Glia instruct developmental neuronal remodeling through TGF-β signaling

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Abstract

Neural circuits are remodeled in response to developmental and environmental cues. Here we show that glia secretes Myoglianin, a TGF-β ligand, to instruct developmental neural remodeling in Drosophila. Glial Myoglianin up-regulates neuronal expression of an ecdysone nuclear receptor that triggers neurite remodeling following the late-larval ecdysone peak. This observation reveals that glia orchestrate developmental neural remodeling not only via engulfment of unwanted neurites but also through enabling neuron remodeling.

Results and Discussions

To establish and refine functional neural circuits, neurons alter connections as the organism matures. In Drosophila, larval brain neural circuits are remodeled into adult ones during metamorphosis¹. Neurons forming functional larval neural circuits prune their neural projections by local degeneration in early metamorphosis and re-extend their neurites to form the adult-specific neural circuits²-⁴. This phenomenon requires activation of the TGF-β signaling in the remodeling neurons. TGF-β signaling up-regulates expression of the B1 isoform of the ecdysone receptor (EcR-B1) at the late larval stage⁵. Then, the pruning of larval projections is triggered by the steroid molting hormone ecdysone⁶.

actin-β (actβ), which encodes a Drosophila Activin/TGF-β family molecule, is expressed in the developing larval brain. Temporal inhibition of Actβ with its dominant-negative form or double-strand RNA (dsRNA) partially suppresses the expression of EcR-B1 in the wandering larvae. Therefore, we proposed in an early study that Actβ is a candidate ligand for the TGF-β signaling in neuronal remodeling⁵. However, we found that the recently isolated actβ null mutant, actβ⁸⁸⁰⁷ has no defect on the developmental remodeling of the

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mushroom body (MB) γ neurons (Supplementary Fig. 1). This observation excludes Actβ as a major ligand for TGF-β-dependent remodeling of MB neurons. In addition, the actβ null mutant grows normally until the pharate adult stage7, contrasting the embryonic lethality due to a ubiquitous induction of the dominant-negative form or dsRNA of actβ. These contradictory phenomena argue presence of off-target effects in our earlier suppression of Actβ with dominant-negative proteins or RNA interference (RNAi).

Notably, myoglianin (myo), which encodes another Drosophila TGF-β ligand8, is temporally expressed in the brain of third instar larvae. While no myo transcripts could be detected in the brain of early larvae, intense signals for myo transcripts were seen in subsets of glial cells in the cortex and inner regions of the central brain after the mid third instar larval stage (Fig. 1a-d and Supplementary Fig. 2). We found that myo is selectively expressed in two subtypes of larval glial cells: the larval cortex and astrocyte-like glial cells (Fig. 1e-h). The cortex glia surround the cell body of each mature neuron, and the astrocyte-like glia infiltrate into brain neuropile (Supplementary Fig. 3). The glial processes of both types are in the vicinity of, if not directly contacting, the larval MB γ neurons.

To determine if myo governs MB remodeling, we silenced the glial expression of myo by targeted RNAi. dsRNA or microRNA (miRNA) against myo was selectively expressed in glia using the pan-glial GAL4 driver, repo-GAL4. myo transcripts were no longer detectable following induction of myo-dsRNA in pan-glial cells (Supplementary Fig. 2). We then followed the pruning and re-extension of MB γ axons by immunostaining with anti-Fasciclin 2 (Fas2) antibody (Fig. 2a-d). In wild type animals, the perpendicular γ axonal branches in the larval MB lobes are completely pruned by 18 hours after puparium formation (APF). γ neurons subsequently re-extend axons horizontally to form the midline-projecting γ lobe in adult brains (Fig. 2a,b). This remodeling was blocked by pan-glial induction of myo RNAi (Fig. 2c,d). The perpendicular axonal branches of γ neurons persisted through early metamorphosis (100%, n=10), and the abnormally retained larval neurites co-existed with the α/β lobes in the adult MBs that failed to remodel (Fig. 2c,d, Supplementary Fig. 4 and 5). Direct visualization of MB γ neurons validated the above observations with anti-Fas2 antibody (Supplementary Fig. 6). The myo-silenced brains, including their glial network, were otherwise grossly normal (Supplementary Fig. 7). These observations indicate that loss of myo expression in glia exerts no detectable effect on glial cells but adversely affects MB remodeling.

