student's two-sided *t*-tests or ANOVA tests were performed using GraphPad Prism (https://www.graphpad.com/ scientific-software/prism). The sample size can be found in the figure legends. Each n represents the number of mice used in the experiment. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$, n.s. (not significant). Error Bars represent SEM.

3. Results

3.1. DLK mediates the neuronal intrinsic immune response to traumatic nerve injury

We previously showed that neurons can elicit cell-autonomous immune response to traumatic nerve injury both *in vivo* and *in vitro* (Wang et al., 2018). Axonal injury results in upregulation of a set of cytokines and chemokines, including *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12*. To investigate whether DLK regulates the neuronal immune response to nerve injury, *Dlk$^{fl/fl}$* mice were generated and crossed with *Wnt1a-Cre* to genetically ablate *Dlk* in the sensory neurons. First, we examined the induction of cytokines and chemokines in cultured sensory neurons from *Dlk$^{fl/fl}$* (WT) and *Wnt1a-Cre*;*Dlk$^{fl/fl}$* (cKO, conditional knock out) mice in response to nerve injury by distal axonal transection. The injured axons were immediately removed from the culture system to exclude the potential effect of axon degeneration on the neuronal immune response. Upregulation of *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12* was greatly reduced in the injured neurons from cKO mice compared to those from the WT littermates (Fig. 1A), suggesting the critical function of DLK in mediating neuronal intrinsic immune response. We then reintroduced DLK into cKO neurons through lentiviral transduction, which delivered genes encoding both DLK and green fluorescent protein (GFP), or GFP alone as control. Nearly all NeuN$^+$ neurons were found to express GFP upon immunohistochemical examination by co-staining GFP and NeuN, indicating the high efficiency of lentiviral transduction (Fig. S1A). The presence of *Dlk* mRNA and DLK protein was verified by qPCR and immunoblotting, respectively (Fig. 1B and Fig. S1B). In fact, the upregulation of *Csf1*, *Ccl2*, *Ccl7* and *Ccl12* was recovered in cKO neurons upon ectopic expression of DLK (Fig. 1B). Interestingly, the induction of *Ccl2*, *Ccl7*, and *Ccl12* could be further enhanced in WT neurons upon ectopic expression of DLK, suggesting a dose-response relationship of DLK in mediating their production (Fig. 1B). Together, the results indicate that DLK played an essential role in mediating neuronal intrinsic immune response.

Then, we examined the role of DLK in mediating the neuronal immune response to the commonly used nerve injury models *in vivo*, sciatic nerve injury (SNI) and chronic constriction injury (CCI). SNI was performed by a distal nerve transection at the mid-thigh, 4.5–5.0 cm from the lumbar (L4 and L5) DRG. Upregulation of *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12* was detected in DRG post-injury (Fig. 1C), but their induction was largely blunted in cKO mice (Fig. 1C). We further determined the neuronal immune response of sciatic nerves to CCI, a relatively mild but chronic nerve injury model *in vivo* (Bennett and Xie, 1988; Sommer and Schafers, 1998). Similarly, the mRNA levels of chemokines and cytokines such as *Csf1* and *Ccl2* were upregulated in WT DRG but not in cKO mice post CCI (Fig. 1D). The data here supported the important function of DLK in mediating the neuronal immune response.

To better understand the role of DLK in regulating the neuronal intrinsic immune capacity at the transcriptional level, we conducted SNI in WT and DLK cKO mice followed by RNA-Seq analysis. A total of 1809 injury-responsive genes showed changes in expression (2-fold change or higher) in WT DRG upon injury (Fig. 1E and Table S2); 827 genes showed differential expression (2-fold change or higher) between those in WT and cKO mice at 24 h after injury (Fig. 1E and Table S3). The Venn map was used to visualize the contribution of DLK to the expression of injury-responsive genes, with 362 injury-responsive genes regulated by neuronal DLK (Fig. 1E and Table S4). Gene Ontology (GO) enrichment analysis of these DLK-dependent injury-responsive genes conducted with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) revealed significant enrichment in several biological processes critical for immune responses, *e.g.*, defense response, inflammatory response, response to cytokines, chemokine-

