1 **Glial response to hypoxia in *trachealess* mutants induces synapse**

2 **remodeling**

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13 **Abstract**

14 Synaptic structure and activity are sensitive to environmental alterations.
15 Modulation of synaptic morphology and function is often induced by signals from
16 glia. However, the process by which glia mediate synaptic responses to environmental
17 perturbations such as hypoxia remains unknown. Here, we report that, in the
18 *Drosophila trachealess (trh)* mutant, smaller synaptic boutons form clusters named
19 bunch boutons appear at larval neuromuscular junctions (NMJs), which is induced by
20 the reduction of internal oxygen levels due to defective tracheal branches. Thus, the
21 bunch bouton phenotype in the *trh* mutant is suppressed by hyperoxia, and
22 recapitulated in wild-type larvae raised under hypoxia. We further show that hypoxia-
23 inducible factor (HIF)-1 α /Similar (Sima) is critical in mediating hypoxia-induced
24 bunch bouton formation. Sima upregulates the level of the Wnt/Wingless (Wg) signal
25 in glia, leading to reorganized microtubule structures within presynaptic sites. Finally,
26 hypoxia-induced bunch boutons maintain normal synaptic transmission at the NMJs,
27 which is crucial for coordinated larval locomotion.

28 **Author summary**

29 Oxygen is essential for animals to maintain their life such as growth, metabolism,
 30 responsiveness, and movement. It is therefore important to understand how animal
 31 cells trigger hypoxia response and adapt to hypoxia thereafter. Both mammalian
 32 vascular and insect tracheal branches are induced to enhance the oxygen delivery.
 33 However, the study of hypoxia response in the nervous system remains limited. In this
 34 study, we assess the morphology of *Drosophila* neuromuscular junctions (NMJs), a
 35 model system to study development and function of synapses, in two hypoxia
 36 conditions, one with raising wild-type larvae in hypoxia, and the other in the
 37 *trachealess* (*trh*) mutant in which the trachea is defective, causing insufficient oxygen
 38 supply. Interestingly, glia, normally wrapping the axons of NMJs, invade into synapse
 39 and trigger Wg signals to reconstitute the synaptic structure under hypoxia. This
 40 synaptic remodeling maintains the synaptic transmission of synapse, which associate
 41 the locomotor behavior of larvae.

42

43 **Introduction**

44 Animals need oxygen and food, not only to sustain life, but also for motility.
 45 In vertebrates, oxygen and nutrients are delivered through the vascular systems to
 46 organs and tissues throughout the body. To maintain proper nutrient and oxygen
 47 supply, and thus physiological functions, the vascular system is also highly
 48 coordinated with the nervous system during development. Indeed, the vascular and
 49 nervous systems resemble each other in terms of their anatomical structures and
 50 developmental processes [1, 2]. In the brain, nerves and vessels, form close
 51 associations and are in physical contact through the third player astrocytes to form
 52 neurovascular units (NVU) [3]. Such organization is essential for controlling oxygen
 53 and glucose delivery through the blood vessels by neuronal activity, and this
 54 regulatory process is mediated through the coupled astrocytes [4]. However, some
 55 invertebrates lack the complex vascular systems [5]. In nematodes, oxygen is supplied
 56 simply by ambient diffusion to inner cells [6]. Insects such as *Drosophila* have
 57 evolved a prototype of the tracheal system to deliver oxygen and a primitive vascular
 58 system, the dorsal vessel, to facilitate nutrient delivery [7]. However, the physical
 59 association of nerves, trachea, and glial processes has also been demonstrated at the
 60 NMJs of adult *Drosophila* flight muscles [8].

61 Animals respond to changing oxygen levels by altering their oxygen delivery
 62 system. Insufficient oxygen levels (hypoxia) activate a broad range of genes to re-
 63 establish body homeostasis. One crucial regulator of these hypoxia-responsive genes
 64 is the sequence-specific DNA-binding transcription factor hypoxia inducible factor 1
 65 (HIF-1) [9]. HIF-1 consists of α and β subunits that form heterodimers [10]. Whereas
 66 HIF-1 β is expressed constitutively, HIF-1 α protein levels are modulated by oxygen
 67 levels [11]. Under normal oxygen conditions (normoxia), oxygen-dependent prolyl

hydroxylases (PHDs) catalyze hydroxylation of a conserved prolyl residue in the central oxygen-dependent degradation (ODD) domain of HIF-1 α [12-14]. Hydroxylation of HIF-1 α promotes interaction with Von Hippel Lindau (VHL), which is the substrate recognition subunit of the cullin2-based E3 ubiquitin ligase, leading to HIF-1 α ubiquitination and proteasomal degradation [15]. Under hypoxia, prolyl hydroxylation does not occur, HIF-1 α proteins are stabilized and are translocated from the cytoplasm to the nucleus where they form heterodimers with HIF-1 β to activate transcription of target genes [16, 17]. One major class of target genes encoding the Fibroblast Growth Factor (FGF) is involved in inducing angiogenesis in mammals. In *Drosophila*, the FGF member encoded by *Branchless* (*Bnl*) induces tracheal branching [18]. When oxygen levels are reduced, oxygen-starved cells express Bnl as a chemo-attractant to guide the growth tracheal terminal branches toward them [19].

In addition to adaptations of the respiratory system, the nervous system also responds to hypoxia. Oxygen levels modulate the survival, proliferation, and differentiation of radial glial cells (RGCs) in the human cerebral cortex. Interestingly, physiological hypoxia (3% O₂) induces neurogenesis and differentiation of RGCs into glutamatergic neurons [20]. Hypoxia induces neurite outgrowth in PC12 cells through activation of A2A receptor [21]. Brief exposure to anoxia and hypoglycemia caused axonal remodeling in hippocampal neurons, including presynaptic protrusion of filopodia and formation of multi-innervated spines [22]. Under hypoxia or upon depletion of PHD2, upregulation of the actin cross-linker Filamin A (FLNA) induces generation of more immature spines [23]. Astrocytes have been shown to play a crucial role in ischemic tolerance via the activation of P2X7 receptors, which trigger upregulation of HIF-1 α [24].

93 Neuronal PAS (NPAS) proteins containing a DNA-binding Per-Arnt-Sim
 94 domain function in vascular and nervous system development. In mice, NPAS1 is
 95 responsible for cortical interneuron generation [25], whereas NPAS3 is required for
 96 adult neurogenesis [26]. NPAS1 and NPAS3 also play key roles in lung development
 97 [27, 28]. The homolog of NPAS1/3 in *Drosophila*, Trachealess (Trh), has been well
 98 studied for its involvement in formation of the respiratory tracheal system. Trh is a
 99 master regulator of tracheal cell fates, activating gene expression to induce tracheal
 100 development [29, 30]. However, the role of Trh in the development of other tissues,
 101 particularly the nervous system, is unknown. In this study, we found altered synaptic
 102 bouton morphology at the NMJs of *trh¹/trh²* mutants. By performing *trh-RNAi*
 103 knockdown and *UAS-trh* transgene rescue experiments, we show that *trh* is required
 104 in tracheal cells for normal bouton formation. Defective tracheal branching in the
 105 *trh¹/trh²* mutant mimics the effect of hypoxic conditions during larval development,
 106 and supplying higher than normal oxygen levels restored normal bouton morphology.
 107 We further show that glial cells respond to hypoxia by elevating Wnt/Wg expression
 108 to mediate synaptic bouton remodeling through HIF1- α /Sima in *Drosophila*. Finally,
 109 we reveal that this synaptic remodeling maintains normal synaptic transmission and it
 110 is required for normal locomotion in larvae.

