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ORIGINAL ARTICLE

Glial-derived TNF/Eiger signaling promotes somatosensory neurite sculpting

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Abstract

The selective elimination of inappropriate projections is essential for sculpting neural circuits during development. The class IV dendritic arborization (C4da) sensory neurons of *Drosophila* remodel the dendritic branches during metamorphosis. Glial cells in the central nervous system (CNS), are required for programmed axonal pruning of mushroom body (MB) γ neurons during metamorphosis in *Drosophila*. However, it is entirely unknown whether the glial cells are involved in controlling the neurite pruning of C4da sensory neurons. Here, we show that glial deletion of Eiger (Egr), orthologous to mammalian tumor necrosis factor TNF superfamily ligand, results in dendrite remodeling deficiency of *Drosophila* C4da sensory neurons. Moreover, the attenuation of neuronal Wengen (Wgn) and Grindelwald (Grnd), the receptors for TNF ligands, is also examined for defects in dendrite remodeling. We further discover that Wgn and Grnd facilitate dendrite elimination through the JNK Signaling. Overall, our findings demonstrate that glial-derived Egr signal links to the neuronal receptor Wgn/Grnd, activating the JNK signaling pathway and promoting developmental neuronal remodeling. Remarkably, our findings reveal a crucial role of peripheral glia in dendritic pruning of C4da neurons.

Keywords Glia · TNF/Eiger · JNK signaling pathway · Neuronal remodeling

Introduction

As it develops, the nervous system begins with a primitive prototype characterized by being over-branched and over-connected. Further remodeling is then required to refine the nervous system to maturity [1, 2]. Neurite pruning is a conserved mechanism used to specify neuronal connections or to remove developmental intermediates and involves the degeneration of neurites without the loss of the parent cells [3–5]. During metamorphosis, holometabolous insects, like *Drosophila*, undergo significant nervous system remodeling to establish an adult-specific nervous system [4, 5]. The motor neurons innervating the larval muscles at the neuromuscular junctions (NMJ) are pruned predominantly through a retraction mechanism to reconstruct the motor nervous system that is specifically designed for adults [6–8]. The γ neurons of the mushroom body shed their larval axons and dendrites in the central nervous system (CNS). Subsequently, they extend their neurites again to form novel circuits that are specific to the adult [9]. By examining their dendritic architecture and central axon projections, four classes of *Drosophila* dendritic arborization (da) neurons can be identified: classes I-IV [10]. During the early pupal phase, the sensory class IV dendritic arborization (C4da) neurons maintain the integrity of their axons while pruning their lengthy and branched larval dendrites specifically and fully [3, 11, 12] (Fig. 1A). Dorsal class I sensory neurons (ddaD and ddaE) sculpt their larval dendrites in a specific manner, similar to that of C4da neurons, without losing their axons [3]. On the other hand, sensory neurons classified as ddaB (class II) and ddaA/F (class III) experience apoptosis at the beginning of the pupal phase [3]. The steroid hormone ecdysone controls dendrite pruning in a cell-intrinsic way by triggering a transcriptional cascade that results in the production of certain pruning factors, including the actin-severing enzyme Mical and the transcription factor Sox14 [11, 13, 14]. Developmental neurite pruning has been also observed in several regions of the mammalian brain, including the hippocampus, cerebral cortex, cerebellum,

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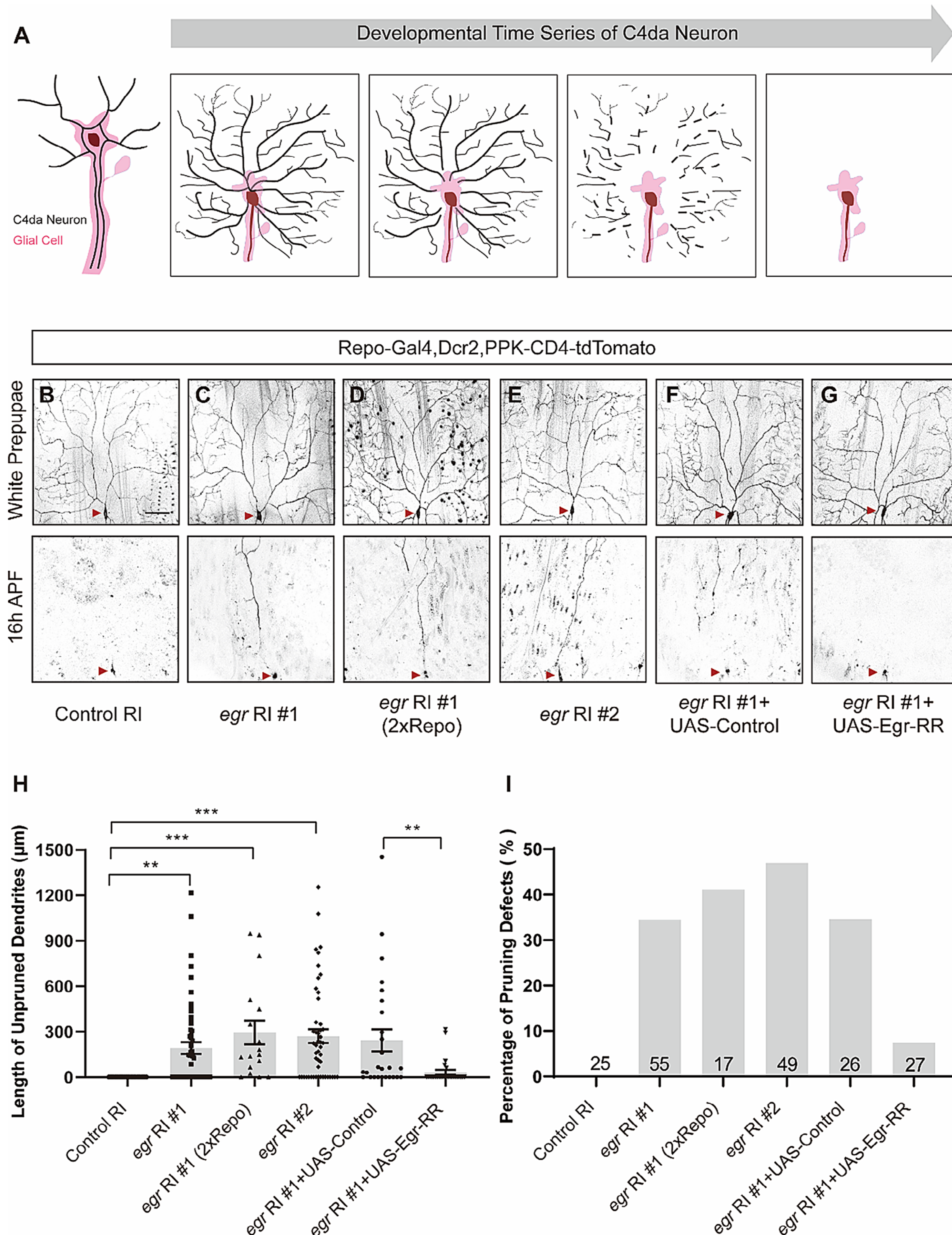

Fig. 1 Glial-Egr is essential for controlling dendritic pruning in C4da neurons. **(A)** A schematic view of glial cells as well as dendritic pruning process in C4da sensory neurons. **(B-G)** Live confocal images of C4da neurons at the WP and 16 h APF stages. The ppk promoter drives CD4-tdTomato endogenously label C4da neurons, Repo-Gal4 was simultaneously utilized to knock down or overexpress Control RNAi **(B)**, *egr* RNAi #1 **(C)**, *egr* RNAi #1 with 2 copies of Repo-Gal4 **(D)**, *egr* RNAi #2 **(E)**, *egr* RNAi #1 + UAS-Control **(F)**, and *egr* RNAi #1 + UAS-Egr-RR **(G)** in glial cells. RNAi is abbreviated as RI, RNAi-resistant is abbreviated as RR, and red arrowheads point to the C4da somas. **(H)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(I)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In H, data are mean ± s.e.m. One-way ANOVA with Bonferroni's test was applied to determine statistical significance. $^{**}P < 0.01$; $^{****}P < 0.0001$. The number of neurons (n) examined in each group is shown on the bars. Scale bar: 50 μm

and olfactory bulb, over the past few decades [1, 2, 15, 16]. Noteworthily, neurological disorders, such as autism spectrum disorder and schizophrenia, are often linked to aberrant pruning, which results in altered dendritic or axonal density in human brains [17, 18]. Gaining knowledge of the mechanisms underlying developmental pruning would be crucial to understanding the etiology of neurological diseases in humans.

A number of cell-autonomous mechanisms have been shown in previous studies to regulate developmental neuronal pruning in *Drosophila*, for instance the ubiquitin-proteosome system and caspases [11, 19–22], the endocytic pathways [23], compartmentalized calcium transients [24, 25], the secretory pathway [12, 26–28], the JNK signaling pathway [29], microtubule stability and polarity [30–36], the energy homeostasis [37], the mechanical tearing [38], and others. It was previously thought that glial cells are responsible for the pruning of synapses in the CNS in both vertebrates and invertebrates [39–42], whereas epithelial cells are the primary phagocytes in clearing degenerating dendrites of C4da sensory neurons [43]. However, these peripheral phagocytes all appear to clear neurites that have undergone pruning in an immune-responsive manner. It is currently unknown whether these perineuronal cells initiate the process of neuronal remodeling during development by sending a specific signal in a transcellular manner.