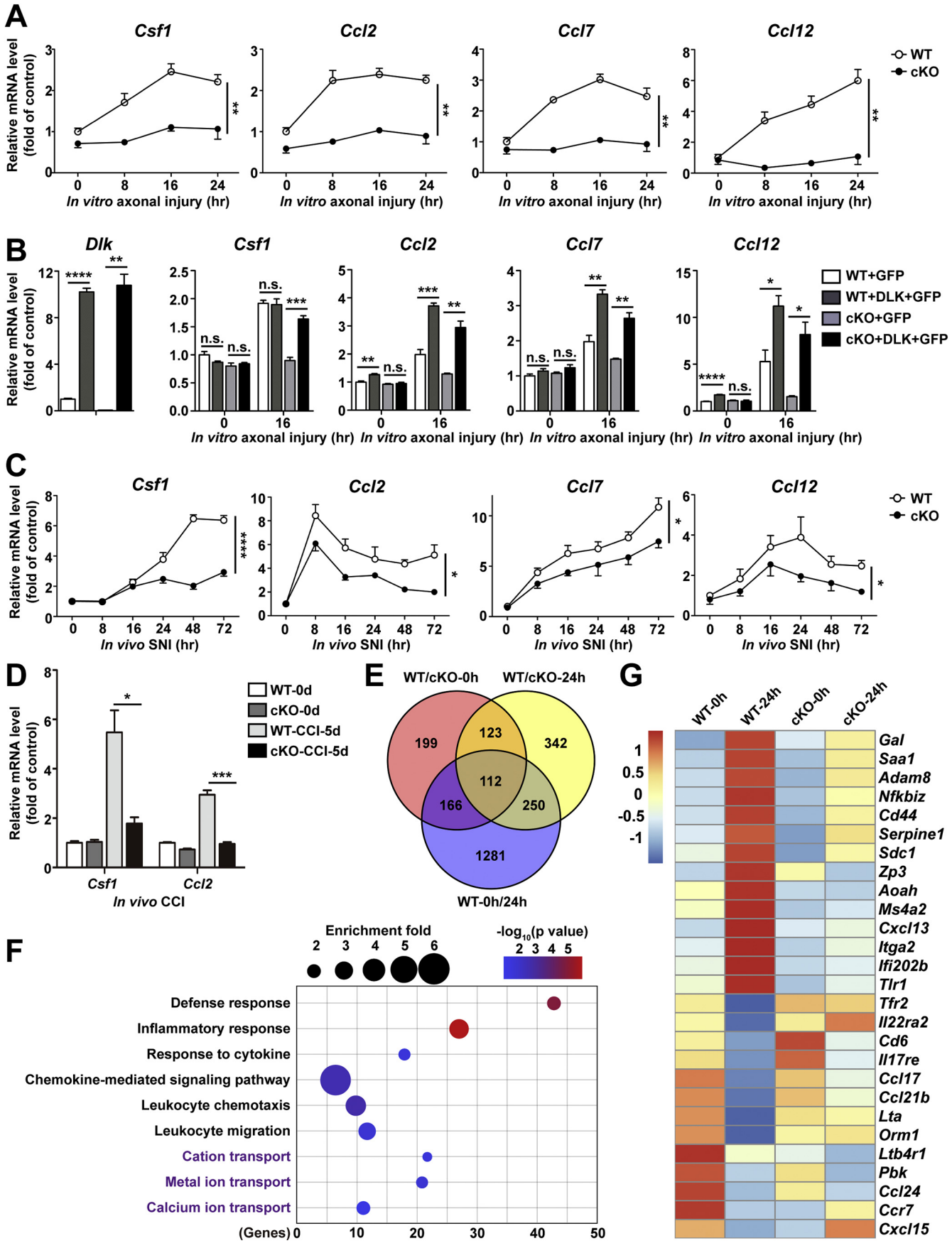

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Fig. 1. Neuronal DLK regulates intrinsic immune response to nerve injury.
(A) Cultured WT and *Dlk* cKO neurons were subjected to traumatic axonal injury, and neurons were harvested for mRNA analysis of *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12* in 24 h. $n = 3–4$. (B) Cultured WT and *Dlk* cKO neurons were transduced with lentivirus encoding GFP alone or DLK-Flag and GFP. Neurons were subjected to axonal injury 5 days post transduction and harvested for mRNA analysis of *Dlk*, *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12* in 16 h. $n = 4$. (C) SNI was performed on WT and *Dlk* cKO mice. Induction of *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12* mRNA in DRG was analyzed by qPCR at indicated time points post-injury. n = 3–6. (D) CCI was performed on WT and *Dlk* cKO mice. Induction of *Csf1* and *Ccl2* mRNA in DRG was analyzed by qPCR at indicated time points post-injury. $n = 5–6$. (E) WT and *Dlk* cKO mice were subject to SNI. RNA was harvested from DRG 24 h post-injury for RNA-Seq. RNA-Seq analysis identified differentially expressed genes in WT mice before and after injury (WT-0 h/24 h), in WT and cKO mice at the basal level (WT/cKO-0 h), and in WT and cKO mice 24 h post-injury (WT/cKO-24 h). The Venn diagram shows the overlap of three groups of differentially expressed genes. (F) The DLK-dependent injury responsive genes were subjected to the functional enrichment test using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Enriched biological pathway terms are shown. Black annotation, immune response related biological pathway terms. Purple annotation, ion transport related biological pathway terms. 27 enriched inflammatory response related genes in (F) were listed in the heat map.

mediated signaling pathway, leukocyte chemotaxis, and leukocyte migration (Fig. 1F and Table S5). Twenty-seven enriched inflammatory response-related genes were listed in the heat map (Fig. 1G). A number of ion transport related genes (including cation transport, metal ion transport, calcium ion transport, *etc.*), which are associated with nerve injury and axon regeneration, were also expressed in a DLK-dependent manner (Fig. 1F). The observed biological processes are consistent with those in the previous study reported by Shin et al. (Shin et al., 2019), supporting the important role of DLK in regulating neuroinflammation among a plethora of biological functions.

3.2. DLK regulates neuronal intrinsic immune response through cJun and STAT3

To understand the signaling events by which DLK mediates the induction of cytokines and chemokines, we used *Wnt1a-Cre;Dlk*$^{fl/fl}$ mice and investigated the role of DLK in the activation of two critical transcription factors, cJun and STAT3, which mediate transcriptional changes in response to nerve injury (Dubovy et al., 2018; Karney-Grobe et al., 2018; Qin et al., 2013; Sanna and Galeotti, 2018; Shin et al., 2012; Wlaschin et al., 2018; Zhong et al., 2018). The activation of cJun has been found to be blunted in DRG neurons upon nerve injury *in vivo*, when *Dlk* is deleted driven by *Wnt1a-Cre* (Shin et al., 2012) or globally deleted in an inducible manner driven by *CAG-CreER* (Wlaschin et al., 2018). By deleting *Dlk* in sensory neurons, we also found that that phosphorylated cJun (p-cJun), the indication for cJun activation, was greatly reduced in cultured cKO neurons (Fig. 2A and B), which could be rescued by ectopic expression of DLK (Fig. 2C and D). Moreover, the activation of cJun was decreased in DRG post SNI (Fig. 2E and F) and CCI *in vivo* (Fig. 2G and H). The data together provide evidence for the neuronal intrinsic regulation of DLK on cJun activation.

Given the important function of DLK in mediating Wallerian degeneration downstream of SARM1 (Yang et al., 2015) and the role of SARM1 in triggering the intrinsic immune response by regulating the transcriptional activity of cJun (Wang et al., 2018), we further determined whether DLK acts downstream of SARM1 to elicit the neuronal immune response. *Sarm1* was knocked down in cultured DRG neurons by lentivirus-delivered shRNA (Fig. S1C and S1D). The reduction of *Sarm1* resulted in decreased p-DLK within minutes post axonal injury (Fig. S1D), revealed by immunoblotting with p-DLK antibody (Fig. S1E), which suggested that SARM1 acted upstream to activate DLK upon axonal injury.

DLK has been shown to mediate translocation of phosphorylated STAT3 (p-STAT3) from the nerve injury site to DRG neuron cell bodies (Shin et al., 2012), and consistently, deletion of DLK in sensory neurons in this study led to reduced p-STAT3 in DRG post SNI (Fig. S2A and S2B) and CCI (Fig. S2C and S2D). We then bred *Stat3*$^{fl/fl}$ mice with *Wnt1a-Cre* mice to genetically delete *Stat3* in sensory neurons. The *Wnt1a-Cre;Stat3*$^{fl/fl}$ mice were embryonal lethal and therefore *Wnt1a-Cre;Stat3*$^{fl/+}$ mice were used in this study. The heterozygous deletion of *Stat3* in sensory neurons resulted in a decrease in upregulation of *Csf1*, *Ccl2,* and *Ccl7* in DRG (Fig. S2E). Together with our previous study showing that SARM1-JNK-cJun signaling regulates the inflammatory response in neurons upon nerve injury (Wang et al., 2018), the results of this study suggest that DLK is a key component in the signal axis mediating neuronal intrinsic immune response through cJun and STAT3.