111 Results

112 *trh* modulates synaptic bouton formation non-cell autonomously

113 To understand the possible role of Trh in synapse formation, we examined
 114 NMJ morphology in the *trh* mutants. Since both *trh*¹ and *trh*² loss-of-function alleles
 115 are homozygous lethal [31-33], we examined the trans-heterozygous *trh*¹/*trh*² mutant
 116 that survive to adult stages and compared it to wild-type (*w*¹¹¹⁸) and heterozygous
 117 *trh*¹/+ controls. Synaptic boutons of *w*¹¹¹⁸ and *trh*¹/+ NMJs were evenly spaced along
 118 the axonal terminals, displaying the typical “beads-on-a-string” pattern (Fig 1A, upper
 119 and middle panels, enlarged images at right). Strikingly, the *trh*¹/*trh*² mutant larvae
 120 exhibited aberrant NMJ morphology in that their synaptic boutons were small and
 121 formed clusters without discernable connections, particularly at the terminals (Fig 1A,
 122 bottom panel); a phenotype described as “bunch boutons” [34]. This bunch bouton
 123 phenotype in the *trh*¹/*trh*² mutant was detected at a high frequency; 18% of total
 124 boutons were bunched compared to 3% for *trh*¹/+ and 0% for *w*¹¹¹⁸ (Fig 1B). Of the
 125 12 *trh*¹/*trh*² NMJs we examined, 11 possessed at least one bunch, with an average of 5
 126 bunches per NMJ. Each bunch consisted of 3 to 10 small boutons (mean = 4.3). The
 127 *trh*¹/+ larvae exhibited a much weaker phenotype; only 4 of 10 examined larvae had 1
 128 or 2 bunches, with an average of 5 small boutons per bunch. We did not observe a
 129 bunch bouton phenotype in any of the nine *w*¹¹¹⁸ NMJs we assayed (Fig 1B).
 130 Although the percentage of bunch boutons in the *trh*¹/*trh*² mutant was greatly
 131 increased, total bouton number was only slightly higher than that observed in controls,
 132 suggesting that bunch boutons form at the expense of normal ones (Fig 1B). We also
 133 assessed the percentage of satellite boutons that are also small ones stemmed from
 134 normal-size boutons, and are often observed in wild-type NMJs. We observed some
 135 satellite boutons in the *w*¹¹¹⁸ control and the *trh*¹/*trh*² mutant, and found no significant

136 differences between them (Fig S1A). Given the small size and clustering of synaptic
 137 boutons in the *trh¹/trh²* mutant, we examined whether these bunch boutons express
 138 synaptic proteins normally. We found that the synaptic vesicle protein Synapsin (Syn
 139 in Fig 1A) was normally distributed relative to control, but the active zone protein
 140 Bruchpilot (Brp) was expressed at higher levels in bunch boutons (Fig S1B). The
 141 postsynaptic glutamate receptor, as revealed by GluRIIA (Fig S1C) and GluRIII (Fig
 142 S1B) signal, as well as dPAK (Fig S1C) were also localized in bunch boutons, which
 143 were surrounded by the subsynaptic reticulum protein Dlg (Fig S1D). Thus, although
 144 the Brp signal intensity in the *trh¹/trh²* mutant was stronger than in the wild-type, the
 145 composition of synaptic proteins in bunch boutons was largely similar to that of
 146 normal-sized boutons.

147 As *trh* is expressed in both tracheal and nervous systems during embryonic
 148 stages [35], altered bouton morphology in the *trh¹/trh²* mutant could be due to a lack
 149 of *trh* in neurons, tracheal cells or other cells/tissues. Therefore, we performed *trh*-
 150 RNAi knockdown by using tissue-specific GAL4 drivers for trachea (*btl-GAL4*),
 151 neurons (*elav-GAL4*), glia (*repo-GAL4*), and muscles (*MHC-GAL4*). We observed a
 152 dramatic increase in bunch boutons upon tracheal *trh* knockdown using *btl-GAL4* (Fig
 153 1C and 1D). In contrast, *trh-RNAi* alone or using *elav-GAL4*, *repo-GAL4* or *MHC*-
 154 *GAL4* failed to replicate the bunch bouton phenotype (Fig 1D).

155 To further confirm the necessity of tracheal *trh* for normal bouton formation,
 156 we performed a rescue experiment of the *trh¹/trh²* phenotype. When we expressed a
 157 *UAS-trh* transgene in the trachea of the *trh¹/trh²* mutant upon using the tracheal *btl*-
 158 *GAL4* driver, the bunch bouton phenotype was suppressed (Fig 1E and 1F). Controls
 159 bearing only the *btl-GAL4* driver or the *UAS-trh* transgene still contained the

comparably high numbers of bunch boutons observed in *trh¹/trh²* (Fig 1B) or upon tracheal *trh-RNAi* knockdown (Fig 1D). These results indicate that *trh* is required in the trachea for normal bouton formation.

Hypoxia induces bunch bouton formation

Apart from specifying the tracheal cell fate, Trh is also involved in the branching of tubular structures during post-embryonic stages [30]. Therefore, we examined the tracheal phenotypes in the *trh¹/trh²* larvae and observed an increase of the number of terminal branches in the dorsal branch of the third segment (Fig S2A and S2B). Furthermore, we identified morphological defects such as tracheal breaks and tangles, suggesting structural defects in the *trh¹/trh²* larvae (arrows in Fig S2A). Tracheal branching activity is enhanced under hypoxia [18]. Thus, the increased number of terminal branches in *trh¹/trh²* could be a compensatory mechanism for defective trachea formation.

To understand whether *trh¹/trh²* mutant cells are under hypoxia, we used the hypoxia biosensor GFP-ODD, in which the GFP is fused to the oxygen-dependent degradation (ODD) domain of Sima, under the control of the *ubiquitin-69E (ubi)* promoter [36]. We first confirmed that GFP-ODD signal was low under normoxia (21% O₂) and enhanced under hypoxia (5% O₂, Fig 2A) in wild-type late-stage embryos when tracheal tubules are already formed and functioning [36]. Indeed, enhanced GFP signal was ubiquitous under hypoxia in wild-type embryos, with some pronounced focal GFP signals (Fig 2A, upper row, and Fig S2C). The signal of mRFP-nls, also under the control of *ubi* promoter as an internal control, remained constant under hypoxia (Fig 2A, bottom row, and Fig S2D) [36]. Quantification of the

184 GFP/RFP ratio revealed a significant difference between normoxia and hypoxia
 185 conditions (Fig 2B). We then examined whether oxygen supply is deficient in the
 186 *trh¹/trh²* mutant by measuring the GFP-ODD signals. We detected higher GFP-ODD
 187 signal under hypoxia in the mutant compared to *w¹¹¹⁸* control (Fig 2A and Fig S2C).
 188 The heterozygous *trh¹/+* presented similar GFP-ODD signal to *w¹¹¹⁸*. Quantification
 189 of the GFP/RFP ratio also demonstrated a significant increase in GFP-ODD signal in
 190 *trh¹/trh²* (Fig 2B), supporting that the *trh¹/trh²* mutant senses reduced oxygen levels.