Tumor necrosis factor (TNF), a crucial cytokine, is responsible for regulating a range of cell processes, including inflammation, proliferation, differentiation, survival, and distinct cell death types, including apoptosis and necrosis in a context-dependent manner [44–48]. Importantly, conditions that include cancer, autoimmune disease, and neurodegenerative disease have been linked to the aberrant function of TNFs [49, 50]. Apart from their well-documented proinflammatory and cancer-related roles, TNFs are believed to be required for coordinating the metabolic rewiring linked to disease states in both mammals and *Drosophila* [49, 51]. It initiates multiple downstream signaling cascades when it engages its cognate receptors. The JNK cassette, formerly known as c-Jun N-terminal kinase (JNK), is a vital downstream regulator of the TNF signaling pathway [52, 53]. After being activated, JNK is moved to the nucleus where it phosphorylates and activates activator protein 1 (AP1) and specificity protein 1 transcription factor complexes. Gene expression is subsequently controlled by these transcription factors, which can lead to various physiological effects [54–57]. Other crucial downstream intracellular signaling cascades of TNF signaling are nuclear Factor-κB (NF-κB) mediated proliferation or caspase-dependent apoptosis [48, 58–60]. 19 ligands that compose the TNF-α superfamily in mammals, and 29 related receptors constitute the TNF receptor (TNFR) superfamily, making the mammalian TNF/TNFR signaling network complicated. Different and sometimes opposing cellular responses can be triggered when multiple TNF ligands activate more than one TNF receptor [47, 61, 62]. The mechanisms that lead to selective activation of a single TNFR over another are not well understood in mammals attributed to functional redundancy and the diversity of TNF ligand/receptor pairs. Eiger (Egr) is the only TNF homolog encoded in the *Drosophila* genome [52, 63]. It belongs to the type II transmembrane protein which, like TNF-α, must be cleaved to be secreted [46, 64]. This can be done by the TNF-α converting enzyme (TACE) in vertebrates, and a homolog of it also exists in *Drosophila* [64–66]. Notably, it has been identified that Wengen (Wgn) and Grindelwald (Grnd) are the two receptors of Egr in *Drosophila* [67–69]. Therefore, it offers an exceptional framework to investigate the relationship between TNF and TNFR and how it is linked to diverse cellular and molecular events. *Drosophila* TNF/Egr, like mammalian TNFs, has multiple roles in controlling cell death, host defense, tumor promotion, tissue growth, regeneration, and nutrient response [45, 59, 65, 70]. Whether and how Egr transduces signals from perineuronal cells to govern neuronal pruning is still largely unclear.

In *Drosophila*, the C4da sensory neurons are positioned between epithelial cells and muscle cells and are encircled by glial cells [71, 72]. Using an in vivo *Drosophila* model of neuronal pruning, we observed transcellular signaling from peripheral glia near C4da sensory neurons. Here we show that the fly TNF, Egr in peripheral glia, initiates dendrite pruning via the conserved TNF receptors Wgn and Grnd, in C4da sensory neurons. Moreover, our study shows that activation of the neuronal JNK signaling can alleviate the dendrite pruning defects in Wgn and Grnd-attenuated neurons. We therefore elucidate a previously unknown transcellular molecular pathway from glial cells to sensory neurons that controls dendrite pruning, and it paves the avenues for exploring novel peripheral regulatory signals of neuronal remodeling.

Results

TNF/Egr in glial cells is required for dendrite pruning of C4da sensory neuron

Selective removal of synaptic connections is necessary for the accurate wiring of neuronal circuits. While the intrinsic mechanisms of the neuron are crucial in this process, it is becoming increasingly perceived that perineural cells also play an important role [73]. The normal development and function of the nervous system depends on the close interaction between glia and neurons, the two primary cell types of

the nervous system. Notably, a range of neurological disorders may be caused by dysfunction in neuron-glia interactions [74–76]. Previous research has shown that mammalian microglia and *Drosophila* astrocytes in the CNS can function as neuroimmune cells, monitoring and phagocytosing pruned or damaged neurites [39, 40, 42]. However, it is not yet well understood whether peripheral glial cells regulate the pruning of sensory neuron dendrites through cross-system signaling. To isolate the non-cell autonomous signaling, we performed a glial RNA-interference (RNAi) screen of signaling molecules representing multiple cellular physiological pathways, by assessing dendritic pruning in C4da sensory neurons.

The expression of dsRNA constructs targeting candidate genes is controlled by the glial-specific GAL4 driver Repo-Gal4 and visualized C4da sensory neurons by endogenously inserted membrane-bound fluorescent protein under the control of pickpocket (PPK) promoter (PPK-CD4-tdTomato). The highly branched dendrites of C4da neurons at the white prepupae (WP) stage are visible after glial expression of control RNAi (against mCherry which is not expressed in glia), which are pruned away at 16 h after puparium formation (16 h APF) (Fig. 1B, H, I). Interestingly, we subsequently isolated two independent RNAi transgenes, TH03055.N (#1) and TH201501125.S (#2), which both target against the same gene, *egr*. RNAi knockdown of *egr* via Repo-Gal4 together with UAS-Dicer2 to enhance RNAi efficacy, led to a deficiency of dendritic pruning in C4da neurons at 16 h APF (Fig. 1C, E, H, I), although this phenotype was less pronounced relative to previous cell-autonomous regulators [28, 77, 78]. Furthermore, expressing Egr RNAi transgene (#1) using two copies of Repo-Gal4 drivers resulted in more severe pruning phenotypes at 16 h APF (Fig. 1D, H, I). Previous studies showed that in the *Drosophila* genome, Egr is closest to the mammalian TNF superfamily ligand. The presence of residual dendrite branches or fragments suggests that neuronal pruning is blocked or delayed when glial Egr function is compromised. We then investigated whether overexpression of Egr in glial cells could rescue the pruning-deficient phenotype. Our data suggest that reintroducing Egr, with mutations in the shRNA-targeted region (RNAi-resistant), into Egr-downregulated glia significantly ameliorated dendritic pruning deficiency (Fig. 1G, H, I). In contrast, overexpression of the control Luciferase did not rescue the phenotype (Fig. 1F, H, I). This confirms that the loss of Egr function in glia causes neurite pruning defects. To further confirm the requirement of Glial-Egr for dendrite pruning, we used two previously published alleles, egr^1 and egr^3 [79]. Either egr^1 or egr^3 mutation exhibited consistent dendrite pruning defects at 16 h APF (Figure S1B, D, F, G), in contrast to the control (Figure S1A, F, G), these phenotypes were able to be rescued by glial overexpression of Egr (Figure S1C, E, F, G). Also, we wondered whether Egr could also modulate dendritic pruning in a cell-autonomous manner, and thus we exploited C4da neuron-specific driver pickpocket (PPK)-Gal4 to express Egr RNAi, which demonstrates that downregulation of Egr in C4da neurons does not seem to have a significant impact on dendritic pruning (Figure S2A-D). Taken together, our results elucidate that perineural Egr in glia is required to regulate neurite remodeling of *Drosophila* somatosensory neurons.

The wrapping, subperineurial, and perineurial glial egr work together to control dendritic pruning

Since Repo-Gal4 is a pan-glial driver, in order to further understand the cellular basis of dendritic remodeling and glial cell interactions, it is necessary to identify the subtypes of glial cells involved in dendritic pruning. Previous studies have revealed three different types of glial cells that are distributed around C4da sensory neurons in *Drosophila*, comprising wrapping glia (WG), subperineurial glia (SPG), and perineurial glia [80]. We next utilized Nrv2-Gal4 to drive Egr RNAi transgene expression in the wrapping glia and showed that downregulation of Egr in the wrapping glia resulted in a weak dendritic pruning-deficient phenotype relative to control (Fig. 2A, B, I, J). NP6293-Gal4 is a perineurial glia-specific driver, and we similarly observed a weak dendritic pruning phenotype after knockdown of Egr in perineurial glia (Fig. 2C, D, I, J). Besides this, Moody-Gal4, which is thought to be a subperineurial glia that also wraps around C4da neurons, driving Egr RNAi expression in such subglia also produced a slight dendritic pruning defect (Fig. 2E, F, I, J). The above results imply that all three types of glial cells wrapping C4da neurons seem to be only partially involved in the regulatory process of dendritic pruning. Then, we wondered whether these three types of peripheral glial cells act synergistically to participate in the dendritic pruning through Egr. To this end, we simultaneously knocked down Egr expression in wrapping, subperineurial, and perineurial glia while endogenously labeling C4da neurons, resulting in a significant dendritic pruning phenotype (Fig. 2G, H, I, J). Taken together, Egr derived from wrapping, subperineurial, and perineurial glia act in concert to facilitate dendritic remodeling in C4da neurons during development.

