Glia delimit shape changes of sensory neuron receptive endings in C. elegans

Carl Procko, Yun Lu and Shai Shaham*

SUMMARY

Neuronal receptive endings, such as dendritic spines and sensory protrusions, are structurally remodeled by experience. How receptive endings acquire their remodeled shapes is not well understood. In response to environmental stressors, the nematode Caenorhabditis elegans enters a diapause state, termed dauer, which is accompanied by remodeling of sensory neuron receptive endings. Here, we demonstrate that sensory receptive endings of the AWC neurons in dauers remodel in the confines of a compartment defined by the amphid sheath (AMsh) glial cell that envelops these endings. AMsh glia remodel concomitantly with and independently of AWC receptive endings to delimit AWC receptive ending growth. Remodeling of AMsh glia requires the OTD/OTX transcription factor TTX-1, the fusogen AFF-1 and probably the vascular endothelial growth factor (VEGFR)-related protein VER-1, all acting within the glial cell. ver-1 expression requires direct binding of TTX-1 to ver-1 regulatory sequences, and is induced in dauers and at high temperatures. Our results demonstrate that stimulus-induced changes in glial compartment size provide spatial constraints on neuronal receptive ending growth.

KEY WORDS: C. elegans, Glia, Sensory receptive ending

INTRODUCTION

Dendritic spines and sensory neuron protrusions are examples of specialized neuronal receptive endings that respond to extracellular cues. Spines detect and propagate signals evoked by presynaptic release of neurotransmitters, and sensory receptive endings process and interpret external sensory stimuli. Both structures exhibit striking similarities in function, shape and molecular constituents (Shaham, 2010), and both structures exhibit morphological plasticity. For example, during synaptogenesis in the mouse cerebellum, dendritic spines of Purkinje cells exhibit dynamic shape changes, with spines rapidly emerging, growing and retracting. The frequency of these shape changes decreases as development proceeds to establish a stable synaptic repertoire (Dunaevsky et al., 1999). Similarly, in the nematode C. elegans, the ciliated receptive endings of the AWB sensory neurons take on different morphologies in response to sensory deprivation (Mukhopadhyay et al., 2008), and perturbation of sensory signaling alters the shapes of receptive endings of AWC sensory neurons (Roayaie et al., 1998). Receptive ending shapes are also regulated by systemic cues. For example, dendritic spine number and density of rat hippocampal neurons is correlated with circulating estrogen levels (Wooley et al., 1990). Likewise, pheromonal cues trigger changes in the shapes of sensory neuron receptive endings in C. elegans (Albert and Riddle, 1983).

In response to environmental stressors, including high population density, low food abundance and high temperature, C. elegans enters a protective developmental stage, termed dauer, which is highly resistant to environmental insults. Dauer development is triggered by dauer pheromone, which promotes systemic changes in physiology through insulin and TGF-β neuroendocrine signaling pathways (Kimura et al., 1997; Ren et al., 1996). Various cellular changes coincide with dauer entry, including remodeling of sensory receptive endings of the major sensory organs, the amphids (Albert and Riddle, 1983). The two amphids consist of bilateral pairs of neurons, each extending a dendrite that terminates in a ciliated sensory receptive ending at the nose-tip (Fig. 1A). In dauers, the spatially separated receptive endings of the AWC amphid neurons expand and overlap extensively (Albert and Riddle, 1983).

Dauer-induced AWC neuron remodeling offers unique advantages for studying receptive ending plasticity: it is inducible, reproducible, and can be studied in an organism with facile genetics and molecular biology.

Ensheathing the sensory ending of each AWC neuron is an amphid sheath (AMsh) glial cell. AMsh glia are also remodeled in dauers, such that the left and right AMsh glia expand and fuse at the nose-tip (Fig. 1B,C) (Albert and Riddle, 1983). Glia are the most abundant cell type in the human brain, and glia contribute extensively to nervous system disease (Canoll and Goldman, 2008; Ozawa et al., 1994; Shaham, 2005). However, the roles played by glia in the nervous system remain largely mysterious. Several observations suggest that glia could influence the shapes of neuronal receptive endings. Electron-microscopy reconstructions demonstrate that glia and glia-like cells associate with most excitatory synapses in the cerebellum and the hippocampus (Spacek, 1985; Ventura and Harris, 1999). Similarly, the endings of diverse sensory cells are ensheathed by glia (Shaham, 2010). The proximity of receptive endings and glial processes might reflect more dynamic interactions between these cellular protrusions. For example, in the cerebellum, Bergmann glia extend and retract processes in concert with the emergence and regression of Purkinje cell dendritic spines (Lippman et al., 2008). Similarly, during lactation in female rats the processes of hypothalamic astrocytic glia retract, and this is accompanied by synaptic remodeling of the glia-ensheathed supraoptic nucleus (SON) neurons (Theodosius and Poulain, 1993).
Mutations in glial ephrin A3 can alter dendritic spine morphology (Carmona et al., 2009; Murai et al., 2003), and ablation of glia in *C. elegans* can affect the maintenance of sensory receptive ending shapes (Bacaj et al., 2008), suggesting that, at least in some cases, glia are required to set up receptive ending morphology.

Some glia express neurotransmitter receptors (Porter and McCarthy, 1996) and, in the case of visual cortex astrocytes, exhibit tuning responses similar to those of nearby neurons (Porter and McCarthy, 1996; Schummers et al., 2008). Thus, glia possess machinery to gauge the environment surrounding receptive endings. Together, these various observations raise the intriguing but speculative possibility that extracellular cues received by glia might promote their shape changes, which in turn could affect the shapes of neurons.