191 Thus, formation of bunch boutons in the *trh¹/trh²* mutant could be caused by
 192 hypoxia. To test this hypothesis, we reared wild-type larvae under hypoxia (5%) and
 193 assessed synaptic bouton morphology at the third instar stage. Consistently, we
 194 observed small clustered boutons, mirroring the bunch bouton phenotype, at NMJs
 195 (Fig 2C and 2D). Furthermore, when we subjected the *trh¹/trh²* mutant to a high
 196 oxygen level (50%), the bunch bouton phenotype was completely suppressed, with
 197 *trh¹/trh²* NMJs exhibiting normal bouton morphology (Fig 2C and 2D). These results
 198 suggest that hypoxia due to the defective tracheal system in the *trh¹/trh²* mutant
 199 induces the bunch bouton phenotype, and that this phenotype can be suppressed by
 200 extra oxygen supply.

201

202 **Glial HIF-1 α /Sima mediates bunch bouton formation**

203 HIF-1 α /Sima mediates the response to low oxygen supply [37, 38]. Protein
 204 levels of Sima are increased in wild-type *Drosophila* embryos subjected to hypoxia
 205 [39], leading to transcriptional activation of downstream target genes and the
 206 induction of tracheal branching [18]. We overexpressed Sima in tracheal cells,
 207 neurons, glia, or muscle cells by tissue-specific drivers to investigate which types of
 208 cells may play a role in modulating synaptic bouton formation. Overexpressing Sima

209 in trachea caused embryonic lethality, preventing us from observing NMJ phenotypes.
 210 Larvae in which Sima was overexpressed in muscles, neurons, and glia could survive
 211 to the third instar stage and we detected a substantial number of bunch boutons upon
 212 glial Sima overexpression (Fig 3A and 3B). This result shows that the hypoxia-
 213 responding factor Sima is capable of inducing bunch bouton formation when
 214 overexpressed in glia.

215 If glial Sima is the factor responsible for responding to hypoxia in the *trh¹/trh²*
 216 mutant, reducing the Sima level in glia would suppress bunch bouton formation.
 217 Accordingly, we expressed the *sima-RNAi* transgene, which could effectively deplete
 218 *sima* expression (Fig S3A), using *repo-GAL4* in the *trh¹/trh²* mutant. As our
 219 prediction, the bunch bouton phenotype was almost completely suppressed upon glial
 220 *sima* knockdown (Fig 3C and 3D). In controls, *trh¹/trh²* mutants carrying only the
 221 *UAS-sima-RNAi* transgene or the *repo-GAL4* driver still exhibited large numbers of
 222 bunch boutons (Fig 3C and 3D). We also tested whether low oxygen level-induced
 223 bunch bouton formation is mediated through Sima in glia. Bunch bouton phenotypes
 224 were detected in controls carrying either *repo-GAL4* or *UAS-sima-RNAi* when raised
 225 in 5% O₂ (Fig 3E). However, almost no bunch boutons were detected in larvae
 226 carrying both *repo-GAL4* and *UAS-sima-RNAi* when raised in the same low-oxygen
 227 environment (Fig 3E and 3F). We also examined Sima protein levels in larvae, and
 228 found ubiquitous increases (including in Repo-positive glia) in the *trh¹/trh²* mutant or
 229 for the control under the 5% O₂ condition (Fig S3B). Thus, glial Sima mediates the
 230 hypoxia response in the *trh¹/trh²* mutant and in the low O₂ condition to modulate
 231 synaptic bouton formation.

232

233 **Glial Wg remodels bouton morphology**

234 Next, we explored possible signals transduced from glia to neurons in
 235 response to hypoxia. The glia-secreted Wingless (Wg) signaling molecule regulates
 236 synaptic growth at *Drosophila* NMJs [40, 41]. Therefore, we examined whether Wg
 237 can be induced under hypoxia in the *trh¹/trh²* mutant. Wg signal was enriched around
 238 the synaptic boutons of wild-type NMJs (Fig 4A), similar to previous reported
 239 staining patterns [40, 42]. Although the pattern of Wg signal at *trh¹/+* NMJs was
 240 similar to that of *w¹¹¹⁸*, we detected much higher levels of Wg at *trh¹/trh²* NMJs (Fig
 241 4A). Quantification of Wg immunofluorescence intensities normalized to co-stained
 242 HRP revealed a ~3-fold increase relative to *w¹¹¹⁸* and *trh¹/+*, respectively (Fig 4B).
 243 We then examined whether Sima is required for the enhanced Wg expression in the
 244 *trh¹/trh²* mutant. In a *trh¹/trh²* mutant carrying *repo-GAL4*, Wg levels were also
 245 increased 3.0-fold relative to the *repo-GAL4* control (Fig 4C, 4D). When we reduced
 246 *sima* levels in the *trh¹/trh²* mutant by *repo-GAL4*-driven *UAS-sima-RNAi*, Wg signal
 247 was suppressed to a level equivalent to that in the *repo-GAL4* control (Fig 4C and 4D).
 248 Interestingly, *sima-RNAi* knockdown in glia of the *repo-GAL4* control had no effect
 249 on the Wg level (Fig 4C and 4D), suggesting that Sima is induced to upregulate Wg
 250 expression in the *trh¹/trh²* mutant but has no role in basal Wg expression in the wild-
 251 type. Taken together, we suggest that glial Sima is required for Wg upregulation at
 252 the NMJs of the *trh¹/trh²* mutant.

253 If glia-secreted Wg is responsible for bunch bouton induction in the *trh¹/trh²*
 254 mutant, then glia-specific knockdown of *wg* in that mutant should suppress bunch
 255 bouton formation. Indeed, the bunch bouton phenotype was almost undetectable in the
 256 *trh¹/trh²* mutant also bearing *repo-GAL4* and the *UAS-wg-RNAi* transgene (Fig 4E and
 257 4F). In control, bunch boutons were still prominent in the *trh¹/trh²* mutant bearing

only *UAS-wg-RNAi* (Fig 4E and 4F). Inactivation of Wg signaling has been shown to induce unbundled filaments and a reduction of the more stabilized loops upon immunostaining for the microtubule-binding protein Futsch [41, 43]. To show that elevated Wg signaling remodels presynaptic bouton structure in the *trh¹/trh²* mutant, we examined Futsch-labeled microtubules within synaptic boutons and found significantly more Futsch-positive loops within the boutons (Fig S4A and S4B), supporting the elevation of presynaptic Wg signaling. Taken together, these results suggest that Wg plays a prominent role in the *trh¹/trh²* mutant to transduce the hypoxia signal from glia to remodel presynaptic bouton structure.

Since glial processes can invade synaptic boutons to match the growth of NMJs [44], we were intrigued to assess whether glia the *trh¹/trh²* mutant exhibits morphological change. In a live preparation of NMJs, we found that glial processes invaded the area of synaptic boutons in the *trh¹/trh²* mutant, whereas glial processes were comparatively restrained in the control (Fig S4C). Quantification of fluorescent signals of glial process overlaying the synaptic bouton area revealed significantly greater area of overlap in the *trh¹/trh²* mutant relative to control (Fig S4D). This increased extent of glial processes in the synaptic area may facilitate signal transduction from glia to synaptic boutons for structural reorganization.