TNFR/Wgn facilitates dendritic pruning in C4da sensory neuron

Subsequently, we want to know how Egr in glia transcellularly governs dendrite sculpting in C4da neurons. We first investigated several common regulators of dendrite pruning, like EcR-B1, Headcase (HDC), and Nrg, in the

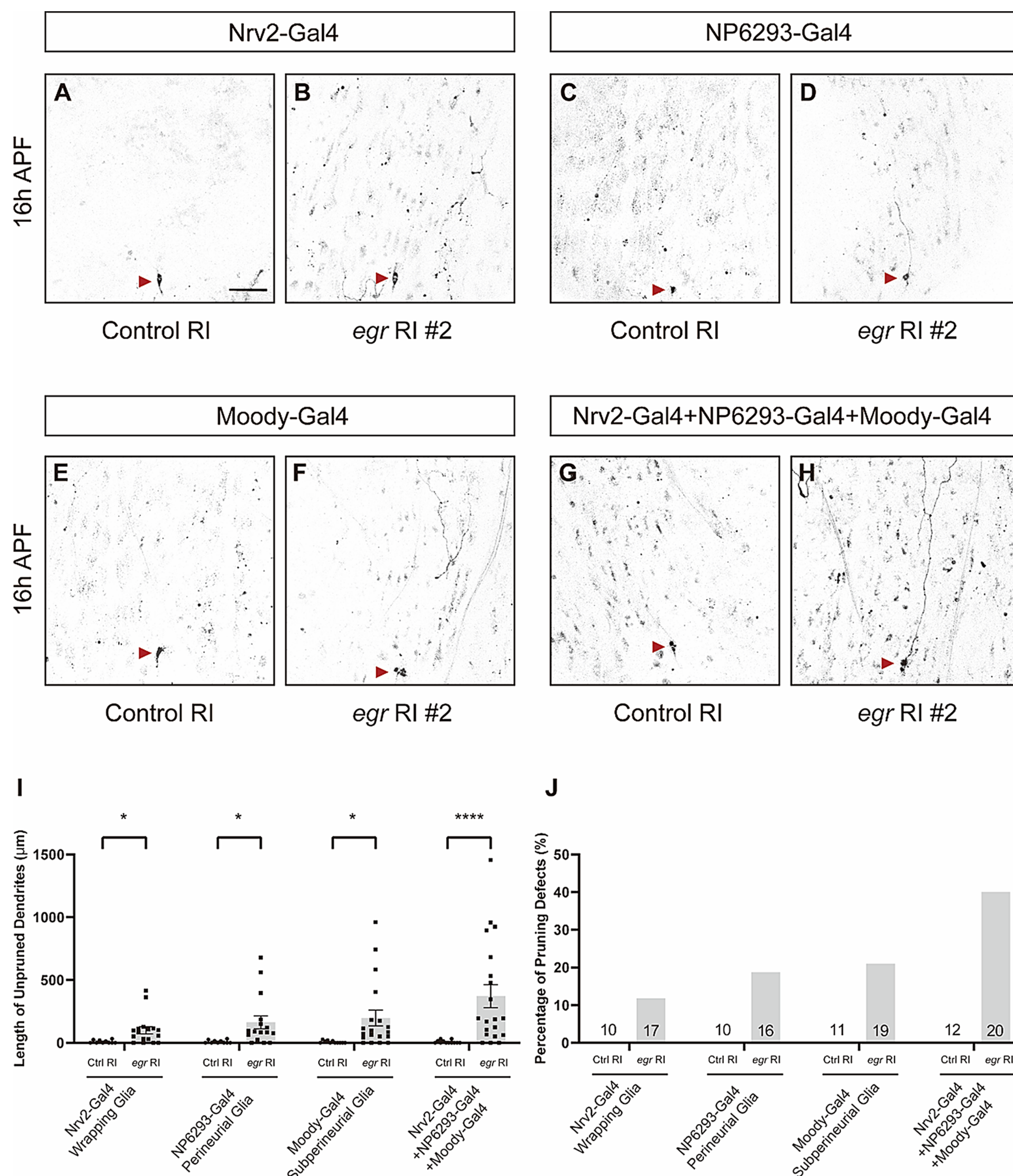

Fig. 2 The wrapping, subperineurial, and perineurial glial Egr synergistically regulate dendritic pruning in C4da neurons. **(A-H)** Live confocal images of C4da neurons at the 16 h APF stage. The ppk promoter drives CD4-tdTomato endogenously label C4da neurons, Nrv2-Gal4 was simultaneously utilized to express Control RNAi **(A)** and *egr* RNAi #2 **(B)** in wrapping glia; NP6293-Gal4 was simultaneously utilized to express Control RNAi **(C)** and *egr* RNAi #2 **(D)** in perineurial glia; Moody-Gal4 was simultaneously utilized to express Control RNAi **(E)** and *egr* RNAi #2 **(F)** in subperineurial glia; Nrv2-Gal4 + NP6293-Gal4 + Moody-Gal4 were simultaneously utilized to express Control RNAi **(G)** and *egr* RNAi #2 **(H)** in the above three types of subglia. RNAi is abbreviated as RI and red arrowheads point to the C4da somas. **(I)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(J)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In I, data are mean ± s.e.m. One-way ANOVA with Bonferroni's test was applied to determine statistical significance. *$P<0.05$; ****$P<0.0001$. The number of neurons (n) examined in each group is shown on the bars. Scale bar: 50 μm

background of glial-Egr RNAi. However, our immunostaining data showed that compared to the controls (Figure S3A, B, C, D, E, F), the levels of EcR-B1, HDC, and Nrg were no obvious differences in glial-Egr knockdown (Figure S3A', B, C', D, E', F). Given that the TNF ligand-receptor system of *Drosophila* is considerably simpler than that of mammals, and it consists of a sole TNF ligand, Egr, and two TNFRs, Wgn and Grnd [52, 63]. We next wonder whether TNF/Egr in glial cells governs the remodeling of dendrites during development by transmitting signal to TNFR/Wgn

on C4da sensory neurons. In an attempt to investigate the potential role of neuronal TNFR/Wgn in neurite remodeling, we subsequently downregulated the expression of Wgn by two independent RNAi lines (#1, TH03054.N; #2, BL55275). Our data showed that RNAi knockdown of Wgn in C4da neurons led to a pronounced deficit in dendrite pruning at 16 h APF (Fig. 3B, D, G, H). Additionally, the utilization of double copies of PPK-Gal4 drivers to express the Wgn RNAi transgene (#1) led to a noteworthy escalation in the severity of pruning defects, as evidenced by a retention in the average dendritic length to 1155 μm (Fig. 3C, G, H). At the same time, the dendrites of the control neurons had been completely removed (Fig. 3A, G, H). Likewise, knockdown with another Wgn RNAi line, V9152 (#3), consistently caused defects of dendrite pruning at 16 h APF (Figure S4B, F, G). Noteworthily, it was previously reported that dendritic pruning defects were not produced by the knockdown of Wgn through Wgn RNAi (BL58994 #4) in C4da neurons [29]. The experiments we carried out using this RNAi strain confirmed this finding (Figure S4C, F, G), which could be explained by the differing knockdown efficiencies of distinct Wgn RNAi lines or by the off-target effect of this RNAi. To further confirm the critical role of Wgn in neurite pruning, we subsequently made use of a p element insertion allele, wgn^{e00637}, and our findings indicated that the wgn^{e00637} mutant displayed consistent defects of neurite pruning, while wild-type neurons fully eliminated their dendrites at 16 h APF (Figure S4A, D, E, F, G). Besides, we found that the overexpression of Wgn modified to be resistant to RNAi in C4da neurons significantly reduced the remaining dendritic branches in Wgn RNAi neurons (Fig. 3F, G, H), whereas induction of Luciferase control could not (Fig. 3E, G, H). Due to the lack of an effective antibody against Wgn, in order to examine the subcellular localization of Wgn in C4da neurons, we next utilized PPK-Gal4 to drive the expression of Wgn-GFP, and found that GFP signals were distributed in a punctate form in the dendrites of C4da neurons (Fig. 3I-K'). However, since Gal4/UAS is an overexpression method, the observed pattern only partially reflects the endogenous localization of the protein. Moreover, we also examined EcR-B1, HDC, and Nrg levels in the context of Wgn RNAi, and the results were analogous to those of Egr, the absence of Wgn in C4da neurons has no significant effect on these pruning modulators (Figure S5A-F). In summary, these results systematically demonstrate the crucial role of neuronal Wgn in dendritic pruning, implying that TNF/Egr in glial cells might contribute to neuronal remodeling during development through the neuronal receptor TNFR/Wgn.