Here, we demonstrate that remodeling of *C. elegans* AMsh glia in response to dauer signaling is required for proper morphological changes of the AWC sensory neuron receptive endings in dauers. Remodeling of AMsh glia requires the AFF-1 fusogen, the OTD/OTX transcription factor TTX-1, and probably also its direct target gene, the VEGFR-related protein VER-1. *ver-1* expression is induced by dauer entry and also by high temperature, a dauer stimulus. Our results suggest that glia can play key roles in delimiting changes in neuronal receptive-ending morphology in response to external cues.

### MATERIALS AND METHODS

#### Strains

*C. elegans* were cultivated using standard methods (Brenner, 1974). The wild-type strain was Bristol (N2). Alleles used were *ttx-2(p691), lin-11(n389, n566), che-1(e1805), daf-16(mu86), ire-1(zc4), daf-12(gk211), ttx-1(p678), unc-86(e1416, n846), daf-7(e1372), daf-18(e1416), osm-9(ky10), ttx-1(p767, ov26), pck-1(gk211, n3, m4), osm-6(p811), ceh-14(e33), pck-2(ok328), ttx-3(ks5, mg158), ceh-37(ok642, ok272), ceh-36(ks640), ceh-2(e1033), daf-7(ua89, m337), daf-12(m20, m25) and lin-15(n765)*. Two *ver-1* alleles were used: *tm1348* and *ok1738*.

#### *ver-1* alleles

The predicted wild-type VER-1 protein is 1083 amino acids and includes an amino-terminal extracellular region, a single transmembrane domain and an intracellular protein kinase domain (Popovici et al., 2002). *tm1348* has a frame-shift deletion that codes for a truncated protein of 267 conserved amino acids lacking the transmembrane and protein kinase domains, and which is followed by an additional non-homologous 21 amino acids (HSPSSETLRETSNEKFTYFZ). A single base mutation also causes an amino acid change at position 266 (Y to C).

#### Transgenic strains

Germline transformations were performed using standard protocols (Mello and Fire, 1995). Co-injection markers were plasmid pRF4 containing the dominant marker rol-6(su1006) (Mello et al., 1991), PJM23 containing wild-type lin-15 (Huang et al., 1994), or fluorescent markers *unc-122 promoter::dsRed* or *gcy-7 promoter::gfp*. *pSL1180* is an empty cloning vector used to increase the DNA concentration of injection mixtures. *ver-1 promoter::gfp* and *ttx-1 rescue* transgenes were integrated by treating animals carrying extrachromosomal arrays with ultraviolet light in combination with psoralen. The generated strains were backcrossed to N2 more than three times. Arrays and integrants are as designated in the text.

#### nsEx1391

**F**

**G**

**H**

**I**

Fig. 1. AWC neuron receptive endings and amphid sheath (AMsh) glia remodel in dauer animals. (A-C) Schematics of amphid remodeling in *C. elegans* dauer larvae. (A) Schematic of the head of the animal, showing the two bilateral AMsh glia and AWC sensory neurons. The horizontal line indicates the position of the transverse sections shown in B and C. (B,C) Sections through the nose tip showing the relative positions of the AWC neuron receptive endings and the ensheathing AMsh glia in non-dauer (B) and dauer (C) animals. AMsh glia fusion can occur on either the ventral or dorsal side (see also Albert and Riddle, 1983). Not to scale. (D-F) Representative electron micrographs (EM) and schematic outlines of amphid sensory organs in non-dauer wild-type adults (D), wild-type dauers induced by starvation (E) and *daf-7(e1372)* dauers (F). Scale bars: 5 μm. Dorsal is up. (G) EM and schematic outline of the amphid sensory organs of a *daf-7(e1372); AWC::yfp* (oy545) dauer, in which both AWC neurons were ablated in the first larval stage. AMsh glia fusion was scored prior to EM analysis by assaying for glial cytoplasmic mixing (see Fig. 2A). In all schematics, left and right AWC (AWCL/R; dark shading) and AMsh glia (AMshL/R; light shading) are indicated.

The initial *ver-1* promoter::*gfp* construct was a gift from R. Roubin and C. Popovici (Molecular Oncology, INSERM, Marseilles). It includes promoter sequence from −2110 to +263 relative to the predicted *ver-1* ATG start site. Additional *ver-1* promoter::*gfp* constructs are as described in the text; promoter regions were ligated into *gfp* vector pPD95.75. *ttx-1 promoter::dsRed* was made by inserting a 7.5 kb upstream *ttx-1* promoter fragment into *dsRed* vector pEP9. *ttx-1 promoter::gfp* includes the ~3.5 kb fragment further upstream, followed by the *myo-2* 160 bp minimal promoter to facilitate
expression (Okkema et al., 1993), inserted into pPD95.75. When injected, all lines exhibited green fluorescent protein (GFP) expression in the amphid and phasmid socket cells. Some also had sheath cell expression. Occasionally, weak fluorescence was also observed in other cell types. To create cell-specific rescue constructs, ttx-1a and ttx-1b splice forms (WormBase release WS206) were PCR amplified from N2-derived cDNA and inserted into heat shock vector pPD95.78. The heat shock promoter was replaced with 2 kb gcy-8 promoter (AFD expression), 5 kb vap-1 promoter (AMsh expression), or 2kb F16F9.3 or T02B11.3 promoters, both of which give specific AMsh and PHsh glia expression (Bacaj et al., 2008). T02B11.3 promoter::egl-1a was integrated to generate strain nsis219. The gcy-8 promoter::egl-1 construct was created similarly. A double insertion of the egl-1 cDNA occurred, generating two consecutive egl-1 cDNAs both running 5′ to 3′ following the promoter. To create glia::Otx1 and glia::Otx2 expression vectors, ttx-1 cDNA was replaced with mouse Otx2 and Otx2 amplified from Marathon-Ready mouse cDNA (Clontech), driven by the F16F9.3 promoter. F16F9.3::gfp and F16F9.3::dsRed fluorescent reporters were created by inserting 2 kb of F16F9.3 promoter into vectors pPD95.75 and pEP9, respectively. Full-length eff-1a and aff-1 cDNAs were inserted in-frame with gfp and following the F16F9.3 promoter to create glia-specific translational fusions. To create GST::TTX-1(1HD), the homeodomain of ttx-1 (lacking the first 154 and last 95 amino acids of the TTX-1A protein) was inserted into GST fusion vector pGEX-5X-1.