3.3. DLK regulates the inflammation in DRG upon nerve injury

To further investigate the function of DLK-controlled neuronal immune response, we examined the inflammation in DRG *in vivo*. In WT mice, significant accumulation of CD11b$^+$ immune cells was observed in DRG at 3 days after SNI (Fig. 3A and B), which was markedly reduced upon deletion of *Dlk* (Fig. 3A and C). The number of activated immune cells, indicated by CD68$^+$ staining, was also decreased in cKO mice (Fig. 3D and E). In the CCI model, the number of CD11b$^+$ cells reached the peak at 5 days post-injury (Fig. 3F and G). Similarly, the accumulation of CD11b$^+$ immune cells and the number of CD68$^+$ cells were reduced in cKO mice 5 days post CCI (Fig. 3F, H, I and J). These results suggested that DLK-controlled neuronal immune response mediated the infiltration and activation of immune cells in DRG in response to nerve injuries of both SNI and CCI.

3.4. DLK regulates glial cell reaction in the spinal dorsal horn

The sensory neurons, with cell bodies located in the DRG outside of the spinal cord, project their peripheral axons through the sciatic nerve and their central axons into the spinal dorsal horn. The axonal injury of peripheral sciatic nerve containing both sensory and motor axons leads to inflammation in both the spinal dorsal horn where the sensory neurons project to, and the ventral horn where the motor neuron cell bodies are located, indicated by the activation and proliferation of microglia and astrocytes, termed as microgliosis and astrogliosis, respectively. To determine the function of the DLK-mediated signal in regulating spinal astrogliosis and microgliosis, we examined GFAP (glial fibrillary acidic protein) and Iba1 (Ionized calcium binding adapter molecule 1), the markers for astrocytes and microglia, respectively, through immunohistochemical analysis in the lumbar (L1/L2) spinal cord 5 days post SNI and CCI (Fig. 4). Astrogliosis and microgliosis were evidenced by the increased intensities and number of cells stained positive for GFAP in the spinal dorsal horn upon SNI (approximately 2.5-fold) and CCI (approximately 2.3-fold) (Fig. 4A and B) and for Iba1 post SNI (approximately 6-fold) and CCI (approximately 4-fold) (Fig. 4A and C), respectively. Of note, no difference between WT and cKO mice in the number or state of microglia and astrocytes in the spinal cord at basal level was observed (Fig. 4A, B and C). Genetic deletion of *Dlk* driven by *Wnt1a-Cre* abolished astrogliosis and microgliosis in the spinal dorsal horn both in SNI and CCI models (Fig. 4A, B and C), suggesting that DLK-mediated injury response could play important role in inducing inflammation in the spinal cord, consistent with the finding by Wlaschin and colleagues that DLK is required for injury-induced spinal microgliosis following a spared-nerve injury (Wlaschin et al., 2018). Next, we examined the astrogliosis and microgliosis in spinal ventral horn induced by the concomitant injury of motor neuron in SNI and CCI models. Interestingly, deficiency of *Dlk*