Wgn acts synergistically with Grnd in the regulation of dendritic pruning

Grnd is commonly thought to be another important receptor for Egr, able to bind to Egr via the TNF homology domain and mediate its pro-apoptotic and signaling functions [67]. When performing Grnd RNAi experiment in C4da neurons, it was observed that Grnd downregulation caused residual dendritic branching at 16 h APF (Fig. 4C, F, G), analogous to those Wgn RNAi neurons (Fig. 4B, F, G). In contrast, the dendritic branch in C4da neurons was almost eliminated at this time in the control group (Fig. 4A, F, G). Given that both Grnd and Wgn receptors in neurons might bind the Egr ligand from glial cells and promote dendritic pruning during development, we examined whether simultaneous downregulation of these two receptor molecules in C4da neurons would significantly increase the defective dendritic pruning phenotype. As expected, silencing either Grnd or Wgn alone did not have the same effect on dendritic pruning in C4da neurons as the simultaneous deletion of both TNFRs, which resulted in more dendritic branches remaining at 16 h APF (Fig. 4B-E, F, G). Thus, our experimental results confirm that Grnd and Wgn work synergistically to regulate developmentally relevant dendritic pruning in C4da neurons.

Glial egr regulates dendritic pruning dependent on its neuronal Wgn/Grnd receptors

To further verify that Egr signaling in glial cells is responsible for Wgn/Grnd's regulation of dendritic pruning in C4da sensory neurons, we next intend to experimentally manipulate gene expression in glia and C4da neurons simultaneously by making use of the dual expression system LexA/LexAop in combination with Gal4/UAS. Therefore, we first constructed transgenic *Drosophila* with LexAop-Wgn and LexAop-Grnd. Next, we overexpressed Wgn and Grnd in C4da neurons via PPK-LexA and simultaneously utilized Repo-Gal4 to drive the expression of UAS-*egr*-RNAi together with UAS-Dicer to enhance the knockdown efficiency of Egr in glial cells. The results showed that relative to the control, inductions of Wgn and Grnd in C4da neurons ameliorate the dendritic pruning defects caused by glial deletion of Egr (Fig. 5A-E). Overall, these results implicate Wgn and Grnd receptors in C4da neurons in receiving Egr ligand signaling from surrounding glial cells to initiate dendritic pruning during development.

Wgn and Grnd modulate dendritic pruning via activating the JNK signaling pathway

The TNF ligand binds to TNFR at the cell membrane, generating an intracellular second messenger signaling cascade

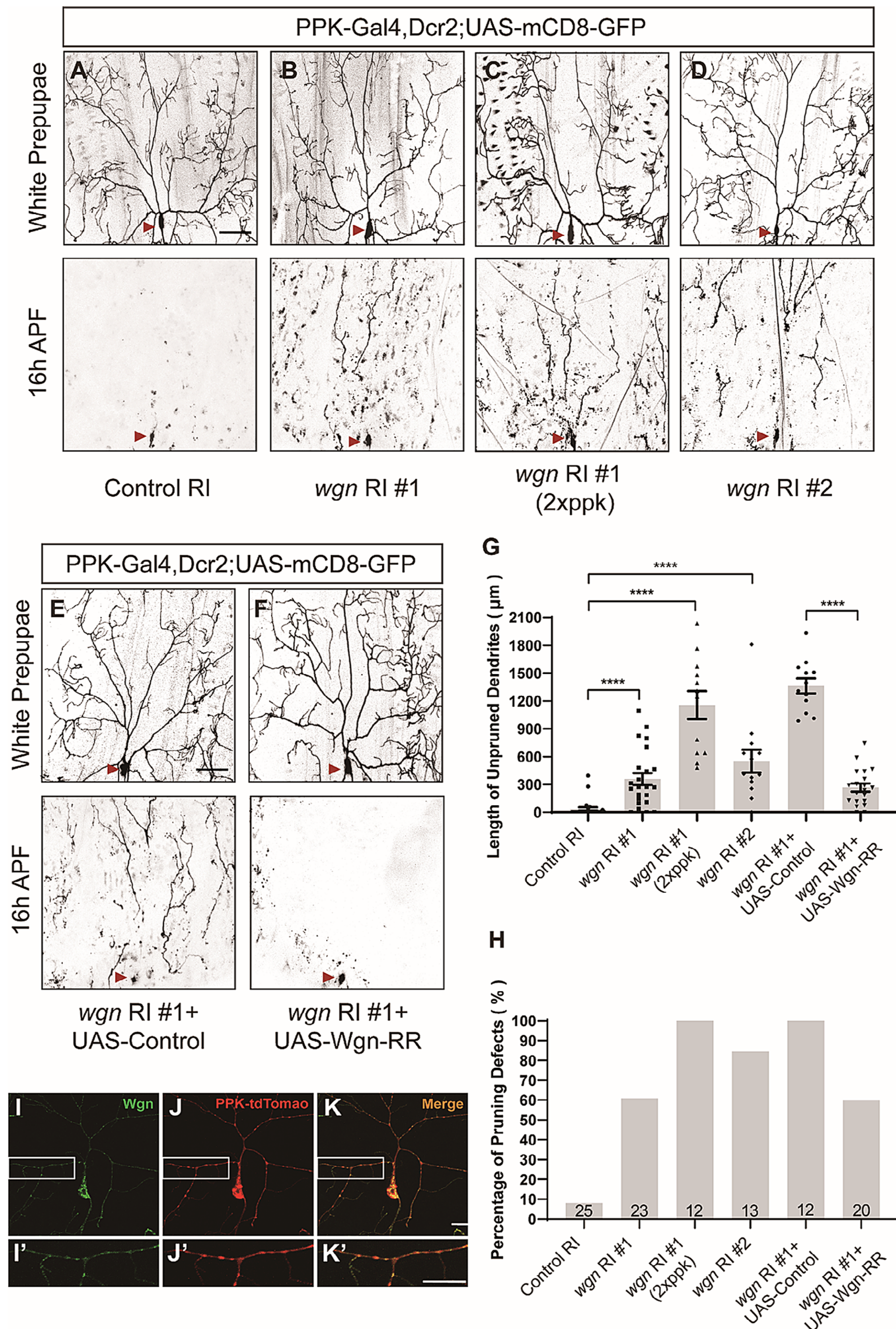

Fig. 3 Neuronal Wgn is required for modulating dendritic pruning in C4da neurons. **(A-F)** Live confocal images of C4da neurons expressing mCD8-GFP driven by PPK-Gal4 at the WP and 16 h APF stages. Dendrites of Control RNAi (A), *wgn* RNAi #1 **(B)**, *wgn* RNAi #1 with 2 copies of PPK-Gal4 **(C)**, *wgn* RNAi #2 **(D)**, *wgn* RNAi #1 + UAS-Control **(E)**, and *wgn* RNAi #1 + UAS-Wgn-RR **(F)** C4da neurons at the WP and 16 h APF stages. RNAi is abbreviated as RI, RNAi-resistant is abbreviated as RR, and red arrowheads point to the C4da somas. **(G)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(H)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In G, data are mean ± s.e.m. One-way ANOVA with Bonferroni's test was applied to determine statistical significance. ***P < 0.001; ****P < 0.0001. The number of neurons (n) examined in each group is shown on the bars. **(I-K')** Confocal images of C4da neurons expressing UAS-Wgn-GFP **(I-I')**, the ppk promoter drives CD4-tdTomato endogenously label C4da neurons **(J-J')**, showing that Wgn is distributed as puncta on the dendrites. Scale bar: 50 μm **(A-F)** and 20 μm **(I-K')**