Microscopy

ver-1 promoter::gfp (nsh22) expression was assayed using a fluorescence dissecting microscope (Leica). Adult hermaphrodites were scored, except as noted. Compound microscope images were taken on an Axiosplan II microscope using an AxiosCam CCD camera (Zeiss) and analyzed using Axiovision software (Zeiss). Additional images were taken on a DeltaVision Image Restoration Microscope (Applied Precision/Olympus) and analyzed using SoftWorx software (Applied Precision). Dauer animals for electron microscopy were grown at 25°C. These were prepared and sectioned using standard methods (Lundquist et al., 2001). Imaging was performed with an FEI Tecnai G2 Spirit BioTwin transmission electron microscope equipped with a Gatan 4K×4K digital camera.

Dauer selection

Animals were starved and dauer selected by treatment with 1% SDS in M9 solution for 20 minutes. Alternatively, animals carrying the daf-7(e1372) mutation were induced to form dauers by incubation at 25°C.

Cytoplasmic mixing assay to score AMsh glia fusion

Adult animals carrying an nse1391 (AMsh glia::gfp) array were picked to plates seeded with OP50 bacteria or bacteria expressing dsRNA, and cultivated at 25°C. L1 and L2 progeny carrying the array in one of the two AMsh glia were picked 24 hours later to freshly seeded plates. Mosaic animals were incubated for 48 hours at 25°C before scoring GFP presence in either one or both AMsh glia. Animals carrying a daf-7(e1372) mutation were only scored if they were dauer larvae at the end of the assay period (Fig. 2A).

Thermotaxis assays

A linear thermal gradient from 18°C to 26°C was established across an aluminum surface using two Peltier feedback devices (Ryu and Samuel, 2002). Staged adult animals cultivated on OP50 bacteria at 15°C, 20°C or 25°C were washed in S-basal medium and transferred to the center of a 10-cm square Petri dish containing 12 ml NGM agar. This dish was placed onto the aluminum surface, with a thin layer of glycerol between the dish and aluminum slab. Animals were allowed to disperse for 45 minutes, fixed with chloroform and counted across six bins, from cold to hot. The center third of the assay plate was removed from the analysis as some animals did not disperse. Fifty to a few hundred worms participated in each assay. Results shown are averages of four independent trials.

Electrophoretic mobility-shift assay (EMSA)

Double-stranded probes covering 40 bp of the ver-1 gene, with the predicted TTX-1 wild-type ATTA core binding sequence at the center, were generated by annealing single-stranded, 5′ biotin end-labeled oligonucleotides. GST::TTX-1(homeodomain) and GST control protein (pGEX-5X-1 empty vector) were induced and purified from BL21(DE3) cells using the Bulk GST Purification Module (GE Healthcare). Binding
reactions were performed using the LightShift Chemiluminescent EMSA Kit (Pierce). Reaction conditions included 400 ng of GST::TTX-1(HD) fusion protein, 1X binding buffer, 2.5% glycerol, 5 mM MgCl2, 1 µg poly (di-dc), 0.05% NP-40 and 20 fmol of biotin-labeled DNA probe. GST control protein was added in excess of 400 ng. In competition reactions, a 200-fold molar excess of unlabeled probe was added. Reactions were performed at 20°C for 20 minutes, then run on a 6% polyacrylamide gel in 0.5X TBE buffer. The reactions were transferred to a positively charged nylon membrane and the biotin-labeled DNA was imaged.

**RESULTS**

**Loss of daf-7 promotes dauer remodeling of AWC neurons**
To characterize remodeling of AWC neuron sensory receptive endings, we examined dauer animals by electron microscopy (EM), we proceeded as follows. AWC remodeling occurs in some but not all dauers. Fourth, complete GFP transfer in mosaic animals observed over time indicated that glia remained unfused in dauer animals expressing GFP in only one AMsh glial cell, whereas dauers expressing GFP in both AMsh glia required molecular mediators of cell fusion. Two C. elegans genes, eff-1 and aff-1, encode fusogen required for somatic cell fusion in the animal (Mohler et al., 2002; Sapir et al., 2007). We found that both genes were expressed in AMsh glia (Fig. 2B; see Fig. S2A in the supplementary material) (Mohler et al., 2002; Sapir et al., 2007) and localized to the apical region that undergoes fusion in dauers (Fig. 1, Fig. 2C; see Fig. S2B in the supplementary material). Furthermore, glia fusion was markedly reduced in aff-1(RNAi) dauers (Fig. 2D), demonstrating an important role for AFF-1 in glia remodeling. Third, EM serial reconstructions confirmed that glia remained unfused in dauer animals expressing GFP in only one AMsh glial cell, whereas dauers expressing GFP in both AMsh glia had fused glia (see below; also data not shown). Fourth, complete GFP transfer in mosaic animals observed over time took less than 2 hours (n=17) and fluorescence was initially more intense at the nose tip (see Fig. S3 in the supplementary material), consistent with cell fusion.