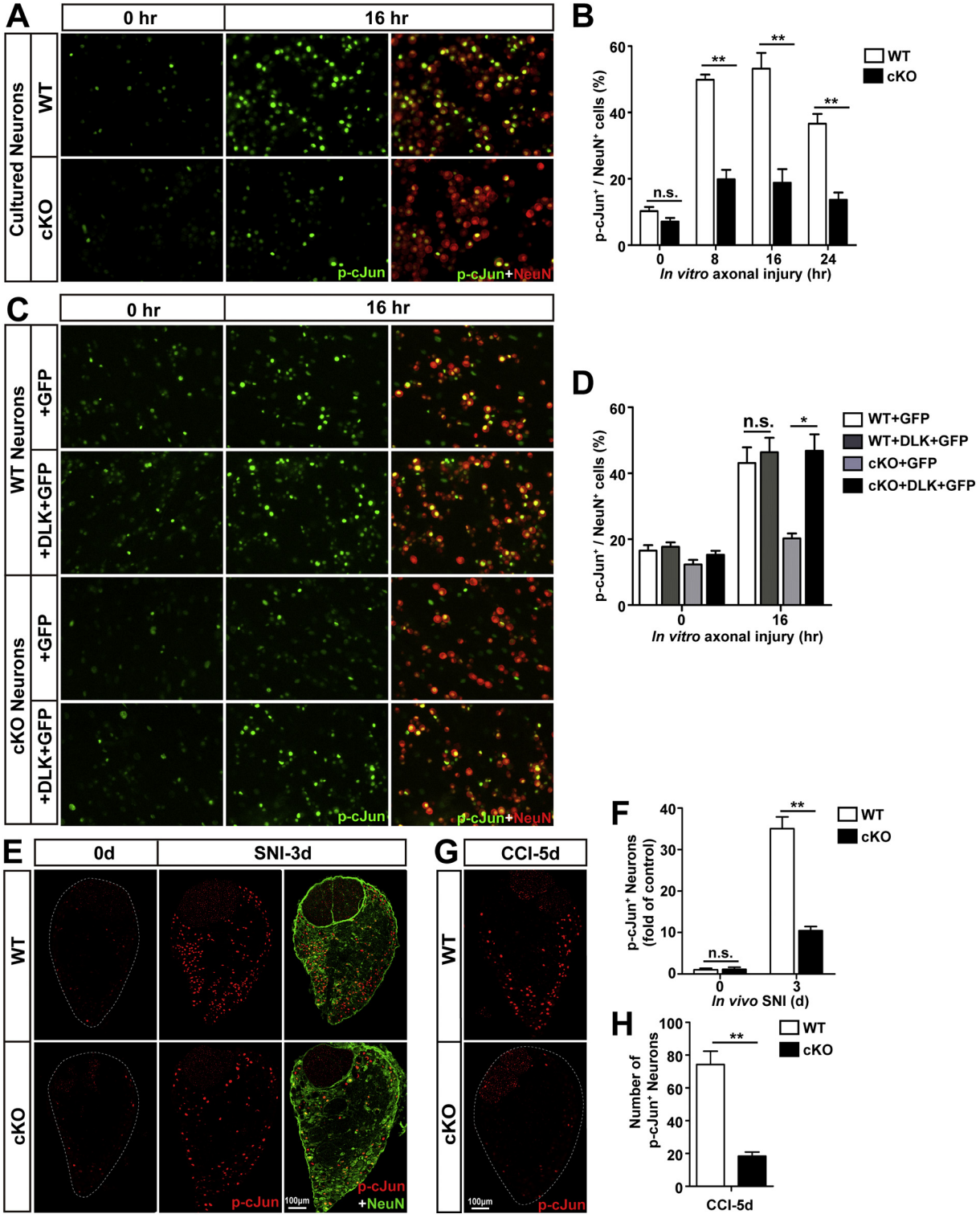

Fig. 2. DLK regulates neuronal intrinsic immune response through cJun.
(A–B) Cultured WT and *Dlk* cKO neurons were subjected to traumatic axonal injury. p-cJun and NeuN were determined by immunohistochemical analysis at indicated time points post-injury (A), and the neurons that stained positive for p-cJun (p-cJun^{+}) were quantified (B). $n = 3$. (C–D) Cultured WT and *Dlk* cKO neurons were transduced with lentivirus for expressing GFP alone or DLK-Flag and GFP. Neurons were subjected to axonal injury 5 days post transduction and harvested for immunohistochemical staining of p-cJun and NeuN at 16 h (C); the neurons that stained positive for p-cJun were quantified (D). n = 3. (E–H) WT and *Dlk* cKO mice were subjected to SNI (E and F) and CCI (G and H). DRG were harvested at indicated time points for immunohistochemical staining of p-cJun (E and G), and the neurons that stained positive for p-cJun (F and H) were quantified. Dashed circle shows the tissue region. n = 3–5.

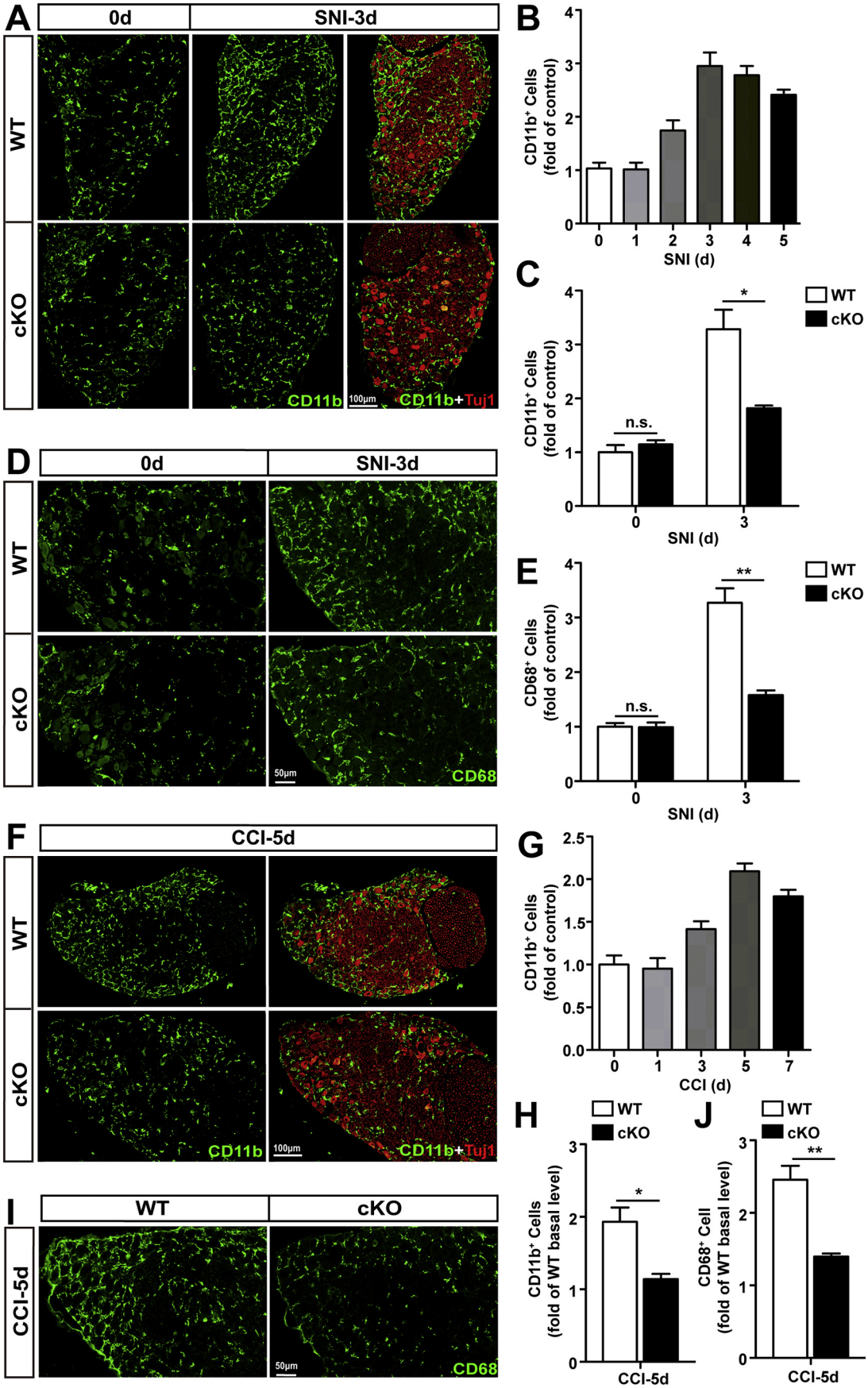

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Fig. 3. DLK regulates the injury-induced inflammation in DRG.
(A-E) SNI was performed on WT and *Dlk* cKO mice. DRG were harvested from day 0 to 5 days (d) post-injury followed by immunohistochemical staining for Tuj1, CD11b and CD68. The representative images were shown for 0 and 3 days (A and D). The time course of the infiltration of $CD11b^+$ immune cells into DRG was determined in WT mice post SNI (B). The cells that stained positive with CD11b (C) and CD68 (E) were quantified. n = 3–4. (F to J) CCI was performed on WT and *Dlk* cKO mice. DRG were harvested from day 0 to 7 days post-injury followed by immunohistochemical staining for Tuj1, CD11b, and CD68. The representative images were shown for 0 and 5 days (F and I). The time course of the infiltration of $CD11b^+$ immune cells into DRG was determined in WT mice post CCI (G). The cells that stained positive with CD11b (H) and CD68 (J) were quantified n = 4–5.

driven by *Wnt1a-Cre* did not perturb the astrogliosis and microgliosis in the spinal ventral horn (Fig. 5A, B, and C). These results suggest that the injury-induced inflammation in the spinal cord was restricted to the regions in which the sensory neurons project their axons in response to peripheral nerve injuries (Fig. 4 and Fig. 5), a notion consistent with that observed on deletion of *Csf1* in sensory neurons driven by *advillin-Cre* (Guan et al., 2016).