Impaired crawling behavior in the *trh* mutant

Given the evident morphological changes at *trh¹/trh²* NMJs, we wondered if locomotion is affected in mutant larvae. We observed larvae crawling under free-movement conditions and found that wild-type control and *trh¹/+* heterozygous larvae presented smooth crawling paths with an average speed of ~0.5 mm/s (Fig 5A and

282 5B), whereas the *trh¹/trh²* larva had shorter paths and a slower speed of 0.14 mm/s.
 283 The head turning frequency in *trh¹/trh²* was comparable to both controls, not
 284 contributing to the slow moving (Fig 5C). Larval crawling is a rhythmic behavior
 285 involving a series of periodic strides (S1 Movie) [45-48]. We noticed uncoordinated
 286 crawling in *trh¹/trh²* larvae, with their posterior body segments failing to follow the
 287 rhythmic movement (S2 Movie). We recorded larval forward crawling and
 288 constructed kymographs to represent the stride cycle. In wild-type larvae, normal and
 289 consistent periodic strides were apparent with regular head and tail displacements (Fig
 290 5D, left panel). Similar to the wild-type, head movements of *trh¹/trh²* larvae were
 291 smooth and periodic, albeit slower. However, tail movements of *trh¹/trh²* larvae were
 292 abrupt (Fig 5D, right panel). Approximately 70% of the strides of *trh¹/trh²* larvae
 293 were uncoordinated (Fig 5E), which might contribute to the slower crawling of the
 294 *trh¹/trh²* mutant.

295 This uncoordinated stride cycle prompted us to examine the bouton
 296 morphology in anterior and posterior segments of *trh¹/trh²* larvae. Strikingly, we
 297 detected the bunch bouton phenotype in segments A2-A4 of individual *trh¹/trh²*
 298 mutant larvae, whereas bouton morphology in their respective posterior A5 and A6
 299 segments was relatively normal (Fig 6A). In fact, we seldom observed the bunch
 300 bouton phenotype in posterior segments (Fig 6B). In wild-type larvae, all segments
 301 we examined had normal bouton morphology (Fig 6A and 6B). While bunch boutons
 302 appeared in A2-A4 segments, the total numbers of boutons were comparable in all
 303 segments between wild-type and *trh¹/trh²*. We further examined whether Wg signal
 304 from glia has any effect on modifying bouton morphology in *trh¹/trh²* larvae. Glial
 305 *wg-RNAi* knockdown suppressed bunch bouton formation in the anterior A3 segment,

306 but had no effect on the morphological phenotype of the posterior A6 segment (Fig
307 6E). Thus, induction of bunch boutons is specific to anterior A2-A4 segments.

308 Given the bunch bouton phenotype in *trh¹/trh²* larvae, we assessed basal
309 synaptic transmission properties, firstly at muscle 6 of anterior A3 segments.
310 Amplitude of evoked junctional potential (EJP), as well as the amplitude and
311 frequency of miniature EJP (mEJP), were comparable among controls and *trh¹/trh²*
312 larvae (**Fig 7A-7E**). The quantal content, calculated by dividing the EJP amplitude
313 with that of mEJP, were also equivalent (**Fig 7F**). We then evaluated the synaptic
314 transmission properties of muscle 6 for posterior A6 segments. Although the
315 amplitude and frequency of mEJP and the EJP amplitude in *trh¹/trh²* larvae remained
316 similar to controls, we did detect a significant reduction in the quantal content of
317 mutant larvae (**Fig 7F**). Thus, the impaired synaptic activity of posterior segments of
318 mutant larvae seems to be consistent with their defective stride cycle.

319 Discussion

320 Here, we demonstrate that *Trh*, a member of the NPAS protein family, non-cell
 321 autonomously regulates synaptic bouton formation at NMJs through a hypoxia
 322 response from glia. We observed small-sized and clustered boutons, so-called bunch
 323 boutons, at the NMJs of *trh* mutant larvae or larvae reared at low oxygen levels. The
 324 abnormal bouton morphology at *trh* NMJs could be suppressed by reducing levels of
 325 the hypoxia response factor Sima in glia. We further showed that Sima enhanced the
 326 Wg signal from glia to cause bunch bouton formation. Although normal synaptic
 327 transmission was detected at NMJs located in anterior segments of larvae bearing
 328 bunch boutons, reduced synaptic transmission was found in posterior segments
 329 lacking bunch boutons of the *trh* mutant, suggesting that glia-induced bunch bouton
 330 formation might be a homeostatic response to restore normal synaptic transmission.
 331 Imbalanced synaptic functioning of mutant NMJs might contribute to the
 332 uncoordinated stride cycles detected in the *trh* mutant, slowing larval crawl speed.
 333 Thus, we provide a model for studying the glial responses that modulate synaptic
 334 remodeling during hypoxia.

335

336 Glia play a critical role in response to hypoxia in the *trh* mutant

337 Animal cells adapt to hypoxia by triggering the expression of HIF-1 α /Sima, the
 338 master transcriptional regulator of the hypoxia response [49]. We observed defective
 339 tracheal structure in the *trh* mutant (Fig S2A), which may result in hypoxic conditions
 340 inside the larval body. The increase in terminal branch number (Fig S2B) may be a
 341 response to oxygen supply deficiency [18]. Moreover, the increases in Sima protein
 342 levels and *ODD-GFP* reporter expression indicate reduced internal oxygen levels (Fig

2A, 2B and S3B). Finally, the bunch bouton phenotype in the *trh* mutant was recapitulated by rearing larvae under hypoxia, and it was suppressed by rearing larvae under hyperoxia. Taken together, these observations suggest that cells in the *trh* mutant sense low oxygen levels caused by the defective tracheal system and respond by elevating Sima protein levels. It is not clear how profound this effect is for other types of larval cells. Based on our ODD-GFP and Sima immunostaining patterns (Fig 2A, 2B and S3B), many types of cells are likely to be affected [50]. Our results also suggest that Trh has a late developmental role in tracheal morphogenesis, in addition to its well-characterized role in early tracheal cell fate specification [29, 30]. Unlike the mammalian homologs NPAS1 and NPAS3 that function in the nervous system, the *Drosophila* NPAS1/3 homolog Trh is more dedicated to tracheal development.

354

355 **Glia induce synaptic remodeling**

356 We suggest that glia is the major cell type mediating bunch bouton formation
357 in the *trh* mutant under hypoxia. HIF-1 α /Sima was ubiquitously, including glia,
358 increased in both the *trh*¹/*trh*² mutant and the control grown under hypoxia (Fig S3B).
359 Importantly, by manipulating Sima expression in glia we could regulate bunch bouton
360 formation (Fig 3). Elevated Sima levels induce tracheal sprouting in tracheal cells, as
361 well as protrusions in non-tracheal cells [18]. Interestingly, we also observed
362 increased overlap of glial processes with synaptic area in the *trh* mutant, indicative of
363 a glial response (Fig S4C, S4D). Several types of cells in *Drosophila* have been
364 shown to respond to hypoxia [39, 51]. For instance, under hypoxia, elevated Sima
365 levels induce the expression of Breathless (Btl, the FGF receptor) in tracheal cells that
366 branch out seeking cells that express Branchless (Bnl)/FGF, with this latter process

also being partially dependent on Sima [18, 19]. In an alternative pathway, atypical soluble guanylyl cyclases that are less sensitive to nitric oxide than conventional soluble guanylyl cyclases can mediate graded and immediate hypoxia responses mainly in neurons [52, 53]. *Drosophila* glia have not been reported to sense and respond to hypoxia, but mammalian astrocytes in the central nervous system have been shown to be involved in these processes. In a middle cerebral artery occlusion mouse model, astrocyte activation was shown to play a crucial role in ischemic tolerance, which is mediated through P2X7 receptor-activated HIF-1 α upregulation [24]. Under physiological hypoxia, reduced mitochondrial respiration leads to the release of intracellular calcium and exocytosis of ATP-containing vesicles, thereby signaling the brainstem to modulate animal breathing [54]. Our results reveal a role for *Drosophila* larval glia in sensing hypoxia via the conventional HIF-1 α /Sima pathway, warranting further detailed study.