Using our validated assay, and consistent with our EM studies, we found that AMsh glia fusion was never observed in wild-type, non-dauer adult animals, whereas 51% of animals induced to enter dauer by the daf-7(e1372) mutation had fused AMsh glia (Fig. 2E; P<0.001, χ² test). To address whether remodeling of AMsh glia was reversible upon dauer exit, we examined post-dauer daf-7(e1372) adult animals recovered by cultivation of dauers at 15°C for 7 days. GFP perdurs in AMsh glia for two days or less (Perens and Shaham, 2005); however, we found equal GFP fluorescence in both glial cells (see Fig. S2C in the supplementary material; n=44). Furthermore, EM serial reconstruction of a single wild-type 2-day post-dauer adult revealed fused AMsh glia (see Fig. S2D in the supplementary material). Together, these results suggest that remodeling induces a permanent change in AMsh glia architecture.

**AMsh glia remodeling is independent of AWC expansion**
In all dauers and normally developed adults that we examined by EM serial reconstructions, regardless of genotype, we found that overlap of AWC sensory receptive endings correlated with AMsh
glia expansion and fusion (8 and 19 animals examined with and without AWC overlap, respectively; Fig. 1E,F; see also below). In one daf-7(e1372) dauer, glia expanded to allow overlap of the bilateral AWC receptive endings, but did not fuse (see Fig. S1 in the supplementary material). These observations suggested to us that the remodeling of AMsh glia, with or without fusion, might be required to define a compartment into which the AWC receptive endings expand in dauers.

Changes in glial architecture to accommodate the remodeling of AWC receptive endings could be induced by the AWC neuron. Alternatively, glia might independently define a compartment that limits the extent of AWC expansion. To distinguish between these possibilities, we ablated the two AWC neurons using a laser microbeam in first-stage daf-7(e1372) larvae that were mosaic for AMsh glia:gfp expression and that expressed an AWC promoter::yfp reporter (oyIs45). Ablated animals were then induced to become dauers by incubation at 25°C for 48 hours, and glial fusion was monitored using the cytoplasmic mixing assay. We found that mock-ablated animals had a high rate of fusion (89%; n=36), perhaps a result of the strain background. Importantly, in animals where both AWC neurons were ablated, we saw no significant decrease in glial cell fusion (81%; n=26; P=0.47, Fisher’s exact test). EM serial reconstructions of ablated animals confirmed both the degradation of the AWC receptive endings and the remodeling of the glia in the absence of AWC expansion (Fig. 1G; n=2).

These results suggest that in response to external dauer signals the AMsh glia, independently of AWC neurons, define a compartment that delimits AWC receptive ending expansion.

ttx-1 is required in AMsh glia for glia remodeling

The observation that glia can remodel in the absence of AWC neurons suggests that active processes within AMsh glia might be required to promote glia remodeling and that interference with these processes should lead to defects in AWC sensory receptive ending shape. To test this prediction and to begin to characterize the molecular basis for glia remodeling, we sought fully penetrant mutants in which glia remodeling was blocked. Importantly, because our previous studies indicated that AMsh glia are required for maintaining AWC receptive ending shape (Bacaj et al., 2008), we aimed to identify mutants that specifically disrupt glia remodeling in dauers, but in which glial shape was unperturbed in non-dauer animals. aff-1 was unsuitable for such an analysis: RNAi knockdown of aff-1 did not have a fully penetrant defect in glial remodeling, and aff-1 genetic lesions had morphological abnormalities in the AMsh glia and other tissues (data not shown) (Sapir et al., 2007).

In the course of our studies, we found that transcription of the AMsh glia gene ver-1 was temperature dependent. We therefore examined the effect of the thermotaxis gene ttx-1, encoding an OTD/OTX transcription factor (Satterlee et al., 2001), on ver-1 expression (see below) and glial fusion. As shown in Fig. 2E, AMsh glia fusion failed to occur in almost all daf-7(e1372); ttx-1(p767) and daf-7(e1372); ttx-1(oy26) double-mutant dauer animals scored using the cytoplasmic mixing assay. Thus, ttx-1 might be a component of the glial remodeling machinery, and ttx-1 mutants might provide a suitable genetic background in which to test the effects of AMsh glia remodeling on AWC neuron shape changes.

However, previous studies suggested that ttx-1 is expressed specifically in the AFD thermosensory neurons of C. elegans. Animals carrying ttx-1 promoter::gfp transgenes express GFP in AFD (Satterlee et al., 2001), and the AFD sensory receptive endings in ttx-1 mutants lack their wild-type microvilli-like protrusions (Perkins et al., 1986). Furthermore, ttx-1 mutants have defects in thermotaxis, an AFD-dependent behavior in which animals placed in a thermal gradient seek the temperature at which they were reared (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). ttx-1 might, therefore, act in AFD to drive AMsh glia fusion in dauers. To test this possibility, we blocked activity in a number of amphid sensory neurons, including AFD, using a mutation in the tax-2 gene, which encodes a subunit of a cyclic nucleotide gated channel required for thermotaxis and other sensory functions (Coburn and Bargmann, 1996; Komatsu et al., 1996). We observed no defect in the fusion of AMsh glia in tax-2(p691) mutants (Fig. 2E; P=0.70, χ² test), suggesting that sensory signaling in AFD was unlikely to be important for glia remodeling. These results suggested the possibility that ttx-1 might act in AMsh glia to promote their remodeling in addition to its previously described roles in AFD.
To test this idea, we examined expression of transgenes containing ttx-1 regulatory sequences fused to sequences encoding fluorescent reporter proteins. As shown in Fig. 3, a 7.5 kb sequence immediately upstream of the ttx-1 transcription start site drives reporter expression in AFD, consistent with previous observations (Satterlee et al., 2001). By contrast, a 3.5 kb sequence further upstream drives expression in AMsh and phasmid sheath (PHsh) glia, as well as in associated socket glia (Fig. 3A,C; data not shown). The PHsh glia are associated with the phasmid sensory organ in the tail. Furthermore, by driving ttx-1 CDNA under cell-specific promoters (see Materials and methods), we observed that, although expression of ttx-1 in AFD was sufficient to confer wild-type AFD sensory ending morphology and thermotaxis to ttx-1 mutants (see Fig. S4A-C,H in the supplementary material) (Satterlee et al., 2001), it did not rescue the AMsh glia fusion defect in dauer animals (Fig. 2E). Restoring ttx-1 in AMsh glia, however, rescued AMsh glia fusion (Fig. 2E) but not thermotaxis or AFD morphology (see Fig. S4A,D,E,I in the supplementary material). These results demonstrate that ttx-1 has separable, cell-autonomous roles in the AFD thermosensory neurons and the AMsh glia.