3.5. DLK is critical for the development of neuropathic pain

Inflammatory response has been increasingly appreciated as a critical factor contributing to the pathological conditions in the somatosensory nervous system, such as neuropathic pain. In this study, DLK was shown to play a critical role in mediating the immune response in DRG and the spinal cord upon CCI, a commonly adopted model for studying neuropathic pain (Figs. 3 and 4). CCI was therefore used to determine whether chronic pain development was affected in *Wnt1a-Cre;*$Dlk^{fl/fl}$ mice (Bennett and Xie, 1988; Campbell and Meyer, 2006; Chaplan et al., 1994; Sommer and Schafers, 1998). CCI causes neuropathic pain characterized by marked mechanical allodynia and thermal hyperalgesia ipsilateral to the injury side, which can be detected by the von Frey hair test and hot plate test, respectively. Alleviated mechanical allodynia and heat hyperalgesia induced by CCI were observed in *Wnt1a-Cre;*$Dlk^{fl/fl}$ mice, indicated by increased 50% paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) (Fig. 6A and B, Fig. 6C and D, respectively). The results here further support the conclusion that DLK plays a critical role in promoting neuropathic pain, in alignment with the findings of previous studies in which DLK was globally deleted in an inducible manner (Wlaschin et al., 2018) or knocked down by intrathecally injected shRNA (Sheu et al., 2018).

In addition to the reduced neuropathic pain post CCI, *Wnt1a-Cre;*$Dlk^{fl/fl}$ mice showed decreased sensitivity to mechanical and thermal stimulation without injury (Fig. 6A and C). To understand the mechanism of reduced pain sensation, we examined the neuronal subpopulations and the differentially expressed genes at basal level in sensory neurons in WT and cKO adult mice. In fact, deletion of *Dlk* led to a higher mRNA level of *Ntrk1* in the DRG, the coding gene for TrkA, but not *Ntrk2* or *Ntrk3*, the coding genes of TrkB and TrkC, respectively (Fig. S3A). The changes in mRNA were due to the increased number and the percentage of neurons expressing TrkA, but not TrkB or TrkC, as examined by immunohistochemical analysis (Fig. S3B, S3C and S3D), which is consistent with the selective reduction of developmental neuron death upon deletion of *Dlk* (Ghosh et al., 2011).The observation that the increased number of $TrkA^+$ neurons did not result in enhanced nociception indicates that changes in the gene expression profile or additional neuronal plasticity occur in the absence of *Dlk* during development. Future investigations are warranted to design improved tools such as inducible deletion of *Dlk* to understand its roles in both developmental and pathological settings fully.

We then analyzed the differentially expressed genes at basal level between WT and cKO mice from the RNA-seq, with a focus on pro-nociception and anti-nociception genes. The Venn map revealed that ten pro-nociception and six anti-nociception genes, known from the Pain Gene Database (Lacroix-Fralish et al., 2007) and manual interrogation (Peng et al., 2017), showed significant up- or down-regulation (2-fold change or higher) in cKO mice (Fig. S3E, Tables S6, and S7). The differentially expressed pro-nociception and anti-nociception genes were listed in the heat map (Fig. S3F and S3G). Among the ten pro-nociception genes, *Lcn2*, *Ltb4r1*, *Mmp9*, and *Ngf* were further analyzed based on their relatively high expression in WT (FPKM value > 1 in RNA-seq data), downregulation of which was confirmed by qPCR analysis (Fig. S3H). Five out of six anti-nociception genes (all with FPKM value > 1 in RNA-seq data), including *Sstr2*, *Sstr4*, *Nts*, *Ntsr2*, and *Sprr1a*, were upregulated in cKO in RNA-seq analysis, upregulation of which could be confirmed by PCR analysis (Fig. S3I). The nociception-related genes were manually interrogated further based on the candidates with well-known functions (Carlton et al., 2001; Diaz-delCastillo et al., 2018; Helyes et al., 2000; Li et al., 2002; Peng et al., 2017; Pinter et al., 2006; Wang, 2004; Wang et al., 2001). Two additional anti-nociception genes, *Npy* and *Sst*, were identified showing increased expression in cKO, although they were not identified through RNA-seq analysis due to a fold change of < 2. The upregulation of *Npy* and *Sst* was verified by qPCR analysis (Fig. S3I). Notably, in alignment with the findings by Wlaschin et al. (Wlaschin et al., 2018), the induction of *Npy* post-injury was reduced upon *Dlk* deletion (FPKM in RNA-seq data, Table S4), indicating a distinct mechanism for *Npy* induction exists in response to injury. The results together suggested that DLK plays pleiotropic roles by regulating both basal pain perception and neuropathic pain development induced by nerve injury. Collectively, the differentially expressed nociception-related genes could contribute to the elevated pain sensation threshold at basal level in cKO mice (Fig. 6).

Of note, the transgenic insertion of *Wnt1a-Cre* did not result in alteration of neuronal intrinsic immune response or pain sensation. Expression of *Csf1*, *Ccl2*, *Ccl7*, or *Ccl12* (Fig. S4A), activation of cJun and STAT3 (Fig. S4B, S4C, S4D, and S4E), infiltration of $CD11b^+$ immune cell in DRG (Fig. S4F and S4G), glial cell reaction at the spinal dorsal horn (Fig. S5A, S5B, and S5C), and CCI-induced pain (Fig. S5D and S5E) did not show detectable changes in comparison with those in WT littermates. These data together supported the critical function of DLK in mediating neuronal immune response in the sensory neurons and regulating glial cell reaction in the spinal dorsal horn and neuropathic pain.

The neuronal immune response mediated by DLK led to production of multiple cytokines and chemokines that could contribute to the inflammation in the spinal dorsal horn and development of neuropathic pain. Among them, CSF1 has been shown to be critical for peripheral nerve injury-induced microgliosis in the spinal cord (Guan et al., 2016), and neuronal derived CCL7 was reported to drive neuropathic pain by promoting the proliferation of astrocytes (Ke et al., 2016). Recently, CCL2 and CCL7 were reported to promote the development of pain-related behaviors revealed by the intrathecal injection of CCL2 and CCL7 proteins or their neutralizing antibodies (Kwiatkowski et al., 2019). To further investigate the potential functions of the neuronal immune response, we have therefore generated *Wnt1a-Cre;*$Ccl2^{fl/fl}$ mice (Fig. S6A). Although extensive molecular or cellular characterizations were carried out, no detectable difference in immune response was observed, *e.g.*, in the recruitment of immune cells in DRG (Fig. S6B and S6C), in the glial reaction in the spinal dorsal horn (S6D, S6E, and S6F), or in CCI-induced mechanical allodynia and heat hyperalgesia (Fig. S6G and S6H). The lack of phenotypic effects could be due to redundancy in functions or cellular sources. Future studies are warranted to investigate further the immune-related genes in regulating inflammation in the somatosensory nervous system and their contributions to chronic pain.