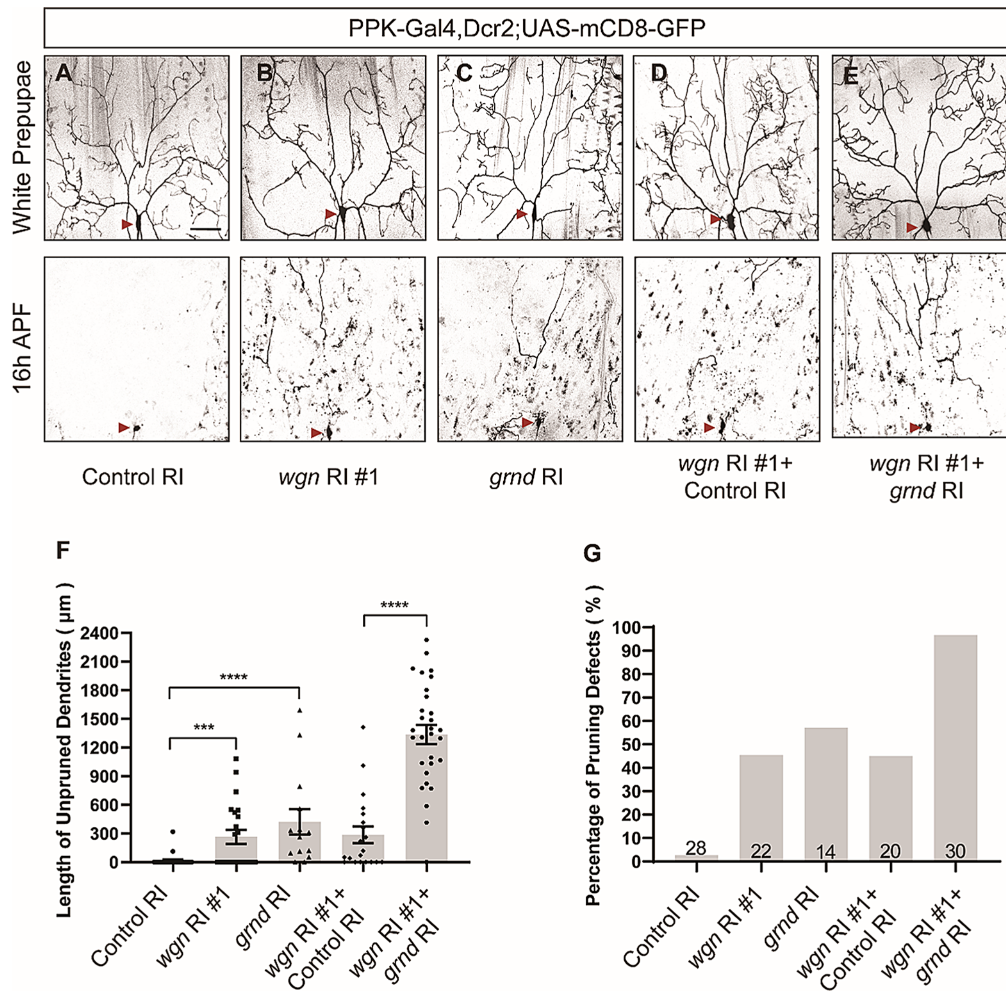

Fig. 4 Neuronal Wgn and Grnd synergistically govern dendritic pruning. **(A-E)** Live confocal images of C4da neurons expressing mCD8-GFP driven by PPK-Gal4 at the WP and 16 h APF stages. Dendrites of Control RNAi **(A)**, *wgn* RNAi #1 **(B)**, *grnd* RNAi **(C)**, *wgn* RNAi #1 + Control RNAi **(D)**, and *wgn* RNAi #1 + *grnd* RNAi **(E)** C4da neurons at the WP and 16 h APF stages. RNAi is abbreviated as RI and red arrowheads point to the C4da somas. **(F)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(G)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In F, data are mean ± s.e.m. One-way ANOVA with Bonferroni's test was applied to determine statistical significance. ***$P < 0.001$; ****$P < 0.0001$. The number of neurons (n) examined in each group is shown on the bars. Scale bar: 50 μm

that drives NF-κB-mediated proliferation or caspase-dependent apoptosis [48, 58, 59]. Based on this, we next wonder whether the control of dendritic pruning by TNF/Egr was dependent on the downstream NF-κB or caspases signaling in neurons. The NF-κB (nuclear factor κB) signaling pathway is an important regulatory system in *Drosophila* melanogaster, which is involved in the control of cellular immune response, inflammatory response, cell apoptosis, cell survival, and proliferation, as well as other biological processes [81–83]. In mammals, there are five NF-κB proteins, including RelAIp65, Rel B, c-Rel, NF-κB1 (P50 and its precursor P105), and NF-κB2 (P52 and its precursor P100). However, there are only three in *Drosophila*, comprising Dorsal, Dorsal-related immunity factor (Dif), and Relish (Rel) [84]. Once released and entering the nucleus, NF-κB protein can bind and activate the transcription of target genes, thereby regulating related biological processes. Herein, we knocked down Rel, by two independent RNAi in C4da neurons, and showed that the attenuation of NF-κB signaling did not appear to have a significant effect on dendritic pruning (data not shown). Simultaneously, we downregulated the expression of Dronc, the sole *Drosophila* initiator caspase needed for apoptosis, via two distinct Dronc RNAi strains V100424 (#1) and V23033 (#2), respectively, with PPK-Gal4 in C4da neurons, and exhibited severe dendritic pruning defects compared to the control group (Figure S6A-C, F, G). This finding is consistent with previous research, which showed that the removal of Dronc in C4da neurons impeded dendritic pruning [22]. Does Egr signaling from glial cells activate Dronc in C4da neurons to facilitate the

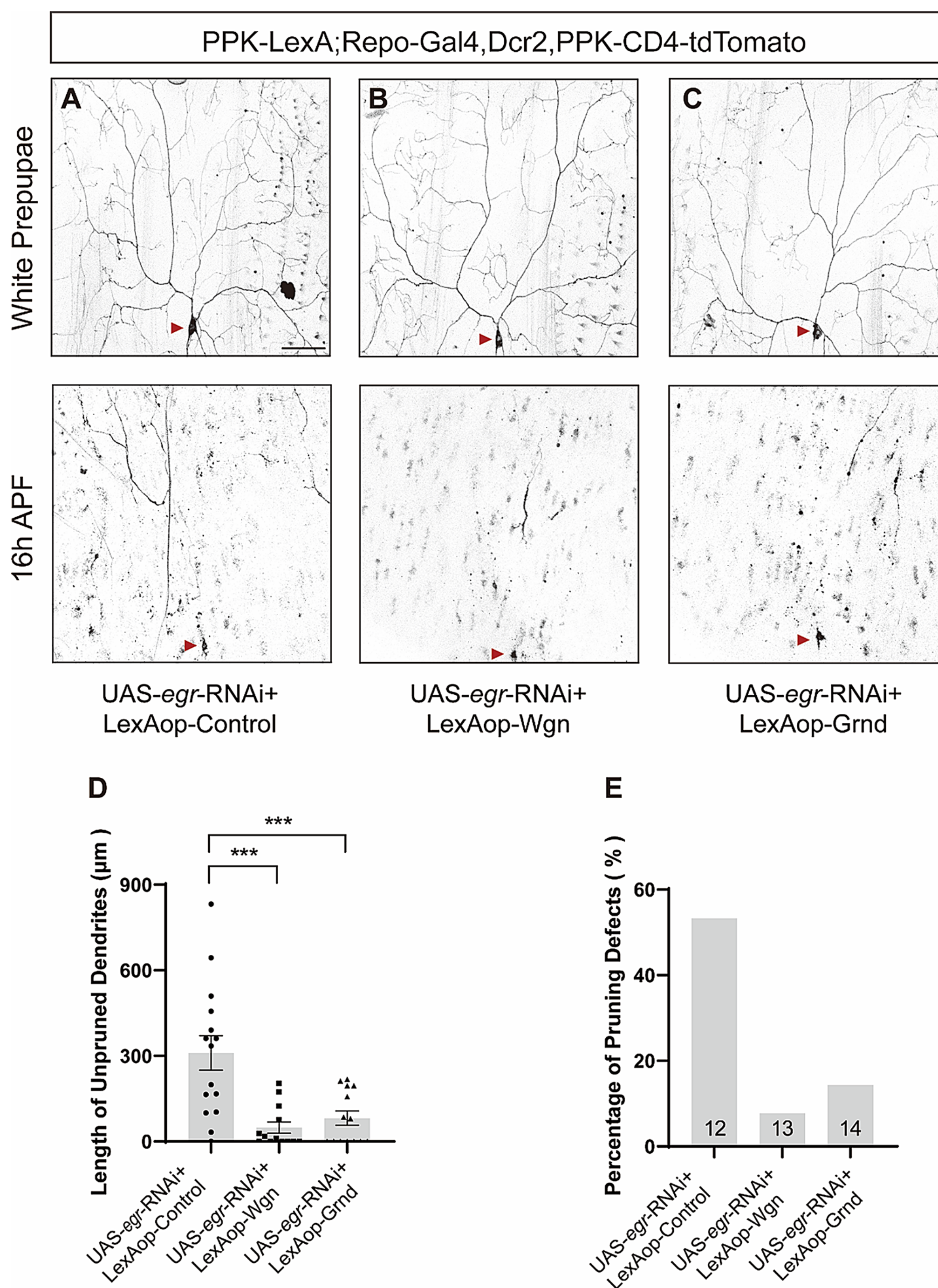

Fig. 5 Glial Egr regulates dendritic pruning via neuronal Wgn and Grnd. **(A-C)** Live confocal images of C4da neurons at WP and 16 h APF stages. The ppk promoter drives CD4-tdTomato to endogenously label C4da neurons, PPK-LexA was utilized to overexpress LexAop-Control **(A)**, LexAop-Wgn **(B)**, and LexAop-Grnd **(C)** in C4da neurons, and Repo-Gal4 was simultaneously utilized to downregulate Egr in glia, showing that the inductions of Wgn and Grnd can alleviate pruning defects in C4da neurons caused by deletion of glial Egr. **(D)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(E)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In D, data are mean ± s.e.m. One-way ANOVA with Bonferroni's test was applied to determine statistical significance. $^{***}P < 0.001$. The number of neurons (n) examined in each group is shown on the bars. Scale bar: 50 μm

developmentally relevant pruning of dendrites? To test this conjecture, we attempted to rescue the dendritic pruning deficit triggered by attenuated TNF signaling by introducing Dronc. Subsequently, we overexpressed Dronc in the context of wgn RNAi #1, however, neuronal death occurred, and dendritic pruning could not be observed in the WP and 16 h APF phases (Figure S6D-E, F, G). This may be attributed to the pro-apoptotic effect of Dronc. Therefore, it is

not possible to clarify whether Dronc acts as a downstream regulator of dendritic pruning by the Wgn receptor. Alternatively, the Dronc can later be down-regulated in the context of wgn RNAi to see if it can exacerbate the dendritic pruning phenotype, demonstrating whether Wgn and Dronc act in the same pathway.