To determine whether ttx-1 was generally required for AMsh glia shape maintenance, we examined the morphology of these cells in non-dauer animals. We found no defects in glial shape. Furthermore, expression of constitutively expressed glial genes was affected weakly or not at all by mutations in ttx-1 (Fig. 3D,E; data not shown). In addition, an aff-1 promoter:gfp reporter (hyEx167) showed GFP expression in AMsh glia of both wild type and ttx-1 mutants [86% of wild-type animals expressed GFP in AMsh glia, compared with 93% of ttx-1(p767) mutants; n=40 for each genotype], suggesting that ttx-1 probably affects glia remodeling and fusion through the transcriptional regulation of genes other than aff-1.

Our data show that ttx-1 has specific cell-autonomous roles in AMsh glia, supporting the idea that active glia-intrinsic processes promote glia remodeling. These results also suggest that animals lacking ttx-1 function provide a suitable setting in which to test the effects of AMsh glia on AWC neuron remodeling.

Proper remodeling of AWC neurons requires glia

To address whether remodeling of AWC receptive endings in dauer animals depends on remodeling of AMsh glia, we examined EM serial sections of ttx-1 mutants. We first noted that ttx-1(p767) starvation-induced dauers as well as ttx-1(p767); daf-7(e1372) dauers did indeed fail to show AMsh glia fusion (Fig. 4A,B; n=6), verifying the results of our cytoplasmic mixing assay. Importantly, in these same animals, AWC receptive endings did not overlap extensively (Fig. 4A,B; n=6, P<0.016). Furthermore, animals in which AMsh glia fusion was rescued by restoring ttx-1 expression in these cells displayed a wild-type pattern of AWC overlap by EM (Fig. 4C; n=2). Interestingly, in two out of the six ttx-1 mutants we examined, AWC appeared to have expanded but, instead of projecting circumferentially around the nose as was always observed when AWC expanded in wild-type dauers, folded back on itself (see Fig. S5 in the supplementary material; see Discussion).

The AWC ablation and ttx-1 studies demonstrate that AMsh glia respond to dauer signals independently of AWC to define a compartment confining AWC receptive endings, and that defects in glial compartment plasticity lead to defects in the morphology of AWC receptive endings.

**ttx-1 might promote AMsh glia remodeling by inducing ver-1 expression**

Although morphological plasticity of glia has been demonstrated in some settings, neither the effects of glial shape changes on neuronal shape, nor the molecular mechanism promoting glial shape changes has been described in any animal. To elaborate further the mechanism by which ttx-1 promotes the remodeling of AMsh glia, we turned our attention to the gene ver-1. ver-1 encodes a receptor tyrosine kinase (RTK) with similarities to the mammalian vascular endothelial growth factor receptor (VEGFR), and was previously reported to be expressed in AMsh and PHsh glia (Popovici et al., 2002). Interestingly, we found that at 15°C, wild-type dauers induced by starvation strongly expressed a ver-1 promoter:gfp reporter in AMsh and PHsh glia (Fig. 5A,C) (Popovici et al., 2002). However, only very weak expression of ver-1 was detected in non-dauer adults raised under the same temperature conditions (Fig. 5B,D). Thus, *C. elegans* AMsh glia respond to dauer signals by modifying gene expression concomitantly with induction of remodeling.

The induction of ver-1 expression in dauers suggested that this gene might be involved in remodeling AMsh glia. To test this idea, we examined two strains carrying different deletions of the ver-1 locus (see Materials and methods) using our fluorescence fusion assay. As shown in Fig. 2E, both strains displayed significantly reduced AMsh glia fusion (P<0.001, χ2 tests; perhaps for technical reasons, we have not been able to obtain transgenic rescue of the mutants). Furthermore, we found that expression of ver-1 in glia was dependent on functional ttx-1, as two independent ttx-1 alleles greatly reduced ver-1 promoter:gfp expression in dauers (Fig. 5E).

To determine how TTX-1 regulates ver-1 expression, we performed deletion studies of the ver-1 promoter with the aim of identifying
a minimal interval required for expression of the gene. We
identified a ~100 bp interval required for ver-1 expression in dauers
(see Table S1 in the supplementary material). Within this interval
we identified a potential TTX-1 binding site based on similarity to
the mammalian Otx2 binding site (Kelley et al., 2000), suggesting

that ttx-1 might directly regulate ver-1. Indeed, a 40 bp
oligonucleotide containing this sequence bound a GST::TTX-1
homeodomain fusion protein, and alteration of the core nucleotides
of the putative binding site from GGATTA
TC to GGGGGG
TC abolished both in vitro binding (Fig. 5F,G) and in vivo expression
of a ver-1 promoter::gfp transgene (see Table S1 in the
supplementary material). Thus, ver-1 is probably a direct TTX-1
target.