We further knocked down myo using glial subtype-specific drivers. Notably, only cortex glia-specific silencing could marginally block MB remodeling and elicit mild MB lobe defects in about 60% of adult MBs (Supplementary Fig. 5 and 9). However, to silence myo in both larval cortex and astrocyte-like glia fully recapitulated the MB remodeling defects caused by the pan-glial induction of myo RNAi (Supplementary Fig. 5 and 8). These subtype-targeted RNAis revealed that Myos of two glial sources act redundantly to govern MB remodeling.

Next, to determine if glial-derived myo is required for up-regulation of EcR-B1 in remodeling MB γ neurons, we compared EcR-B1 expression in late-larval MBs in wild-type larvae to those expressing myo RNAi in glia. We did not detect the characteristic pattern of
EcR-B1 enrichment following silencing of glial myo (Fig. 2e,f). For example, the strong nuclear signal of EcR-B1 in the MB γ neurons was no longer discernible (Fig. 2h,k,i,l and Supplementary Fig. 9). When EcR-B1 expression was selectively restored in the MB γ neurons of glial myo RNAi animals, no defect in MB remodeling could be detected (Supplementary Fig. 4 and 5). This reveals that the neuronal phenotypes resulting from glial myo RNAi can be effectively rescued by neuronal induction of EcR-B1. These results indicate that the glia-derived Myo instructs MB remodeling through up-regulation of neuronal EcR-B1.

Larval olfactory projection neurons (PNs) also remodel their neural projections under the control of the same TGF-β and ecdysone signalings as the MB γ neurons. Interestingly, we found that loss of glial myo blocked EcR-B1 expression and neurite remodeling of PNs and the remodeling defect was significantly rescued by PN-specific induction of transgenic EcR-B1 (Supplementary Fig. 10). These results suggest that glia-derived Myo acts broadly to pattern neuronal remodeling through up-regulation of EcR-B1.

To rule out off-targeting effects of RNAi, we tried to rescue the RNAi phenotypes by co-induction of various myo transgenes with myo-specific miRNA (myo-miRNA). Whereas co-induction of “wild-type myo” with myo-miRNA in glia did not rescue the neural EcR-B1 expression or the MB remodeling defect (Supplementary Fig. 5 and 9), complete rescue was obtained in the presence of “miRNA-resistant myo” that possesses altered codon usage in the miRNA target sites (Fig. 2g,j,m, and Supplementary Fig. 5 and 9). This ascribes the myo-miRNA-induced neuronal remodeling defects to the loss of endogenous myo.

Using an FRT-mediated inter-chromosomal recombination technique, we generated a deletion mutant, myoA1 (Fig. 3a,b). Organisms homozygous for myoA1 showed no developmental delay through the feeding third instar larval stage. However, mutant larvae prepupate on the surface of or in the food and are developmentally arrested prior to head inversion. In the myo mutant pupae (0h APF) or prepupae (2-6h APF), we could not detect any enhancement of EcR-B1 expression within the clustered cell bodies of MB γ neurons (Fig. 3c-h and Supplementary Fig. 9). When myo expression was restored by using a myo-promoter-GAL4 (myo-GAL4) to drive UAS-myo, the myo mutants grow into pharate or eclosed adults. These animals showed enriched EcR-B1 in the larval brain and they underwent normal MB remodeling (Fig. 3i-k). On the other hand, when myo expression was restored with myo-GAL4 subtracted by repo-GAL80 (Supplementary Fig. 11), we could not detect any enrichment of EcR-B1 in these late larval or prepupal brains and saw no evidence of neuronal remodeling in these pharate adults (Fig. 3l-n and Supplementary Fig. 5). These results with myo null mutants substantiate the notion that myo expression in glia governs neuronal remodeling through up-regulation of neuronal EcR-B1 expression.

