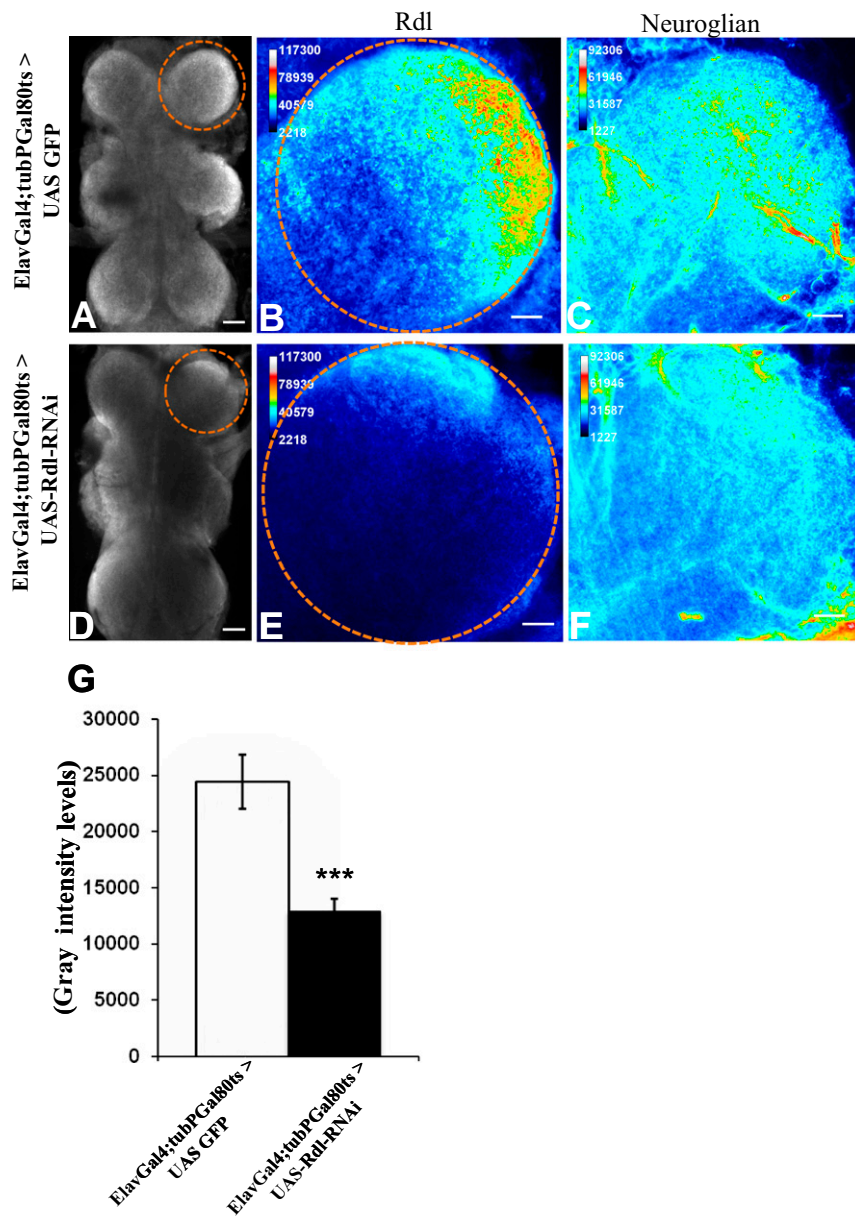
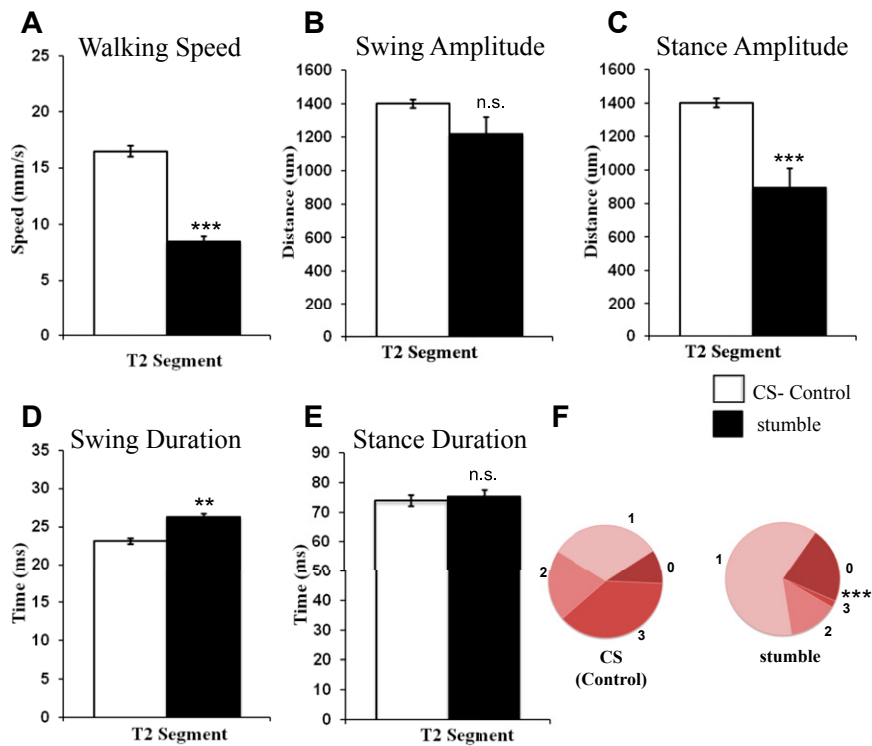