Taken together, these results suggest that ttx-1 might act to
regulate AMsh glia fusion in dauer animals in part by promoting
ver-1 expression.

ver-1 exhibits temperature-dependent expression
in dauers and non-dauers
Although ver-1 expression was strongly induced by dauer entry, we
also noticed induction of expression by high ambient temperature
in non-dauer animals. Over a temperature range of 15-25°C, adult
animals exhibited a graded increase in ver-1 promoter::gfp expression intensity (Fig. 6A; data not shown). The increase in
expression was not a general transcriptional response, as reporters
for three other glial genes demonstrated only mild temperature
effects and were not upregulated by dauer entry (see Fig. S6 in the
supplementary material). Furthermore, neither the heat-shock nor
the unfolded protein response pathways affected ver-1 expression
(see Table S2 in the supplementary material; data not shown),
indicating that the expression pattern observed was not a result of
a general stress response.

The induction of ver-1 expression by temperature was, however,
dependent on ttx-1, as animals containing ttx-1 mutations failed to
express the ver-1 promoter::gfp reporter at high temperatures (Fig.
6B). Restoring ttx-1 specifically in glia rescued temperature-
dependent ver-1 expression, whereas restoring ttx-1 in AFD
neurons did not (Table 1). In addition, the same promoter region
and TTX-1 binding site required for expression of ver-1 in dauers
were required for temperature-dependent expression of ver-1 (see
Table S1 in the supplementary material).
Interestingly, restoring ttx-1 to adult mutant animals using a heat-inducible promoter rescued ver-1 expression at 25°C (see Table S3 in the supplementary material). In addition, we found that the ttx-1(oy26) allele was temperature-sensitive (Fig. 5E; see Fig. S7 in the supplementary material), and that shifting ttx-1(oy26) animals reared at 15°C (the permissive temperature) to 25°C (the restrictive temperature) at any larval stage abrogated ver-1 promoter::gfp expression in glia (Fig. 6C), suggesting that ttx-1 is continuously required for ver-1 expression at 25°C. Strikingly, we also observed a continuous requirement for ttx-1 in maintaining AFD sensory ending morphology: ttx-1(oy26) mutants shifted to the non-permissive temperature acquired an aberrant, single elongated cilium (Fig. 6D; see Fig. S7 in the supplementary material). Thus, in both glia and neurons, ttx-1 functions continuously to control specialized aspects of terminal differentiation.

To determine whether temperature control of ver-1 expression was mediated by the sensory neurons associated with the AMsh glia, we genetically ablated both thermosensory AFD neurons and observed no defect in ver-1 expression (Table 1). Likewise, perturbation of all amphi and phasmid sensory neuron signaling activity by mutations in the tax-2, tax-4 and osm-9 sensory transduction ion channels (Coburn and Bargmann, 1996; Colbert et al., 1997; Komatsu et al., 1996) had little effect on the induction of ver-1 expression at high temperature (Table 1). Importantly, these mutations also had no effect on ver-1 expression in dauer animals (Table 1, footnote). Finally, mutations disrupting sensory neuron cilia morphology and/or circuitry, or mutations in dauer-related TGF-β (Ren et al., 1996) or insulin (Kimura et al., 1997) neuroendocrine signaling components, also had minor or no effects on ver-1 expression at 25°C (see Table S2 in the supplementary material). These observations suggest that AMsh and PHsh glia respond to temperature independently of sensory neuron input, and that the induction of ver-1 expression in dauers and at high temperature is mediated, at least in part, by similar programs.

To assess whether increased ver-1 expression was sufficient to drive AMsh glia remodeling, we examined non-dauer adults raised at 25°C for fusion of AMsh glia. We found that fusion does not occur in these animals (Fig. 2E; see above), even though ver-1 is highly expressed, suggesting that dauer entry provides additional necessary conditions for AMsh glia remodeling.

High ambient temperature is one of the environmental stressors that regulate dauer entry (Golden and Riddle, 1984), raising the possibility that increased expression of ver-1 at high temperatures might facilitate AMsh glia remodeling. However, robust expression of ver-1 is still detected in dauers raised at 15°C, confounding a functional analysis of the effects of temperature on the remodeling of these glia. Nonetheless, we still observed a temperature dependence of ver-1 expression in dauers raised at different temperatures (see Table S1 in the supplementary material). Thus, although temperature and dauer signals both require TTX-1 to induce ver-1 expression, these signals must also converge independently on the ver-1 promoter to induce expression in dauer animals. These results raise the speculative possibility that temperature, in addition to dauer cues, might play a modulatory role in remodeling of AMsh glia.

**DISCUSSION**

**Glia as regulators of neuronal receptive ending shape**

The results described here show that the extent of remodeling of a glial compartment in *C. elegans* dauers restricts the shape of remodeled AWC receptive endings: when ttx-1 function in glia is