We also demonstrated that under hypoxia, glia modulate the formation of synaptic boutons (Fig 3). These results clearly place the glia-modulated morphology of synaptic boutons in the context of hypoxia responses. Several studies have suggested that glia play important roles in regulating synaptic morphology at developmental stages or in response to neural insult. Perisynaptic Schwann cells surround nerve terminals and express neurotransmitter receptors, modulating synaptic efficacy upon nerve stimulation at mouse NMJs [55]. After nerve injury, Schwann cells participate in synaptic homeostasis and remodeling during NMJ re-innervation [56]. Chronic hypoxia causes hypomyelination, leading to synaptic reduction in the mouse cortex, which could be rescued by genetically-induced or drug-enhanced hypermyelination [57]. In *Drosophila*, expansion of glial structures to synaptic boutons matches synaptic growth [44]. Glial invasion of the synaptic region is also

suggested to clear presynaptic debris of unstable boutons during activity-dependent synaptic growth [58]. Our study further establishes that in response to hypoxia, Wg is a glial signal that modulates synaptic bouton formation. Two sources of Wg, from presynaptic motor neurons and from glia, are involved in synapse growth and remodeling [40]. Our results suggest that Sima upregulates the level of Wg secreted from glia to modulate synapse formation in the *trh* mutant or in control larvae grown under hypoxia. In hypoxic macrophages, HIF-1 α mediates the induction of Wnt1, which is a mammalian homolog of Wg [59-61]. Although it is unclear whether Wg is a direct target of the hypoxia signal Sima in *Drosophila*, we show that the level of Wg is controlled by glial Sima (Fig 4C, 4D) and that it mediates Sima-induced bunch bouton formation at *trh* NMJs (Fig 4E, 4F). As a secreted morphogen, Wg functions in both pre-synaptic and post-synaptic sites [41]. At presynaptic terminals, the canonical Wg pathway induces microtubule loop formation to regulate synaptogenesis [43]. We also detected an increase in microtubule loops in the *trh* mutant (Fig S4A, S4B), consistent with a role for Wg in mediating the hypoxia response by modulating synapse formation. Postsynaptic Wg signaling leads to subsynaptic reticulum differentiation [41], which was not apparent in the *trh* mutant (Fig S1D), suggesting that Wg might be a component of the complex hypoxia response that induces synapse remodeling. Brief exposure to hypoxia induces immature spines and impaired synaptic function in hippocampal neurons [23]. The morphological change to bunch boutons at *trh* NMJs (Fig 1A and 1B) was not accompanied by altered synaptic transmission (Fig 7A-F), which may reflect a compensatory effect during long-term hypoxia.

The bunch bouton phenotype has also been described in *spastin* mutants [34, 62]. As an AAA ATPase, Spastin severs microtubules to facilitate transport to distal axon

417 segments [63, 64]. Accordingly, the *spastin* mutant also exhibits a lack of
 418 microtubules at terminal boutons [62]. In contrast, the *trh* mutant presented an
 419 increase of microtubule loops (Fig S4A, S4B). Microtubule loops have been linked to
 420 synaptic bouton stabilization, and an excess of microtubule loops has been associated
 421 with increased synaptic bouton formation [65, 66]. The altered morphology of bunch
 422 boutons may be part of the structural changes necessary to maintain normal synaptic
 423 transmission under hypoxia. The *trh* and *spastin* mutants also exhibit differences in
 424 synaptic function, with loss of *spastin* function slightly enhancing spontaneous
 425 synaptic transmission release but reducing evoked synaptic transmission [62]. Thus,
 426 although the morphology of synaptic boutons at *trh* NMJs resembles that of *spastin*
 427 mutants, bunch boutons at *trh* NMJs retain synaptic functions, unlike the impaired
 428 synaptic transmission of *spastin* mutant boutons.

429

430 **Difference of *trh* NMJs in anterior and posterior segments**

431 The size of NMJs in muscles 6/7 decreases from the anterior to posterior
 432 segments, which could represent a coupling with muscle growth [67, 68], thereby
 433 maintaining similar electrophysiological efficacy at anterior and posterior NMJs (Fig
 434 7A-F). Interestingly, our findings show that synaptic responses in the *trh* mutant
 435 differ, with bunch boutons only appearing in anterior segments (Fig 6A and 6B).
 436 Furthermore, synaptic transmission at *trh* NMJs remained normal in anterior A3
 437 segments but was impaired in posterior A6 segments (Fig 7A-F). These observations
 438 are consistent with bunch bouton formation being part of a homeostatic response to
 439 restore synaptic activity. Why synapses were not remodeled in posterior segments
 440 remains unclear. Motor neurons in the ventral nerve cord project much longer axons

441 to muscles in posterior segments compared to anterior ones. It has been shown that
 442 axonal transport to posterior segments is more vulnerable to inefficient transport
 443 conditions. For example, mutation of long-chain Acyl-CoA synthetase impairs the
 444 balance between anterograde and retrograde transport, causing distally-biased axonal
 445 aggregations and affecting the growth and functioning of synapses [69]. In addition,
 446 larval forward locomotion, propelled by peristaltic contraction, is controlled by
 447 different circuits targeting anterior and posterior segments. The GABAergic SEZ-
 448 LN1 neurons specifically control posterior A6 and A7 segmental muscle contraction
 449 by inhibiting A27h premotor neurons, which promotes longitudinal muscle
 450 contraction during larval forward crawling [70]. It is possible that glia-derived Wg
 451 signals from cell bodies located in the ventral nerve cord of the *trh* mutant may not be
 452 efficiently transported to posterior segments during hypoxia. This polar difference in
 453 synaptic activity and bouton morphology may contribute to the uncoordinated
 454 peristaltic movements of *trh* larvae.

455 **Methods**

456 **Fly stocks**

457 All flies were reared at 25 °C. *w¹¹¹⁸* was used as wild-type control and to backcross
 458 with *trh¹* or *trh²*. Fly strains are as follows: *trh²*, *elav-GAL4*, *MHC-GAL4*, *repo-GAL4*,
 459 *ok371-GAL4*, *UAS-trh*, *UAS-sima*, and *UAS-sima-RNAi* from Bloomington
 460 Drosophila Stock Center (BDSC); *trh¹* and *UAS-wg-RNAi* from Kyoto Stock Center,
 461 and *UAS-trh-RNAi* from Vienna Drosophila Resource Center (VDRC). Also used
 462 were *btl-GAL4* [71], *GFP-ODD* [36], and the *repo-cyto-GFP* line
 463 was generated to drive cytoplasmic GFP expression under the control of the
 464 4.3kb *repo* promoter, which recapitulates the full *repo* expression pattern. [72, 73]

465 **Hypoxia or hyperoxia rearing conditions**

466 Larvae in a food vial were transferred at 1 day after egg laying (AEL) to a ProOx
 467 (model 110, BioSpherix, Lacona, NY) oxygen-controlled chamber. Oxygen or
 468 nitrogen was infused into the chamber to a desired concentration (here, 5% or 50%),
 469 which was maintained until assay.