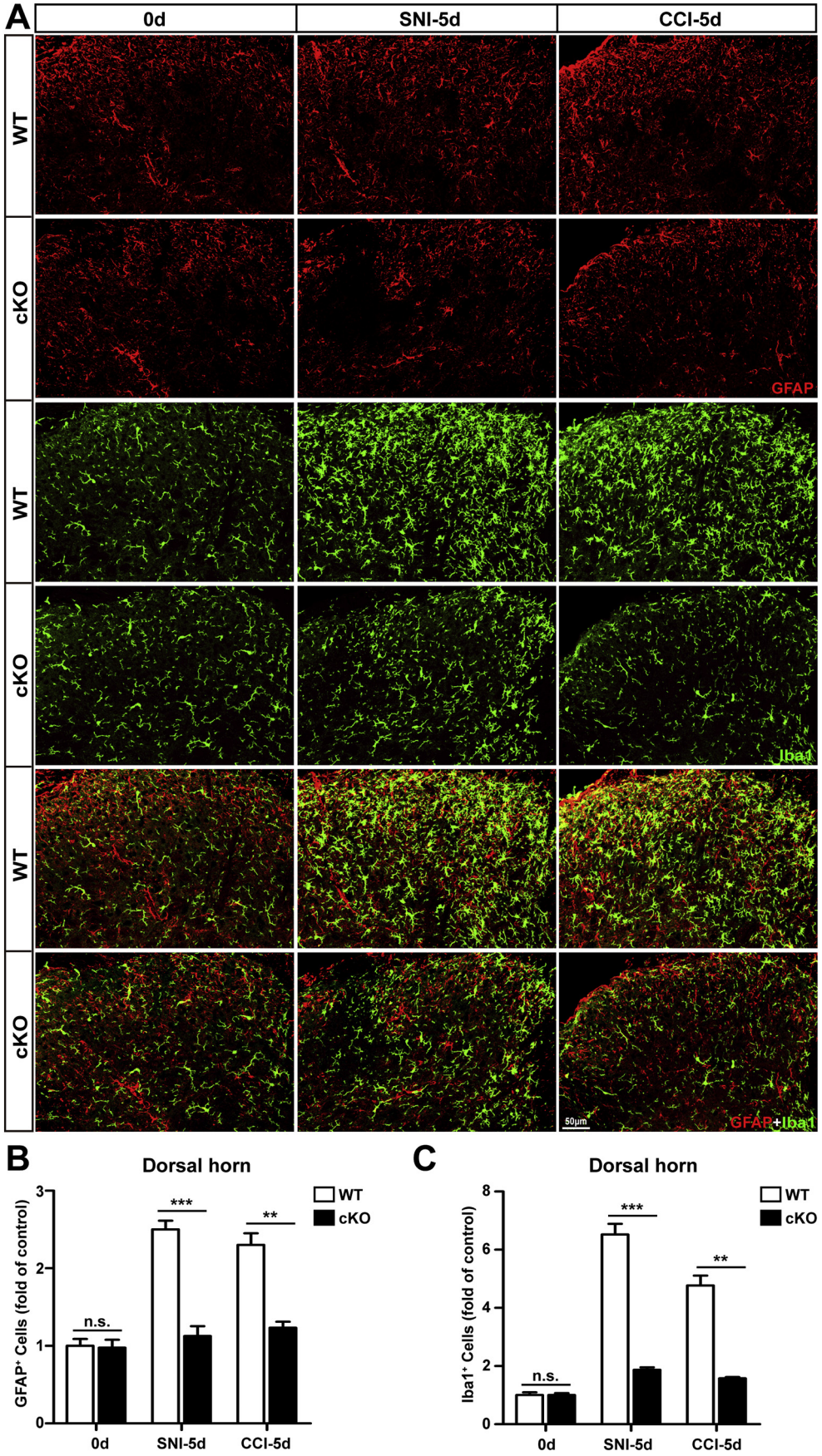

Fig. 4. DLK regulates glial cell reaction in the spinal dorsal horn.
(A to C) SNI or CCI was performed on WT and *Dlk* cKO mice. Spinal cords were harvested 5 days post-injury followed by immunohistochemical staining for GFAP and Iba1 (A), and the cells that stained positive for GFAP ($GFAP^+$) (B) or Iba1 ($Iba1^+$) (C) were quantified. n = 3–4.