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**Fig. 6. TTX-1 is required continuously for temperature-dependent expression of ver-1 in amphid sheath (AMsh) glia and for AFD sensory ending morphology.** (A) Representative differential interference contrast (DIC) and fluorescence merged images (top), and fluorescence only images (below) of ver-1 promoter::gfp (nds22) expression in one of the two AMsh glial cells of wild-type animals at 15-25°C. (B) DIC and fluorescence merged (top), and fluorescence only (below) images of ver-1 promoter::gfp expression in a ttx-1(p767) mutant adult at 25°C. (C) Percentage of wild-type and temperature-sensitive ttx-1(oy26) mutant larvae (L1-L4 stages) expressing ver-1 promoter::gfp in phasmid sheath (PHsh) glia over time (hours, h) following a temperature shift from 15°C to 25°C (n=15 for each data point). ver-1 promoter::gfp expression in AMsh glia showed a similar trend; however, PHsh glial GFP fluorescence was greater (data not shown). (D) The percentage of wild-type and ttx-1(oy26) L4 animals in which AFD dendrite endings do not exhibit an aberrant single cilium morphology at 0 and 48 hours (h) following a temperature shift from 15°C to 25°C. In some animals possessing a single aberrant cilium, some microvilli were still observable at the base of the cilium. Results are comparable with second- and third-stage larvae (data not shown). n=15 for both genotypes. A gcy-8 promoter::gfp transgene (oxyIs17) was used to visualize the AFD neurons. For a description of the effect of the ttx-1(oy26) allele on AFD morphology at different temperatures, see Fig. S7 in the supplementary material. In A and B, exposure time for ver-1 promoter::gfp was 800 ms. Scale bar: 50 μm. In all images, anterior is up.
impaired, the AWC sensory protrusions fail to take on their wild-type overlapping appearance. Glia or glia-like cells are intimately associated with sensory receptive endings from *C. elegans* to humans (Shaham, 2010), suggesting that the roles they play in delimiting receptive ending morphological plasticity might be conserved. Furthermore, the many similarities between sensory receptive endings and dendritic spines, which serve as receptive endings for presynaptic neurons (Shaham, 2010), suggest the interesting possibility that glia could be in a position to define compartments that will constrain dendritic spine shape also. Indeed, all dendritic spines examined by EM in the cerebellum are tightly ensheathed by Bergmann glia (Spacek, 1985), and most spines examined by EM in the hippocampus are ensheathed by astrocytes (Ventura and Harris, 1999). Furthermore, Bergmann glia have been shown to affect neuronal receptive ending shapes in the cerebellum (Lippman et al., 2008), and astrocytic glia affect dendritic spine morphology of hippocampal neurons via ephrin-A3/EphA4 signaling (Murai et al., 2003).

At least two possible mechanisms by which the glial compartment can delimit receptive-ending shape are possible. Glia might form an inelastic physical barrier against expansion of neuronal receptive endings. Alternatively, glia might provide specific signals that induce receptive-ending growth or retraction. Although both mechanisms could apply, our finding that in some *ttx-1* mutants AWC attempts to remodel but forms membrane whorls confined to the space defined by the AMsh glia (see Fig. S5 in the supplementary material) is consistent with a physical barrier role for these cells. A similar role for astrocytic glia in remodeling SON neuron synapses in the mammalian hypothalamus has been proposed: the retraction of astrocytic processes from postsynaptic surfaces might facilitate the increased number of synapses that occur onto a single postsynaptic element during lactation in female rats (Theodosis and Poulain, 1993). In both of these cases, remodeling of the glia could be permissive for neuronal shape changes.

Our studies demonstrate that neuronal remodeling must be tightly coordinated with glia remodeling in dauer animals, predicting the existence of proteins tasked with executing this coordination. That *ver-1* encodes a receptor tyrosine kinase is intriguing, suggesting the speculative possibility that it normally responds to as yet unidentified neuronal cues to promote coordinated glia-neuron remodeling. Expression of *ver-1* in non-dauer animals might serve to prime glia for remodeling, in preparation of pending dauer entry. In addition, *ver-1* expression might represent a neuroprotective or stress response of the glia. Supporting this possibility, the VER-1-related VEGF receptor Flk-1 (also known as VEGFR-2; Kdr – Mouse Genome Informatics) is expressed in neurons and glia of the mammalian hippocampus following nerve injury stress (Wang et al., 2005), and VEGF signaling has neurotrophic and neuroprotective roles in some contexts (Sondell et al., 1999). Furthermore, the predicted *ver-1* kinase domain is similar to kinase domains encoded by the *C. elegans* genes *old-1* and *old-2* (~40% identity), and *old-1* has been implicated in longevity and resistance to environmental insults (Murakami and Johnson, 2001).

The functional outcome of AWC receptive-ending remodeling in dauers is unclear; however, previous studies have correlated AWC sensory ending shape with function (Perkins et al., 1986). It is possible, therefore, that the morphological changes in AWC promote a change in the behavior of the animal. To date, no AWC-

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**Table 1. TTX-1 acts in glia, and not AFD, to control temperature-dependent ver-1 expression**