Additionally, the evolutionarily conserved TNF-α-like signaling has also been reported to regulate cell survival in the *Drosophila* eye and wing induced by activation of the JNK signaling pathway [52, 53]. Notably, a previous report revealed an important role for JNK signaling in dendritic pruning in C4da neurons [29]. A similar phenotype was observed by knocking down *basket* (*bsk*), which encodes JNK in the *Drosophila* genome, or overexpressing a dominant-negative form of Bsk (Bsk^{DN}) in C4da neurons (Fig. 6A-D, G, H). To further confirm whether Bsk acts as a Wgn receptor downstream to regulate dendritic pruning, we attempted to overexpress Bsk in Wgn down-regulated neurons to see if it could improve the dendritic pruning phenotype. Interestingly, the introduction of Bsk substantially rescued the dendritic pruning defect caused by Wgn deletion (Fig. 6F, G, H) while UAS-Control could not (Fig. 6E, G, H). We next wondered whether Grnd, another receptor for Egr, also promotes dendritic pruning by activating downstream JNK signaling. Our results showed that dendritic pruning defects caused by Grnd deletion were also remarkably rescued after overexpression of Bsk in Grnd downregulated C4da neurons (Fig. 7B, C, D). In comparison to the average length of dendritic branching residues of 750 µm in the control group at 16 h APF (Fig. 7A, C, D), the introduction of Bsk resulted in a significant reduction of Grnd deletion-induced dendritic branching residues to only 150 µm, and the percentage of dendritic pruning defects was dropped from 84 to 38% (Fig. 7B, C, D). It is noteworthy that the failure of Bsk to completely rescue the phenotypes of Wgn and Grnd illustrates that there may be other downstream signals of TNF/TNFR in *Drosophila* that regulate developmentally relevant dendritic remodeling or the compensatory effects between Wgn and Grnd. Collectively, these results illustrate that the JNK pathway in C4da neurons acts as a pivotal downstream target of glial TNF/Egr signaling to trigger dendrite elimination during development (Fig. 8). Remarkably, this study reveals for the first time a regulatory signal for C4da dendritic pruning from the surrounding glial cells.

Discussion

The maturation of both the central and peripheral nervous systems (PNS) require the removal of superfluous or excess synaptic connections. It was previously believed that synaptic elimination was mainly caused by cell-intrinsic mechanisms in neurons. In recent years, it has become apparent that central and peripheral glial cells play a crucial role in regulating the synaptic structure, function, and survival during development, including the elimination or degeneration of synapses [42, 64, 85]. The superabundant neural projections in adulthood can be caused by either expurgation or alteration of the characteristics of these cells, which can lead to corresponding modifications to brain circuits and behaviors. Numerous molecules and signaling pathways found in glial cells are involved in either promoting or inhibiting synaptic deletion, according to several mechanistic investigations conducted on model animals [52, 86, 87]. In the end, it results in the formation of precise neuronal circuits and matured functions. Here, we present evidence for the discovery of a novel neuronal sculpting signaling pathway. This pathway originates from peripheral glial cells that are in close proximity to C4da sensory neurons. We showed that glia-specific knockdown of Egr, the single *Drosophila* TNF ligand, causes residual dendrites at 16 h APF, however wild-type dendrites clear almost all dendritic branches at this time point. Our findings further reveal that the functional deficits in Egr do not appear to affect overall glial cell morphology (data not shown). How does the glial cell-initiated Egr signal translocate to C4da neurons to promote dendritic pruning? Previous studies have shown that Egr acts as a secreted ligand in *Drosophila* with two receptors, Wgn and Grnd [67–69]. In addition, it has been demonstrated that Egr has a different affinity to Wgn and Grnd and that they are all highly expressed in the nervous system [89]. We then wonder whether glial Egr triggers dendritic pruning by Wgn and Grnd receptors on the surface of C4da neurons. The results show that Wgn and Grnd act synergistically in C4da neurons to promote dendritic pruning. Importantly, overexpression of Wgn and Grnd in C4da neurons ameliorated the dendritic pruning phenotype triggered by glial cell deletion of Egr. It implies that glial-derived Egr is indeed involved in regulating the neural remodeling process by activating receptors on the neuronal surface in a transcellular manner. Nevertheless, we found that the dendritic pruning phenotypes of neuronal Wgn and Grnd appear to be more severe than glial Egr, suggesting that Wgn and Grnd may receive other ligand signals. This signal may originate from glial cells or other neighboring tissues, like muscle cells, epithelial cells, and others.

In the *Drosophila* CNS, cortex and astrocyte-like glia are involved in the regulation of axonal pruning of MB γ neurons through the secretion of Myo, a transforming growth factor that acts on the corresponding neuronal receptor Babo [88] to trigger axonal remodeling. How is glial-derived Egr secreted and released? Are there ligand proteins involved in Egr secretion and how do they modulate Egr secretion? In the

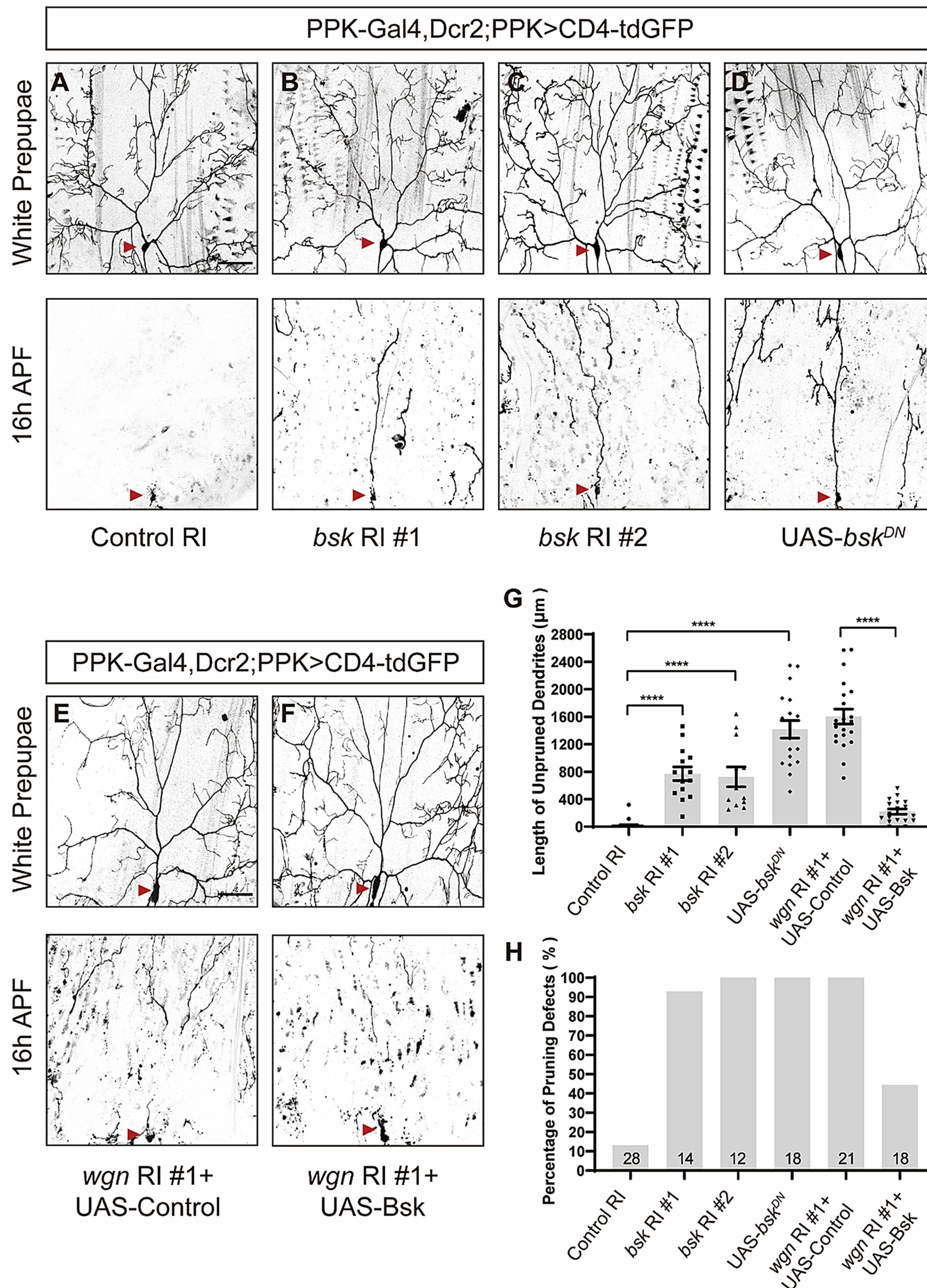

Fig. 6 Wgn regulates dendritic pruning partially through JNK signaling. **(A-F)** Live confocal images of C4da neurons expressing CD4-tdGFP driven by PPK-Gal4 at the WP and 16 h APF stages. Dendrites of Control RNAi **(A)**, *bsk* RNAi #1 **(B)**, *bsk* RNAi #2 **(C)**, UAS-Bsk^{DN}**(D)**, *wgn* RNAi #1 + UAS-Control **(E)**, and *wgn* RNAi #1 + UAS-Bsk (F) C4da neurons at the WP and 16 h APF stages. RNAi is abbreviated as RI and red arrowheads point to the C4da somas. **(G)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(H)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In G, data are mean ± s.e.m. One-way ANOVA with Bonferroni's test was applied to determine statistical significance. ****$P < 0.0001$. The number of neurons (n) examined in each group is shown on the bars. Scale bar: 50 μm