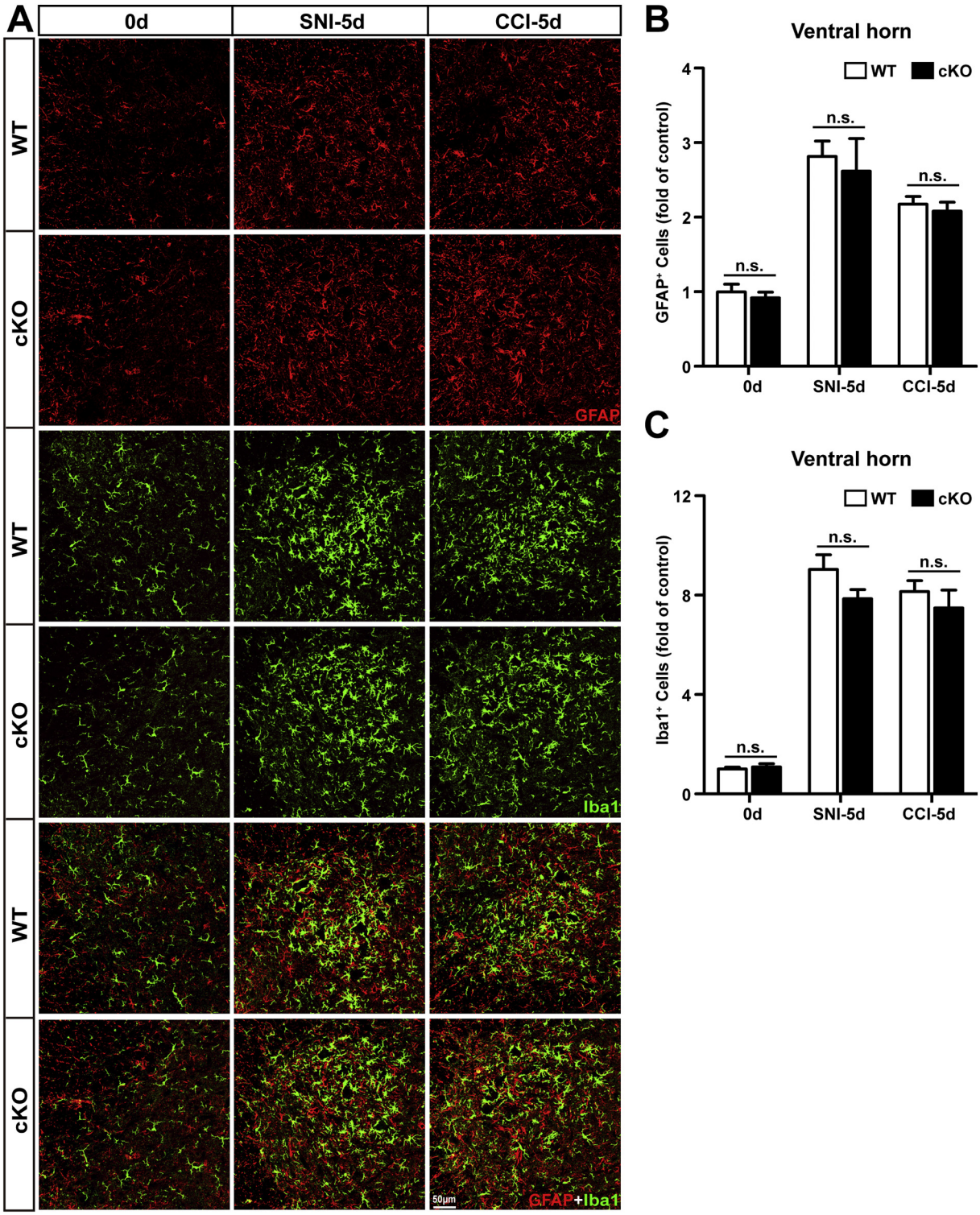

Fig. 5. Deletion of *Dlk* in sensory neurons does not perturb glial cell reaction in the spinal ventral horn.
(A to C) SNI or CCI was performed on WT and *Dlk* cKO mice. Spinal cords were harvested 5 days post-injury followed by immunohistochemical staining for GFAP and Iba1 (A), and the cells that stained positive for GFAP ($GFAP^+$) (B) or Iba1 ($Iba1^+$) (C) were quantified. n = 3–4.

In summary, we showed that DLK in the sensory neurons was the key molecule to induce neuronal intrinsic immune response and to regulate the progression of nerve injury-induced inflammation in the DRG and spinal cord. Deletion of *Dlk* driven by *Wnt1a-Cre* resulted in reduced inflammation in the spinal dorsal horn upon acute and chronic nerve injury and alleviated the neuropathic pain elicited by CCI (Fig. 6E). The findings here may lay the foundation for new therapeutic design in targeting inflammation in chronic pain.

4. Discussion

Inflammatory response has been demonstrated as an important factor regulating the onset and progression of pathological conditions in the nervous system (Becher et al., 2017; Pavlov et al., 2018; Talbot