470 **Immunostaining**

471 The NMJ phenotypes were analyzed as previously described [74]. For live tissue
 472 preparation, non-fixed larvae were dissected as previously described, before directly
 473 incubating larval fillets with anti-horseradish peroxidase (HRP, 1:10) in phosphate
 474 buffered saline (PBS) for 10 minutes. Primary antibodies used were against Synapsin
 475 (3C11, mouse, 1:100; Developmental Studies Hybridoma Bank, DSHB), HRP-Cy5
 476 (rabbit, 1:100; Jackson ImmunoResearch), Dlg (mouse, 1:100, DSHB), GluRIIA
 477 (mouse, 1:100, DSHB), dPAK (rabbit, 1:1000)[75], GluRIII (rabbit, 1:1000)[76], Brp
 478 (nc82, mouse, 1:100, DSHB), Sima (guinea pig, 1:1000)[77], Repo (mouse, 1:1000,

DSHB), Wg (4D4, mouse, 1:10, DSHB), and Futsch (22C10, mouse, 1:100, DSHB).
Secondary antibodies used were anti-rabbit or -mouse 488, Cy3, or Cy5 (1:1000,
Jackson ImmunoResearch).

Image acquisition and processing

Unless specified otherwise, NMJs in muscle 6/7 of A3 segments of wandering third-instar larvae were analyzed. Confocal images were acquired via LSM510 confocal microscopy (Carl Zeiss) using 40x water, 40x water immersion, or 100x oil objectives. All presented images are projections of confocal z-stacks. Numbers of bunch boutons, total boutons, and microtubule loops were counted manually. The immunofluorescence intensities of Wg and HRP, as well as the areas of GFP and HRP signal, were analyzed in ImageJ. Each dot in the bar graph represents the data from a single NMJ of a larva, and 8-10 NMJs from 2-5 independent experiments were pooled for quantification. Embryos were acquired by means of LSM510 confocal microscopy (Carl Zeiss) using a 20x objective, and were analyzed as previously described [36]. Each dot in the bar graph represents data from a single embryo in which fluorescence was measured in at least 35 cells.

Electrophysiological recordings

Basal transmission properties were analyzed at NMJs of muscle 6/7 in specified segments of wandering third-instar larvae as previously described [78], with some modifications. The larval body wall was dissected in cold calcium-free HL3 solution and recorded in HL3 solution containing 0.4 mM CaCl₂ at room temperature.

Crawling behavior

Mid third instar larvae (feeding stage) were placed on black agar plates (2% agar with black food coloring in 25 × 20 cm² dishes) at room temperature for filming. Video

503 recording by a Sony Xperia Z1 camera started after 1 min habituation and lasted for 5
 504 min, and it was analyzed using Ctrax software [79]. The (x, y) positions were used to
 505 calculate the crawling distance between two successive frames, and crawling speed
 506 was derived by dividing total distance travelled by time. The change in angle of larvae
 507 between two frames was divided by time to represent rotational speed. Our forward
 508 crawling assay was a modification of a previous study [48]. Larvae were transferred
 509 into a tunnel (~1 mm width) made in 2% black agar. Specimens were video-recorded
 510 for 3-10 mins using a Leica S8 APO microscope. Kymographs were constructed using
 511 the MultipleKymograph plug-in for ImageJ (NIH). Only forward crawling was
 512 counted, and 7-10 steps for each of ten larvae were analyzed for each genotype.

513

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521

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847

848 Figure legends

849 Fig 1. Bunch bouton formation at *trh* NMJs

850 (A, C, E) Immunostaining images show NMJs of muscle 6/7 for Syn (green) and HRP
851 (red) in w^{1118} , $trh^1/+$, and trh^1/trh^2 (A), *btl-GAL4* and *btl-GAL4/trh-RNAi* (C), and
852 *UAS-trh trh¹/trh²*, *btl-GAL4 trh¹/trh²*, *btl-GAL4/UAS-trh trh¹/trh²* (E). Dashed squares
853 in (A) are enlarged in right panels. White arrows indicate clusters of bunch boutons.
854 Scale bars are 10 μ m. (B, D, F) Bar graphs with data dots within each bar show
855 percentages (mean $\pm$ standard error of mean, SEM) of bunch boutons to total boutons.
856 (B) w^{1118} , $0 \pm 0\%$, $n = 10$; $trh^1/+$, $3.64 \pm 1.74\%$, $n = 10$; trh^1/trh^2 , $18.04 \pm 3.65\%$, $n =$
857 10 . (D) *trh-RNAi*, $0 \pm 0\%$, $n = 10$; *btl-GAL4*, $0 \pm 0\%$, $n = 10$; *btl>trh-RNAi*, $8.64 \pm$
858 2.81% , $n = 9$; *elav-GAL4*, $0 \pm 0\%$, $n = 10$; *elav>trh-RNAi*, $0 \pm 0\%$, $n = 10$; *repo-GAL4*,
859 $2.14 \pm 1.56\%$, $n = 10$; *repo>trh-RNAi*, $0 \pm 0\%$, $n = 10$; *MHC-GAL4*, $0 \pm 0\%$, $n = 10$;
860 *MHC>trh-RNAi*, $0.77 \pm 0.77\%$, $n = 10$. (F) *UAS-trh trh¹/trh²*, $13.80 \pm 3.00\%$, $n = 10$;
861 *btl-GAL4 trh¹/trh²*, $13.56 \pm 2.95\%$, $n = 10$; *btl-GAL4/UAS-trh trh¹/trh²*, $0.88 \pm 0.64\%$,
862 $n = 8$. (B, bottom panel) Total bouton numbers in w^{1118} (74.10 ± 4.72 , $n = 10$), $trh^1/+$
863 (89.60 ± 5.16 , $n = 10$), and trh^1/trh^2 (95.00 ± 11.21 , $n = 9$). Statistical significance by
864 Mann-Whitney test is shown as n.s., no significance; *, $p < 0.5$; **, $p < 0.01$; ***, $p <$
865 0.001 .

867 Fig 2. Induction of bunch boutons in *trh* mutants by hypoxia

868 (A) Images of late-stage embryos of w^{1118} , $trh^1/+$, and trh^1/trh^2 carrying the *GFP-*
869 *ODD* reporter (top row) and *mRFP-nls* control (bottom row) under normoxia (21% O₂)
870 or hypoxia (5% O₂). Scale bar represents 40 μ m. (B) Bar graphs show mean $\pm$ SEM of
871 the GFP/RFP immunofluorescence intensity ratio (w^{1118} at 21% O₂, 0.18 ± 0.05 , $n = 6$;

872 w^{1118} at 5% O₂, 0.84 ± 0.15 , n = 6; $trh^1/+$ at 21% O₂, 0.09 ± 0.02 , n = 6; trh^1/trh^2 at
 873 21% O₂, 0.82 ± 0.15 , n = 6). Statistical significance by Mann-Whitney test is shown
 874 as n.s., no significance; **, p < 0.01; ***, p < 0.001. (C) Images of NMJs from
 875 muscle 6/7 immunostained for Syn (green) and HRP (red) from w^{1118} at 21% or 5%
 876 O₂ (left panels) and trh^1/trh^2 (right panels) at 21% or 50% O₂. White arrows indicate
 877 clusters of bunch boutons. Scale bar represents 10 μ m. (D) Bar graph shows
 878 percentages (mean $\pm$ SEM) of bunch boutons to total boutons (w^{1118} at 21% O₂, 0 $\pm$
 879 0%, n = 10; w^{1118} at 5% O₂, $8.47 \pm 1.78\%$, n = 10; trh^1/trh^2 at 21% O₂, $12.91 \pm 2.30\%$,
 880 n = 10; trh^1/trh^2 at 50% O₂, 0 $\pm$ 0%, n = 10). Statistical significance by Mann-
 881 Whitney test is shown as ***, p < 0.001.