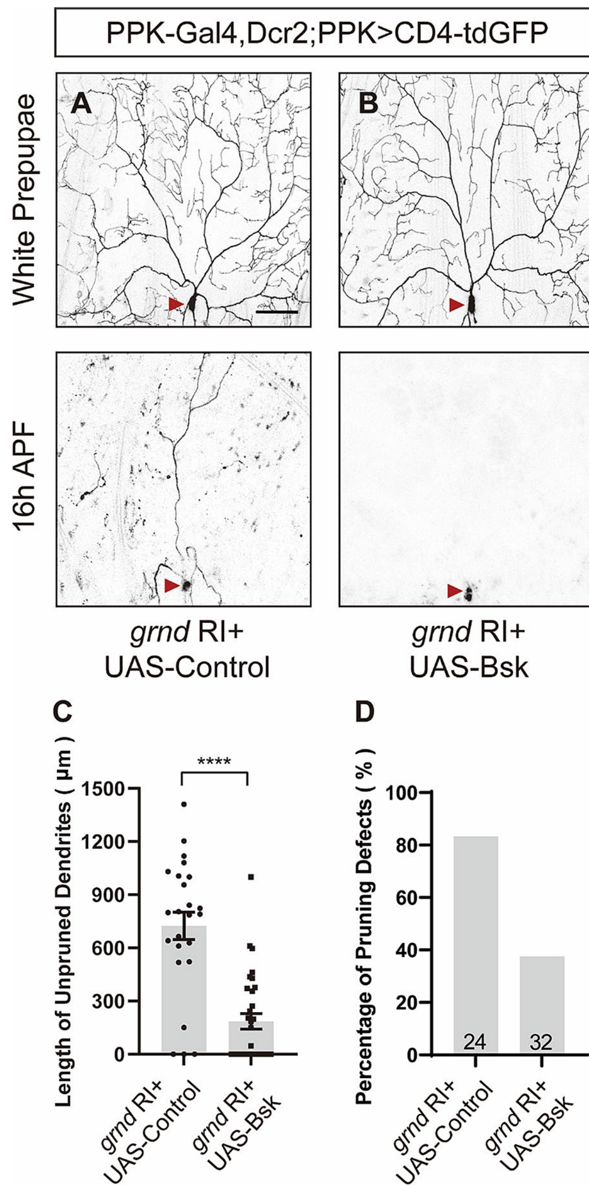

Fig. 7 Grnd regulates dendritic pruning partially through JNK signaling. **(A-B)** Live confocal images of C4da neurons expressing CD4-tdGFP driven by PPK-Gal4 at the WP and 16 h APF stages. Dendrites of *grnd* RNAi + UAS-Control **(A)** and *grnd* RNAi + UAS-Bsk **(B)** C4da neurons at the WP and 16 h APF stages. RNAi is abbreviated as RI and red arrowheads point to the C4da somas. **(C)** Quantitative analysis of unpruned dendrite lengths of the indicated genotypes. **(D)** Percentages of C4da sensory neurons with pruning defects of the denoted genotypes. In C, data are mean ± s.e.m. A two-tailed Student's t-test was applied to determine statistical significance. $****P < 0.0001$. The number of neurons (n) examined in each group is shown on the bars. Scale bar: 50 μm

future, it will be interesting to screen the regulators involved in the secretory pathway to see whether they are required for Egr secretion and dendritic pruning of C4da neurons, and then further explore how glial cells regulate dendritic pruning through Egr. Noteworthily, TNF is commonly present in a membrane-bound manner, although some TNF can be produced in a soluble form after cleavage by tumor necrosis factor converting enzyme (TNF-converting enzyme, TACE) [62, 89]. *Drosophila* TNF/Egr is available in two forms, Egr-L and Egr-S, where Egr-L is the membrane-bound form and Egr-S is the soluble form that can function by circulating in the hemolymph [63, 90]. It has been reported that Egr in the adipose body can be secreted for efficacy after cleavage by TACE [65], but the process of how TACE cleaves Egr has not been described in detail. Thus, we conjectured that Egr, a type II transmembrane protein, originates from glial cells, is cleaved by TACE, secreted into intercellular compartments, and acts on target neurons to function in regulating dendritic pruning. However, whether glial cell-derived Egr is functional after cleavage by TACE requires further experimental verification. On the one hand, it is possible to observe whether deletion of TACE in glial cells causes similar dendritic pruning defects, and on the other hand, we can examine the co-localization of glial-derived tagged Egr with the receptors in C4da neurons by immunofluorescence to more directly demonstrate the mode of action.

How do Wgn and Grnd membrane receptors transmit external signals to C4da sensory neurons? Our study shows that Egr in wrapping glia, perineurial glia, and subperineurial glia promotes dendritic pruning during development via the Wgn and Grnd receptors. Notably, these neuronal receptors could also be exposed to hemolymph circulating glia-derived Egr in addition to receiving C4da-encapsulated glia-derived ligand signaling. Previous studies in *Drosophila* and mammals have shown that TNFR mainly activates three downstream signaling pathways, including NF-κB, caspase, and JNK, to govern a range of cellular physiological functions [59, 60]. We then analyzed all three pathways and found that perturbing Dronc and Bsk in neurons resulted in defective dendritic pruning, consistent with previous reports. However, attenuation of NF-κB signaling seems to have a negligible effect on dendritic pruning (data not shown). Interestingly, we found that activation of JNK signaling in neurons rescued dendritic pruning defects caused by Wgn and Grnd knockdown, yet overexpression of Dronc in the background of Wgn knockdown led to apoptosis, which seems understandable since Dronc is a pro-apoptotic protein. Collectively, these findings demonstrate that glial-derived Egr ultimately promotes developmental neuronal remodeling by activating downstream JNK signaling via binding to C4da sensory neuron surface receptors. However, considering that activation of JNK signaling cannot completely rescue the Wgn and Grnd phenotype, another previous report in mammals showed that muscle-derived TNF activates caspase at the neuromuscular junction, thereby promoting synaptic deletion [91]. Therefore, further studies are needed to confirm whether Dronc plays

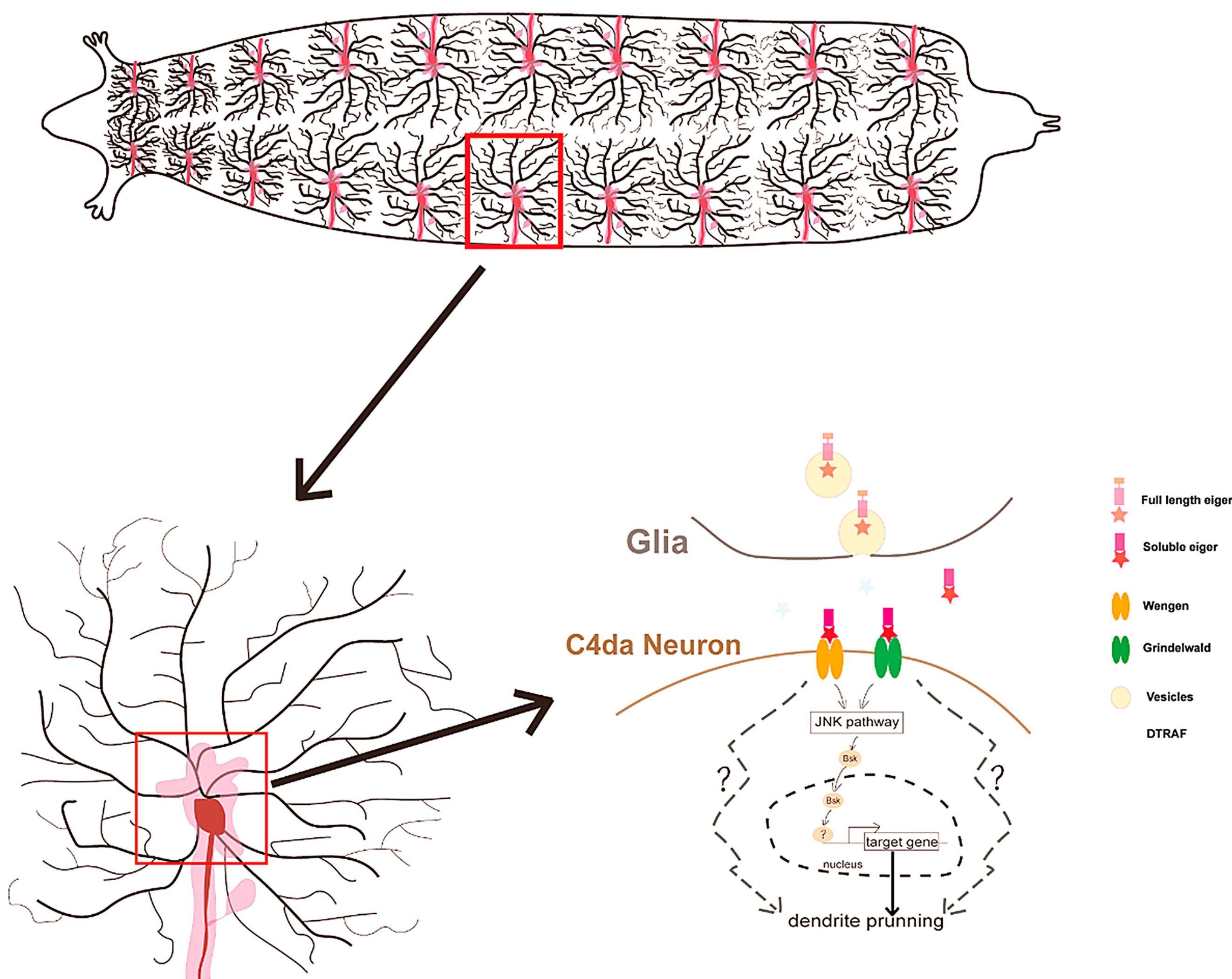

Fig. 8 A schematic diagram of glial-initiated Egr signaling promoting developmentally relevant dendritic remodeling through neuronal Wgn/Grnd receptor activation of JNK signaling