So, does Myo activate TGF-β signaling through the Baboon (Babo) receptor that, contrasting with Myo, acts cell-autonomously to enable neuronal remodeling? There exist three Babo isoforms with different ligand-binding domains. Babo-a has been implicated in governing neuron remodeling. To explore if Myo activity requires Babo-a, we determined their relationship by epistasis. A ubiquitous expression of Myo caused larval lethality (Supplementary Table 1). If Myo signals through Babo-a, silencing babo-a should suppress

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the Myo-induced larval lethality. We tried to deplete specific Babo isoforms by miRNAs against isoform-specific exons. Targeted induction of babo-a miRNA, but not babo-b or babo-c miRNA, could effectively block MB remodeling (Supplementary Fig. 12). When such isoform-specific miRNAs were co-induced with the myo transgene, only miRNA against babo-a potently suppressed the larval lethality due to ectopic Myo (Supplementary Table 1). These epistasis results provide in vivo evidence that Myo and Babo-a act in a linear pathway to upregulate EcR-B1 and enable neuronal remodeling.

Remodeling of larval neurons occurs promptly as the larvae cease activity and become pupae. The tight temporal control of this developmental neuronal remodeling requires a timely induction of the EcR-B1 in these neurons. Three pathways, including TGF-β signaling, the cohesin protein complex and the FTZ-F1 nuclear receptor, are essential for the late-larval upregulation of EcR-B1\(^2\). The nature and source of the TGF-β signaling become increasingly clear after the identification of Myo, on top of Babo and dSmad2, playing an indispensable role in the upregulation of EcR-B1. Myo can bind with the Babo/Wit receptor complex in culture\(^3\). Interestingly, Myo is made by glia and required in glia for neuronal expression of EcR-B1. Namely, glial cells directly instruct the neural remodeling through secretion of Myo (Supplementary Fig. 13). Glial cells further participate in the execution of neuronal remodeling through facilitation of neurite pruning by engulfment of the unwanted neuronal processes\(^2, 14, 15\). Glial cells thus orchestrate developmental neural remodeling, and may play active roles in dynamically modulating mature neuronal connections.

Supplementary Material

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Figure 1. Expression of myo transcripts in the larval brain
(a-d) Expression of myo transcripts detected by *in situ* hybridization in brains of early third (a), middle third (b) and wandering third instar larvae (c-d). Cortex layer (c) and inner brain region (d) are shown.
(e-h) Signals of myo transcripts (arrows) co-localized with the cell bodies of cortex glia (f) and astrocyte-like glia (h), which were labeled with nuclear-lacZ (NZ, magenta). Scale bars = 50 μm.
**Figure 2. Effect of glial silencing of myo on MB remodeling**

(a-d) Remodeling of MB axonal lobes during metamorphosis. MB lobes labeled with anti-Fas2 (b,d) and their schematic illustration (a,c) in control (a,b) and repo>myo-RNAi (dsRNA) (c,d) at WL, 18h APF and adult. Arrows show larval lobes of γ neurons (b,d). Note that, in repo>myo-RNAi (d), larval γ lobes persist at 18h APF (arrows with asterisk) and adult (γ*).

(e-m) Expression of EcR-B1 in wandering larva of control (e,h,k), repo>myo- miRNA (f,g,i,j,l,m). The myo-miRNA resistant myo was co-expressed in glia (g,j,m). Arrows show bi-lateral clusters of MB γ neurons (e-g). High magnification view of larval MB neurons stained with anti-EcR-B1 antibody (h-m). Cell bodies of MB neurons were counter-labeled with MB247>rCD2::GFP in lower panels (k-m). Scale bars = 50 μm.
Figure 3. Effect of loss of function mutation of myo on MB remodeling
(a) Genome organization of myo deletion mutant.
(b) RT-PCR of myo transcript in the myoΔ1 mutant.
(c-h) Expression of EcR-B1 in MB γ neurons in myoΔ1 white pupa (c,f) and prepupa (d,g) and control prepupa (e,h). Counter-labeling of cell bodies of MB neurons with MB247>rCD2::GFP are shown (f-h).
(i-n) Effect of myo overexpression in the myoΔ1 mutant with (i-k) and without (l-n) glial expression. Expression of EcR-B1 in MB γ neurons in white pupae (i,l). MB lobes of 18h APF (j,m) and adult brain (k,n) labeled with anti-Fas2. The myo overexpression was induced by myo-GAL4 (i-n) and glial expression was suppressed with repo-GAL80 (l-n).
Scale bars = 50 μm.