882

883 **Fig 3. Bunch bouton induction by glial Sima at *trh* NMJs**

884 (A, C, E) Images showing NMJs from muscle 6/7 immunostained for Syn (green) and
 885 HRP (red) in *repo-GAL4*, and *repo-GAL4/UAS-sima* (A), *sima-RNAi trh¹/trh²*, *repo-*
 886 *GAL4 trh¹/trh²*, and *repo-GAL4/sima-RNAi trh¹/trh²* (C), *sima-RNAi*, *repo-GAL4*, and
 887 *repo-GAL4/sima-RNAi* under hypoxia at 5% O₂ (E). White arrows indicate bunch
 888 boutons. Scale bars represent 10 μ m. (B, D, F) Bar graphs show percentages (mean $\pm$
 889 SEM) of bunch boutons (B) *ok371-GAL4/UAS-sima*, $0.31 \pm 0.31\%$, n = 10; *repo-*
 890 *GAL4/UAS-sima*, $8.81 \pm 2.22\%$, n = 10; *MHC-GAL4/UAS-sima*, $1.19 \pm 0.64\%$, n = 10.
 891 (D) *sima-RNAi trh¹/trh²*, $7.48 \pm 1.35\%$, n = 10; *repo-GAL4 trh¹/trh²*, $11.56 \pm 2.17\%$, n
 892 = 10; *repo-GAL4/sima-RNAi trh¹/trh²*, $1.58 \pm 0.81\%$, n = 10. (F) *sima-RNAi* at 5% O₂,
 893 $11.14 \pm 2.8\%$, n = 11; *repo-GAL4* at 5% O₂, $12.65 \pm 2.66\%$, n = 10; *repo-GAL4/sima-*
 894 *RNAi* at 5% O₂, $0.45 \pm 0.45\%$, n = 11. Statistical significance by one-way ANOVA
 895 with Tukey's Multiple Comparison post test is shown as ***, p < 0.001 (B) or Mann-
 896 Whitney test as n.s., no significance; **, p < 0.01; ***, p < 0.001 (D, F).

897

898 **Fig 4. Wg induction from glia modulates synaptogenesis at *trh* NMJs**

899 (A, C, E) Images showing NMJs of muscle 6/7 immunostained for Wg (green) and
 900 HRP (red) in w^{1118} , $trh^1/+$, and trh^1/trh^2 (A); $repo-GAL4$, $repo-GAL4/sima-RNAi$,
 901 $repo-GAL4\ trh^1/trh^2$, and $repo-GAL4/sima-RNAi\ trh^1/trh^2$ (C); and for Syn (green)
 902 and HRP (red) in $wg-RNAi\ trh^1/trh^2$ and $repo-GAL4/wg-RNAi\ trh^1/trh^2$ (E). White
 903 arrows indicate clusters of bunch boutons. Scale bars represent 10 μ m. (B, D, F) Bar
 904 graphs show averages (mean $\pm$ SEM) of normalized Wg to HRP intensities. w^{1118} ,
 905 0.18 ± 0.03 , n = 8; $trh^1/+$, 0.15 ± 0.02 , n = 9; trh^1/trh^2 , 0.49 ± 0.07 , n = 9 (B), $repo-$
 906 $GAL4$, 0.23 ± 0.04 , n = 11; $repo-GAL4/sima-RNAi$, 0.25 ± 0.02 , n = 10; $repo-GAL4$
 907 trh^1/trh^2 , 0.68 ± 0.16 , n = 8; $repo-GAL4/sima-RNAi\ trh^1/trh^2$, 0.25 ± 0.05 , n = 9 (D),
 908 and percentages (mean $\pm$ SEM) of bunch boutons in $wg-RNAi\ trh^1/trh^2$, $17.19 \pm$
 909 3.52% , n = 8; $repo-GAL4/wg-RNAi\ trh^1/trh^2$, $0.68 \pm 0.68\%$, n = 11 (F). Statistical
 910 significance by Mann-Whitney test is shown as n.s., no significance; *, $p < 0.05$; **, p
 911 < 0.01 ; ***, $p < 0.001$.

912

913 **Fig 5. Impaired crawling behavior of the *trh* mutant**

914 (A) Representative 5-minute crawling traces for w^{1118} , $trh^1/+$, and trh^1/trh^2 . (B) Bar
 915 graphs show average speeds (mean $\pm$ SEM in mm/s) for w^{1118} , 0.64 ± 0.09 , n = 13;
 916 $trh^1/+$, 0.45 ± 0.08 , n = 12; trh^1/trh^2 , 0.14 ± 0.02 , n = 14, and average rotational
 917 speeds (mean $\pm$ SEM in degree/s) for w^{1118} , 7.80 ± 0.62 , n = 13; $trh^1/+$, 9.73 ± 1.19 , n
 918 = 12; trh^1/trh^2 , 11.26 ± 1.86 , n = 14. Statistical significance by Mann-Whitney test is
 919 shown as n.s., no significance; ***, $p < 0.001$. (D) Kymographs show four larval
 920 forward crawling strides for w^{1118} and trh^1/trh^2 . The green lines show smooth
 921 transitions between successive strides, whereas orange lines show abrupt transitions.

(E) Bar graph shows uncoordinated stride percentages (mean $\pm$ SEM) in w^{1118} ($0 \pm 0\%$, $n = 10$) and trh^1/trh^2 ($69.22 \pm 9.57\%$, $n = 10$). Statistical significance by Mann-Whitney test is shown as ***, $p < 0.001$.

Fig 6. Selective bunch bouton formation at *trh* NMJs of anterior segments

(A) Images show NMJs of muscle 6/7 from abdominal segments A2-A6 immunostained for Syn (green) and HRP (red) in w^{1118} and trh^1/trh^2 . White arrows indicate clusters of bunch boutons. Scale bar represents 10 μ m. (B, C) Line graphs show average percentages (mean $\pm$ SEM) of (B) bunch bouton (w^{1118} : A2, $0 \pm 0\%$, $n = 10$; A3, $0 \pm 0\%$, $n = 9$; A4, $0 \pm 0\%$, $n = 10$; A5, $0 \pm 0\%$, $n = 10$; A6, $0 \pm 0\%$, $n = 9$; trh^1/trh^2 : A2, $15.40 \pm 3.59\%$, $n = 10$; A3, $19.80 \pm 3.09\%$, $n = 10$; A4, $5.11 \pm 1.86\%$, $n = 9$; A5, $0.80 \pm 0.80\%$, $n = 10$; A6, $1.33 \pm 1.33\%$, $n = 9$) and total bouton numbers (w^{1118} : A2, 176.60 ± 15.00 , $n = 10$; A3, 76.11 ± 4.77 , $n = 9$; A4, 85.80 ± 4.13 , $n = 10$; A5, 66.10 ± 5.39 , $n = 10$; A6, 40.00 ± 3.72 , $n = 9$; trh^1/trh^2 : A2, 173.20 ± 8.76 , $n = 10$; A3, 106.60 ± 15.33 , $n = 10$; A4, 74.11 ± 9.90 , $n = 9$; A5, 60.70 ± 9.10 , $n = 10$; A6, 33.11 ± 3.56 , $n = 9$) for w^{1118} (magenta lines) and trh^1/trh^2 (blue lines). Statistical significance by Mann-Whitney test is shown as n.s., no significance; *, $p < 0.05$; ***, $p < 0.001$. (D) Images showing NMJs of muscle 6/7 immunostained for Syn (green) and HRP (red) in $wg-RNAi\ trh^1/trh^2$ (top panel) and $repo-GAL4/wg-RNAi\ trh^1/trh^2$ (bottom panel) in A6. (D) Bar graph shows percentages (mean $\pm$ SEM) of bunch boutons ($wg-RNAi\ trh^1/trh^2$, $1.60 \pm 1.60\%$, $n = 10$; $repo-GAL4/wg-RNAi\ trh^1/trh^2$, $0 \pm 0\%$, $n = 10$). Statistical significance by Mann-Whitney test is shown as n.s., no significance.