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>% PHsh on</th>
<th>% AMsh on</th>
<th>n</th>
<th>% PHsh on</th>
<th>% AMsh on</th>
<th>n</th>
</tr>
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<tr>
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<td></td>
<td>80</td>
<td>100</td>
<td>93</td>
<td>80</td>
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<td></td>
<td>70</td>
<td>0</td>
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<td>70</td>
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<tr>
<td><em>ttx-1(p767); AFD::ttx-1</em></td>
<td></td>
<td></td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td><em>ttx-1(p767); AMsh::ttx-1</em>‡</td>
<td></td>
<td></td>
<td>25</td>
<td>0</td>
<td>50</td>
<td>22</td>
</tr>
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<td></td>
<td></td>
<td>30</td>
<td>87</td>
<td>97</td>
<td>30</td>
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<td>0</td>
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<td>30</td>
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<td>0</td>
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<td>0</td>
<td>21</td>
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<td>0</td>
<td>48</td>
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<td>83</td>
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<tr>
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<tr>
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<tr>
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<td>40</td>
<td>100</td>
<td>90</td>
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*All strains contained the *ver-1* promoter:gfp transgene (ns252).§That *ttx-1* expression only in AMsh glia rescued *ver-1* expression only in AMsh glia supports a cell autonomous role for *ttx-1*.¶The glia promoter drives expression in both AMsh and PHsh glia.†That *ver-1* expression 15°C expressed only in AMsh glia supports a cell autonomous role for *ver-1*.‡The glia promoter drives expression in both AMsh and PHsh glia.¶¶The *tax-2(p691); osm-9(ky10)* genotype also failed to affect *ver-1* expression in dauers. 100% of *tax-2(p691); osm-9(ky10)* starvation-induced dauers at 15°C expressed *ver-1* promoter::gfp in PHsh and 82% in AMsh, compared with 100% of wild-type dauers in PHsh and 88% in AMsh (*n*=50 for each genotype). Two *ttx-1* DNAs, *ttx-1a* and *ttx-1b*, were isolated and gave equivalent rescue. TTX-1A and TTX-1B proteins are identical, except for an additional 53 amino acid residues in TTX-1A. Only *ttx-1a* rescue transgenes are shown. Transgenes were injected at 60 ng/µl of *ttx-1* or Otx plasmid, with 60 ng/µl of pRF4. Lines shown are nsEx899, nsEx895, nsEx893, nsEx1661, nsEx897, nsEx939, nsEx937, nsEx1661 and nsEx1662, and are representative of others. AMsh, amphid sheath; PHsh, phasmid sheath.
mediated behaviors have been described for dauers. Indeed, in dauer animals the expression of at least one AWC odorant receptor is repressed (Peckol et al., 2001), and the classical AWC-sensed attractants benzaldehyde and isoamylalcohol act instead as repulsive cues (C.P. and S.S., unpublished; data not shown). These observations suggest that assessing the functional consequences of dauer remodeling will require a more thorough description of sensory neuron function and molecular biology in dauers than is presently available.

The receptive endings of the amphid AFD thermosensory neurons might also remodel in dauer animals, as the number of AFD microvilli has been reported to increase upon dauer entry (Albert and Riddle, 1983). Thus, it is possible that AMsh glia play a role in this remodeling process also. However, EM serial reconstructions of young larva and adults from our laboratory demonstrate that adults always possess a larger number of microvilli (Y.L. and S.S., unpublished), suggesting that the increase in microvilli number observed in dauers could also be explained by a constant rate of microvilli addition that is independent of the dauer state. In addition, we are unable to use ttx-1 mutants to assess the effects of AMsh glia on AFD remodeling, as ttx-1 also functions within AFD to control morphology.

At excitatory synapses, changes in dendritic spine volume are positively correlated with the extent of presynaptic activity (Feldman, 2009; Holtmaat and Svoboda, 2009), suggesting that extracellular cues are important in driving changes in receptive ending shape. Glia can directly sense extracellular stimuli such as Na+ levels (Shimizu et al., 2007), neurotransmitters (Porter and McCarthy, 1996) and perhaps protons (Wang et al., 2008). Our studies demonstrate that the AMsh glia can respond to dauer entry and temperature, both exogenous stimuli, and do so independently of sensory neuron input. Furthermore, at least one gene, ver-1, is dynamically regulated by both cues. It is therefore reasonable to speculate that glia sense these stimuli, and in turn modulate their shapes to accommodate morphological changes of nearby neuronal receptive endings.

**ttx-1 regulates cell shape in thermoresponsive cells**

It is not known whether the temperature responses of AFD and AMsh glia are mediated by the same or different sensors. A sensor has not yet been identified in either cell; however, three guanylyl cyclases are important for temperature sensation in AFD, suggesting a possible involvement of a G-protein coupled receptor (Inada et al., 2006). Reporter constructs for these guanylyl cyclases are not expressed in AMsh glia (Inada et al., 2006), suggesting that glia might employ a different sensor. However, it is also possible that these cyclases function permissively in AFD, and do not directly interact with the sensor. In *Arabidopsis*, changes in ambient temperature cause fluctuations in the constituents of nucleosomes (Kumar and Wigge, 2010). Thus, chromatin might act as a direct thermal sensor. Such a sensor is unlikely to be employed in AFD neurons, as it is not compatible with the fast calcium transients observed in AFD neurons following temperature shifts (Kimura et al., 2004). A DNA-bound sensor would, however, be consistent with the kinetics of temperature-induced changes in ver-1 expression that we observe in glia (see Fig. 6C). If such a sensor exists, TTX-1 would be unlikely to be its temperature sensitive component, as constitutive expression of TTX-1 or its mammalian homolog Otx2, in glia does not perturb temperature sensitivity of ver-1 expression (Table 1; see Fig. S3 in the supplementary material).

It is of note that ttx-1 is required for determining the morphology of process endings of both glia (fusion/expansion) and neurons (AFD microvilli). Interestingly, ectopic expression of TTX-1 in non-AFD neurons induces protrusions in these cells (Satterlee et al., 2001). Thus, TTX-1 might have specific roles in regulating cell shape. Furthermore, the role of the protein as a terminal cell fate gene might be limited to thermoresponsive cells, as TTX-1 is required in two seemingly unrelated temperature-dependent processes in *C. elegans*: AFD neuron function and glial ver-1 expression. Intriguingly, in the adult mouse brain, some hypothalamic neurons of the preoptic area, which senses body temperature (Boulant, 2000), express the TTX-1 related protein Otx2 (Kelley et al., 2000). Moreover, in the developing olfactory sensory epithelium Otx2 is expressed and largely restricted to the ensheathing glia (Mallamaci et al., 1996). Our finding that murine Otx2, but not Otx1, robustly restored ver-1 expression to ttx-1 mutants in *C. elegans* might, therefore, indicate that Otx2 has conserved roles in thermosensation and/or the function of glia in sensory organs, in addition to its other well-studied roles in development.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058305/-/DC1

**References**