a role in neuronal remodeling as one of the downstream branches of glial Egr signaling.

Of particular importance is the fact that the two factors we identified as affecting dendritic pruning, TNF/Egr and TNFR/Wgn/Grnd, are both conserved and have corresponding homologous genes in mammals. Noteworthily, the clinical introduction of TNF inhibitors can greatly improve the treatment of autoimmune inflammatory diseases, including ankylosing spondylitis [92, 93]. Although several clinical trials have demonstrated the efficacy of TNF inhibitors, there are still some issues with anti-TNF treatment regimens, such as serious adverse effects in some patients related to limited treatment response, which may require us to go further with our studies on TNF/TNFR. The role of TNF in the regulation of neural pruning can be further explored in the future in mammals such as mice, as well as in organoids to screen for suitable drugs for disease treatment, which can not only find potential drug targets for the treatment of related neurological diseases but also bring novel insights into the application of TNF-related proteins in the treatment of immunological and neurodevelopmental diseases. In conclusion, our study has identified for the first time a regulatory signal for C4da neurons dendritic pruning from glia, a finding that highlights the importance of the enveloping glia for dendritic remodeling in the PNS. We suggest that glial cells and epithelial cells might act synergistically to participate in the dendritic pruning process of C4da neurons. First, glial cells transmit signals to C4da neurons to induce the initiation of dendritic pruning, after the pruning process has begun, dendrites break into discontinuous fragmented structures, and then the epithelial cells act as phagocytes, and with the assistance of chemokine-like protein Orion [94], recognize the phosphatidylserine (PS) signal on the surface of the dendrites through the

engulfment receptor Draper (Drpr) to initiate phagocytosis and eventually complete the removal [43, 95]. In the future, uncovering more key molecules, cytokines, and signaling pathways involved in dendritic pruning outside the neuron, is expected to reveal the precise peripheral regulatory network in the process of neurite remodeling. This study aims to provide new perspectives for understanding the formation of nervous system functions and the neural basis of related neurodevelopmental diseases.

Materials and methods

Drosophila stocks

The following stocks were obtained from Bloomington Stock Centre (BSC, Bloomington, IN, USA): W^{1118} (BL5905), Control RNAi/mCherry RNAi (BL35785), UAS-Control/UAS-Luciferase (BL35788), UAS-*wgn*-RNAi #2 (BL55275), UAS-*wgn*-RNAi #4 (BL58994), wgn^{e00637} (BL17874), UAS-Bsk (BL9310), UAS-Bsk^{DN} (BL6409), UAS-Dronc (BL56759), PPK-CD4-tdTomato (BL35845). The following stocks were obtained from the Vienna *Drosophila* RNAi Centre (VDRC): UAS-*wgn*-RNAi #3 (V9152), UAS-*grnd*-RNAi (V104538), UAS-*dronc*-RNAi #1 (V100424), UAS-*dronc*-RNAi #2 (V23033). The following stocks were obtained from the Tsinghua RNAi Stock Center: UAS-*egr*-RNAi #1 (TH03055.N), UAS-*egr*-RNAi #2 (TH201501125.S), UAS-*wgn*-RNAi #1 (TH03054.N), UAS-*bsk*-RNAi #1 (TH04355.N), UAS-*bsk*-RNAi #2 (TH201500082.S). The following stocks were obtained from the Kyoto *Drosophila* Stock Center: egr^1 (D118635), egr^3 (D118634). The UAS-CD4-tdGFP and PPK-LexA are gifts from Prof. Wei Xie (Southeast University, China); The UAS-Dicer2, UAS-mCD8-GFP, Repo-GAL4, Nrv2-Gal4, NP6293-GAL4, and Moody-GAL4 are gifts from Prof. Su Wang (Southeast University, China); The PPK-Gal4 (II) and PPK-Gal4 (III) are gifts from Dr. Yan Zhu (Institute of Biophysics, Chinese Academy of Sciences). All the flies were raised at 25 ˚C on standard medium.

Generation of transgenes

For the rescue experiment, the full-length of UAS-Egr, the full-length of UAS-Wgn, UAS-Wgn-GFP, the RNAi-resistant of UAS-Egr, and the RNAi-resistant of UAS-Wgn were made by cloning the full-length and full-length with modified to be resistant to RNAi cDNA into the transformation vector pUAST-attB. Transgenic fly lines were generated by standard germline transformation. cDNA of Egr and Wgn were obtained from reverse transcription of genomic mRNA. Fly head mRNA was extracted using TRIzol (Life Technologies), and reverse transcribed using cDNA Reverse Transcriptase Kit (Applied Biosystems). Besides, the LexAop-Wgn and LexAop-Grnd plasmid were constructed by digesting the vector plasmid LexAop-Luciferase (LexAop-Control) kept in our laboratory and replacing Luciferase CDS with the amplified Wgn and Grnd CDS. The transgenic lines were generated by the UniHuaii Inc.

Live imaging analysis

Drosophila at the white prepupal (WP) stage was first washed in PBS buffer briefly for three times and subsequently immersed with 90% glycerol, pupal cases at the 16 h APF were carefully removed before mounted with 90% glycerol. Images of C4da neurons were captured with Zeiss LSM 900 confocal microscope.

Immunohistochemistry and antibodies

For immunostaining, white prepupae were dissected in precooling PBS and fixed with 4% formaldehyde for 30 min. The experimental and control samples were incubated synergistically in the same tubes. Mounting was conducted in the mounting medium of VectaShield, and the samples were directly visualized by a Zeiss LSM 900 confocal microscope. The following primary antibodies were used for immunohistochemistry at the indicated dilution: mouse anti-Nrg (1:50, BP104, DSHB, Iowa City, IA, USA), mouse anti-EcR-B1 (1:20, DSHB, Iowa City, IA, USA), and mouse anti-Headcase (1:20, DSHB, Iowa City, IA, USA). The following secondary antibodies were used for immunohistochemistry at the indicated dilution: Alexa Fluor 488- and Alexa Fluor 555- conjugated secondary antibodies (1:500, Invitrogen, Waltham, MA, USA).

Quantification of dendrites

The structure of the C4da neuron dendrite at the 3rd instar larvae, WP, and 16 h APF stages were captured through live confocal imaging. The length of unpruned dendrites and percentage of pruning defects are utilized to evaluate the neural pruning phenotype. The length of unpruned dendrites is the sum length of remaining dendrites at 16 h APF, measured in a 275 μm × 275 μm region derived from the dorsal dendritic field of C4da neurons, ranging from the abdominal segments 2–4. The pruning defect of dendrite is defined by the uncleared length of dendrite that is more than 200 μm at 16 h APF, whereas wild-type dendrites have almost no dendritic residues at this stage. The percentage of dendrite pruning defects refers to the proportion of neurons with pruning defects relative to all observed neurons.

Statistics

For pairwise comparison, two-tailed Student's t-test was used to determine statistical significance. For multiple-group comparison, one-way ANOVA with Bonferroni's test was used to determine significance. Error bars in all graphs represent s.e.m. Statistical significance was defined as ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$, and ns, not significant. The number of neurons (n) in each group is shown on the bars.

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Author contributions Conceptualization: M.R.; Methodology: T.Z.; M.R.; Software: T.Z.; M.R.; Validation: T.Z.; K.L.; M.R.; Formal analysis: T.Z.; M.R.; Investigation: T.Z.; M.R.; Resources: T.Z.; S.W.; M.R.; Data curation: T.Z.; M.R.; Writing—original draft: M.R.; Writing—review & editing: M.R.; Visualization: M.R.; Supervision: M.R.; Project administration: M.R.; Funding acquisition: M.R. All authors have read and agreed to the published version of the manuscript.

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Data availability The authors declare that all data supporting the findings of this study are available within the paper in the main text or the supplementary materials.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing or financial interests.

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