Fig 7. Synaptic transmission at *trh* NMJs.

947 (A, B) Representative traces of mEJPs (A) and EJPs (B) recorded from NMJs of
 948 muscle 6 of segment A3 (left panels) and A6 (right panels) for w^{1118} and trh^1/trh^2 . The
 949 scales for both mEJP and EJP traces are depicted at right. (C-F) Bar graphs show
 950 averages (mean $\pm$ SEM) for (C) mEJP amplitudes ($w^{1118}/A3$, 1.43 ± 0.11 , $n = 10$;
 951 $trh^1/trh^2/A3$, 1.19 ± 0.12 , $n = 13$; $w^{1118}/A6$, 1.25 ± 0.11 , $n = 9$; $trh^1/trh^2/A6$, $1.51 \pm$
 952 0.20 , $n = 8$); (D) mEJP frequencies ($w^{1118}/A3$, 1.92 ± 0.13 , $n = 10$; $trh^1/trh^2/A3$, $2.38 \pm$
 953 0.27 , $n = 13$; $w^{1118}/A6$, 2.02 ± 0.34 , $n = 9$; $trh^1/trh^2/A6$, 1.41 ± 0.21 , $n = 8$); (E) EJP
 954 amplitudes ($w^{1118}/A3$, 54.61 ± 2.06 , $n = 10$; $trh^1/trh^2/A3$, 50.89 ± 4.18 , $n = 13$;
 955 $w^{1118}/A6$, 46.24 ± 3.71 , $n = 9$; $trh^1/trh^2/A6$, 41.64 ± 5.71 , $n = 8$); and (F) quantal
 956 contents (QC) calculated as EJP/mEJP ($w^{1118}/A3$, 40.23 ± 3.33 , $n = 10$; $trh^1/trh^2/A3$,
 957 49.63 ± 3.91 , $n = 13$; $w^{1118}/A6$, 38.90 ± 3.75 , $n = 9$; $trh^1/trh^2/A6$, 27.88 ± 1.53 , $n = 8$).
 958 Statistical significance by Mann-Whitney test is shown as n.s., no significance; *, $p <$
 959 0.05 ; **, $p < 0.01$.
 960

961 **Supporting information**

962 **S1 Fig. Presynaptic and postsynaptic structures in *trh* mutants**

963 (A) Bar graphs show percentages (mean $\pm$ SEM) of satellite boutons to total boutons
 964 (w^{1118} , $5.13 \pm 1.66\%$, $n = 10$; $trh^1/+$, $0.66 \pm 0.66\%$, $n = 10$; trh^1/trh^2 , $2.63 \pm 0.97\%$, n
 965 $= 10$). Statistical significance by Mann-Whitney test is shown as n.s., no significance;
 966 *, $p < 0.5$. (B, C, D) Images showing NMJs of muscle 6/7 immunostained for Brp,
 967 GluRIII, and HRP (B), GluRIIA, dPAK, and HRP (C), and Dlg and HRP (D) in w^{1118} ,
 968 $trh^1/+$, and trh^1/trh^2 . Scale bars are 10 μm .

969

970 **S2 Fig. Tracheal defects in *trh* mutants**

971 (A) A bright-field view of tracheal dorsal branches of $trh^1/+$ (left top panel) and
 972 trh^1/trh^2 (right top panel), and tracheal dorsal trunks of trh^1/trh^2 (bottom panels).
 973 Numbers denote terminal branches, and arrows indicate a tracheal break (bottom left)
 974 and a tracheal tangle (bottom right). Scale bar represents 50 μm . (B) Bar graph shows
 975 mean $\pm$ SEM of dorsal terminal branches ($trh^1/+$, 5.70 ± 0.15 , $n = 10$; trh^1/trh^2 , $7.55 \pm$
 976 0.28 , $n = 11$). (C) Bar graphs show mean $\pm$ SEM of the GFP immunofluorescence
 977 intensity (w^{1118} at 21% O_2 , 20.33 ± 7.54 , $n = 6$; w^{1118} at 5% O_2 , 79.31 ± 17.98 , $n = 6$;
 978 $trh^1/+$ at 21% O_2 , 13.29 ± 3.64 , $n = 6$; trh^1/trh^2 at 21% O_2 , 108.2 ± 14.70 , $n = 6$). (D)
 979 Bar graphs show mean $\pm$ SEM of RFP immunofluorescence intensity (w^{1118} at 21% O_2 ,
 980 102.00 ± 15.55 , $n = 6$; w^{1118} at 5% O_2 , 109.60 ± 13.12 , $n = 6$; $trh^1/+$ at 21% O_2 ,
 981 143.00 ± 15.67 , $n = 6$; trh^1/trh^2 at 21% O_2 , 151.50 ± 16.77 , $n = 6$). Statistical
 982 significance by Mann-Whitney test is shown as n.s., no significance; **, $p < 0.01$; ***,
 983 $p < 0.001$.

984

985 **S3 Fig. Efficiency of *sima*-RNAi knockdown and Sima upregulation**

986 (A) Assay for *sima* mRNA levels in *da-GAL4* (control) or *da-GAL4/sima-RNAi* shows
987 a reduction upon *sima-RNAi* knockdown. The *Rpl19* mRNA levels are comparable in
988 both genotypes. (B) Images showing central nerve cord immunostained for Sima
989 (green) and Repo (red) in *w¹¹¹⁸* at 21% O₂, *w¹¹¹⁸* at 5% O₂, and *trh¹/trh²* at 21% O₂.
990 Arrows indicate glia with high Sima protein levels. Scale bar represents 20 μm.

991

992 **S4 Fig. Microtubule loops and glial invasion of *trh* NMJs**

993 (A) Images showing NMJs of muscle 6/7 immunostained for HRP (red) and Futsch
994 (green) in *trh¹/+* and *trh¹/trh²*. White arrows indicate microtubule loops. Dashed
995 squares are enlarged in right panels. Scale bar represents 10 μm. (B) Bar graphs show
996 averages (mean ± SEM) of Futsch loops (*trh¹/+*, 2.92 ± 0.29, n = 12; *trh¹/trh²*, 9.56 ±
997 1.11, n = 9). (C) 3D images of live tissues showing NMJs of muscle 6/7 carrying
998 *repo-cyto-GFP* (green) labeled for HRP (red) in *trh¹/+* and *trh¹/trh²* by using Imaris.
999 Synaptic overlap by glia is represented in yellow. Scale bar represents 10 μm. (D) Bar
1000 graph shows percentages (mean ± SEM) of synaptic areas overlapping with glia in
1001 *trh¹/+*, 6.2 ± 0.99%, n = 10; *trh¹/trh²*, 16.7 ± 3.66, n = 10. Statistical significance by
1002 Mann-Whitney test is shown as *, p < 0.05 or ***, p < 0.001.

1003

1004 **S1 Movie. Forward crawling of *w¹¹¹⁸***

1005

1006 **S2 Movie. Forward crawling of *trh¹/trh²***

Fig. 1

Fig. 2

Fig. 3

Fig. 6

A

B

C

E