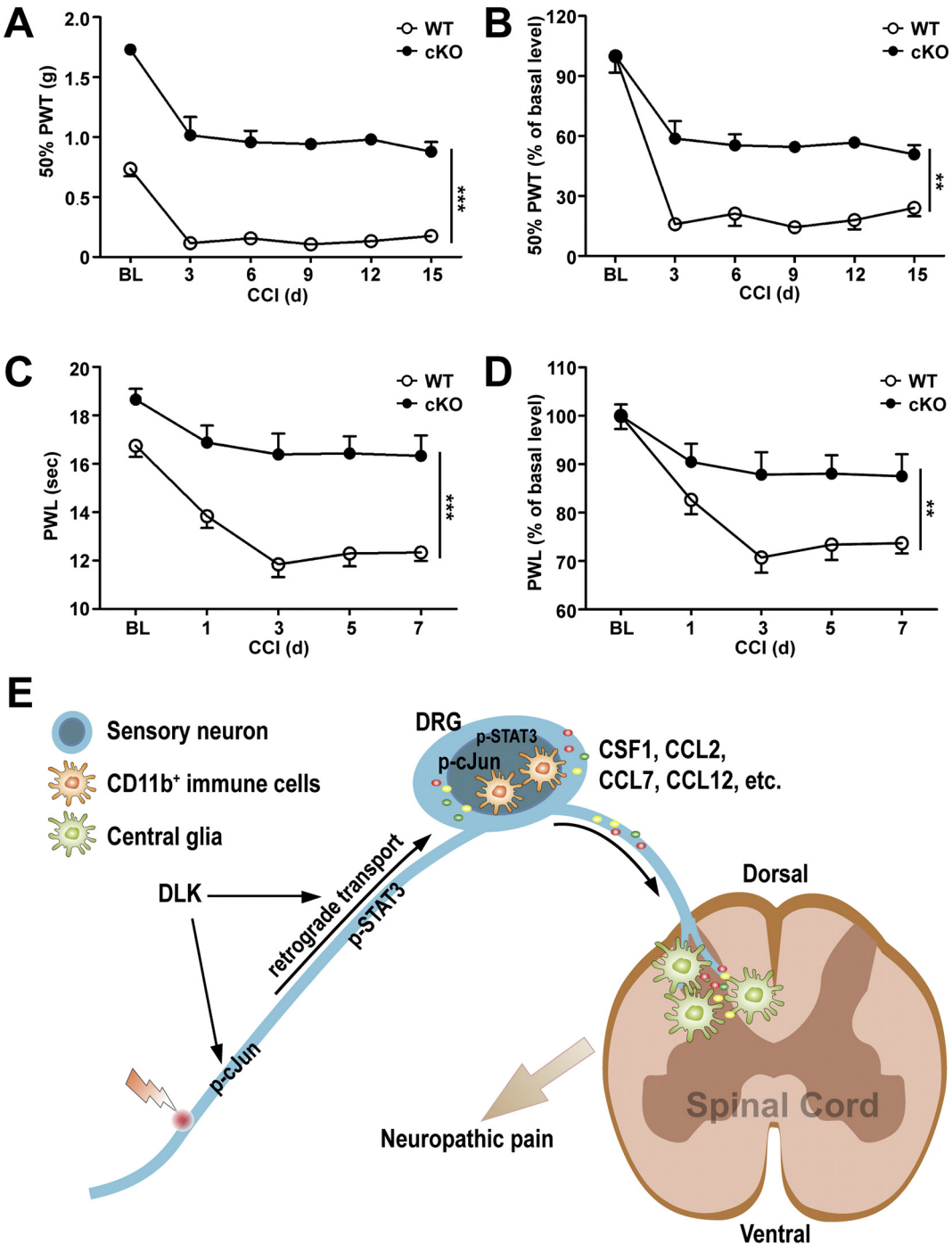

Fig. 6. DLK is critical for development of neuropathic pain induced by CCI.
(A to D) WT and *Dlk* cKO mice were subjected to CCI. Mechanical allodynia was assessed by measuring PWT with the von Frey hair test at indicated time points post-surgery (A and B), and the PWT normalized to the basal level is shown in (B). n = 5–6. Heat hyperalgesia was assessed with PWL in response to a 52.5 °C hot plate (C and D). PWL normalized to the basal level is shown in (D). *n* = 11–13. Proposed model of DLK-mediated neuroinflammation in DRG and spinal dorsal horn post peripheral nerve injuries. The injury response mediated by DLK through cJun and STAT3 induces the production of cytokines and chemokines, including CSF1, CCL2, CCL7 and CCL12, and regulates the infiltration of $CD11b^+$ immune cells into DRG, the glial reaction in the spinal dorsal horn and the development of neuropathic pain.

et al., 2016). In this study, by exploiting cultured neurons *in vitro*, and acute and chronic sciatic nerve injury models *in vivo,* in combination with genetic deletion of *Dlk* driven by *Wnt1a-Cre,* we found that DLK mediated the neuronal intrinsic immune response to nerve injury. Further, the DLK-controlled injury response regulated inflammation in the DRG and glial activation in the central nervous system.

Global ablation of *Dlk* in an inducible manner using *CAG-CreER* alleviates mechanical allodynia after spared nerve injury and reduces neuronal *Csf1* induction, which contributes to decreased microgliosis in the spinal cord (Wlaschin et al., 2018). Utilization of cell type-specific Cre allowed us to show that DLK mediated neuronal intrinsic immune response, leading to the induction of cytokines and chemokines, including *Csf1*, *Ccl2*, *Ccl7*, and *Ccl12*, in sensory neurons. The observation of decreased astrogliosis and microgliosis in the spinal cord upon

deletion of *Dlk* driven by *Wnt1a-Cre* in the peripheral sensory neurons (Chai et al., 2000; Danielian et al., 1998) led us to speculate that the peripheral neuronal immune response played an important role in regulating central inflammation. It remains to be determined whether DLK is involved in inducing neuronal immune signaling in different neuron types upon various insults, given that cytokines and chemokines can be produced by neurons in the central nervous system as well (Murphy et al., 1995; Tanaka et al., 2004; Wang et al., 2018; White et al., 2005; Zhang and De Koninck, 2006).

The defective DLK activation upon knockdown of *Sarm1* supported the signal cascade of SARM1-DLK-cJun/STAT3 in mediating neuronal immune response. Notably, *Sarm1* deficiency delayed the activation of cJun for up to 24 h upon SNI and did not cause a significant change in the induction of *Csf1* (Wang et al., 2018). Deletion of *Dlk* blocked the activation of cJun to a greater extent, *e.g.*, for > 3 days post SNI and 5 days post CCI (Fig. 2), and decreased the induction of CSF1 by a significant level (Fig. 1). The phenotypic differences indicated that additional molecules or mechanisms could function redundantly with SARM1, which converge on DLK activation to mediate the neuronal immune response.

Injury to the sciatic nerve in the periphery could lead to inflammation in the spinal cord, suggesting the role of microenvironmental remodeling in the central nervous system by the peripheral nerve integrity. The deletion of *Dlk* driven by *Wnt1a-Cre* resulted in decreased inflammation at the spinal dorsal horn where the sensory neurons project their central axons but not the ventral horn where motor neuron cell bodies are located. The drastic distinction of region-specific inflammation in the spinal cord warrants further study to determine whether a tightly controlled immune axis exists from the peripheral to the central nervous system in transmitting damage signals. Moreover, inflammation in DRG proceeded the spinal glial reaction, *e.g.*, infiltration of $CD11b^+$ immune cells into DRG peaked at 3 days post sciatic nerve injury (Fig. 3B), while glial reaction in the spinal cord increases progressively from 3 to 5 days (Wlaschin et al., 2018). Though highly speculative, the infiltration of immune cells into DRG could potentially reshape the immune homeostasis of DRG neurons, which would further regulate the inflammation through projected axons in the spinal dorsal horn. Also, though the spinal cord inflammation precipitates the pain conditions, it remains to be determined whether the immune remodeling in DRG could instead influence the regenerative capacity of the sensory nerves post-injury.

Neuropathic pain is a debilitating disease with a high prevalence estimated above 7% in the general population (Bouhassira et al., 2008; Colloca et al., 2017). With the accumulating evidence of the role of neuron-immune interactions in promoting pain development, the inflammatory reaction in the nervous system has emerged as an attractive target (Grace et al., 2014; Ji et al., 2016; Milligan and Watkins, 2009; Old et al., 2015; Skaper et al., 2018). The role of neuronal DLK in regulating both astrogliosis and microgliosis indicates that the neuronal intrinsic immune response could play important roles in inducing inflammation across multiple cell types in the nervous system. The results support DLK as a potential therapeutic target for neuropathic pain by dampening neuronal inflammation, though caution should be taken given that DLK is also essential for axon regeneration after injury (Hammarlund et al., 2009; Shin et al., 2012; Watkins et al., 2013; Yan et al., 2009).

Taken together, our study has provided evidence for the critical function of DLK in controlling the neuronal intrinsic immune response induced by acute and chronic nerve injuries and mediating immune cell infiltration into DRG and the glial cell reaction in the spinal dorsal horn. In addition, DLK signaling regulates the neuropathic pain induced by CCI by mediating the inflammation in the spinal dorsal horn. Future study is awaited to investigate the molecular and cellular mechanisms underlying immune homeostasis and dysregulation mediated by neurons, which would provide important insights into interventions for neurological disorders.

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Author contributions

Z.H. and N.D. performed and analyzed the experiments. K.L. carried out analysis on RNA-Seq data. W.Z. directed the project, conceived experiments and provided supervision. The manuscript was written by W.Z. and Z.H.

Declaration of Competing Interest

The authors declare no competing interests.

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