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Glia instruct developmental neuronal remodeling through TGF-βsignaling

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Abstract

Neural circuits are remodeled in response to developmental and environmental cues. Here we show that glia secrets 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 metamorphosis[1]. 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 circuits[2-4]. This phenomenon requires activation of the TGF-β signaling in the remodeling neurons. TGF-βsignaling up-regulates expression of the B1 isoform of the ecdysone receptor (EcR-B1) at the late larval stage[5]. Then, the pruning of larval projections is triggered by the steroid molting hormone ecdysone[6].

activin-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 remodeling[5]. However, we found that the recently isolated *actβ* null mutant, $act\beta^{ed80}$ [7] 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 stage[7], 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 ligand[8], 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

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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[9]. 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-B1expression 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, $myo^{\Delta 1}$ (Fig. 3a,b). Organisms homozygous for $myo^{\Delta 1}$ 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[5]? There exist three Babo isoforms with different ligand-binding domains[10]. Babo-a has been implicated in governing neuron remodeling[11]. 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[12]. 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[13]. 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

Refer to Web version on PubMed Central for supplementary material.

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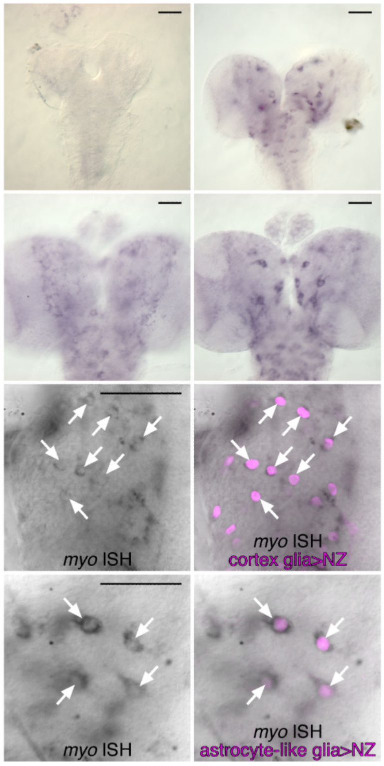

Figure 1. Expression of *myo* transcripts in the larval brain

(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.

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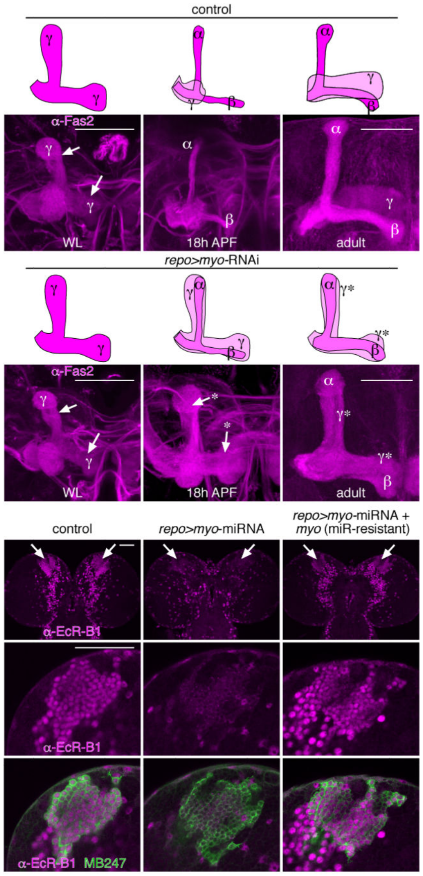

Figure 2. Effect of glial silencing of *myo* on MB remodeling

(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.

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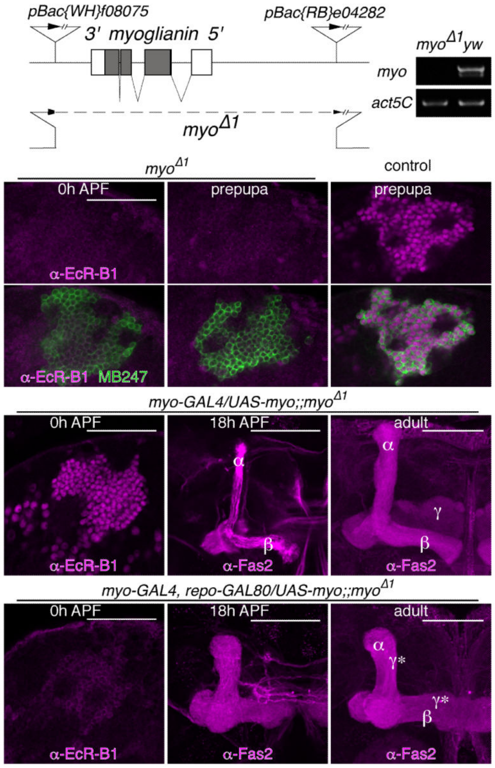

Figure 3. Effect of loss of function mutation of *myo* on MB remodeling

(a) Genome organization of *myo* deletion mutant.

(b) RT-PCR of *myo* transcript in the $myo^{\Delta1}$ mutant.

(c-h) Expression of EcR-B1 in MB γ neurons in $myo^{\Delta1}$ 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 $myo^{\Delta1}$ 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.