# Supporting Information

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**Fig. S1.** Rdl expression in VNC neuropile and upon knockdown with panneuronal Elav Gal4 driver. (A) Gray scale representative image of Rdl expression in VNC (ventral nerve cord) using UAS-GFP as reporter of Elav Gal4 driver. (B) Magnified view of the T1 neuropile showing Rdl expression in summed intensity z projection, represented as heat map (orange dashed circle). (C) Neuroglian staining representing untargeted protein expression as a heat map in the same region as B. (D) Knockdown of Rdl in all neurons using Elav Gal4 shows significant reduction of Rdl expression in the VNC. Region encircled with an orange dashed line is expanded in E. (E) Expanded region of T1 neuropile from D shows significant reduction of Rdl expression, represented as a heat map. (F) Neuroglian staining representing untargeted protein expression as a heat map in the same region as E. (G) Fluorescence intensity quantification of Rdl between controls and Elav-driven Rdl knockdown represented as gray intensity levels. We observe significant reduction in the gray intensity levels in Elav-driven Rdl knockdown flies compared with control Elav Gal4; +; tubPGal80ts flies ( $n = 10$ ). Quantitative analysis for bar plots represented was performed using Student's  $t$  test ( $***P < 0.001$ ). [Scale bar: 30  $\mu$ m (A and D) and 10  $\mu$ m (B, C, E, and F).] Gradient vertical bars in heat maps (B, C, E, and F) indicate the scale of fluorescence intensity; maximum intensity is red, and minimum intensity is blue.

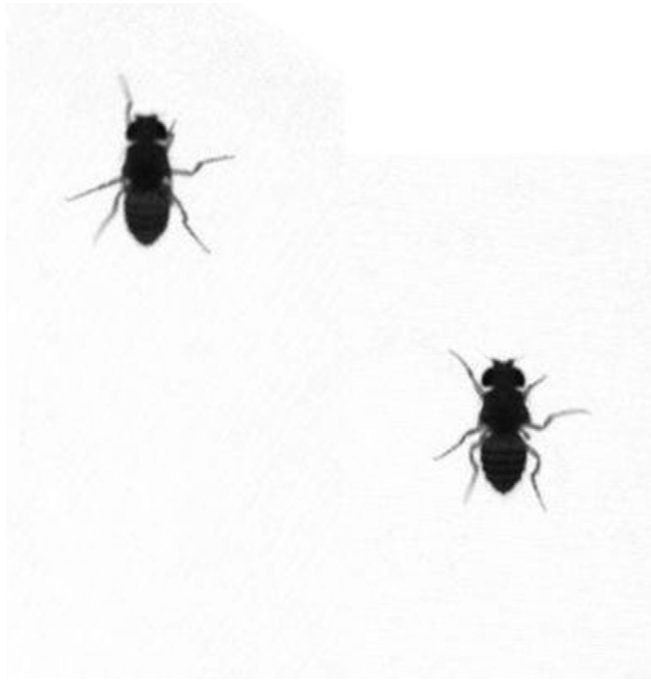


**Fig. S2.** Characterization of stumble mutants for uncoordinated walking behavior. (A) Video analysis of stumble mutants shows significant reduction in walking speed. (B) stumble mutants do not show any change in swing amplitude. (C) The stance amplitude is significantly shortened in stumble mutants compared with wild-type controls. (D) stumble mutants show a significant increase in swing duration. (E) Stance duration is unaffected in stumble mutant flies. In this figure, the left bar indicates control, and the right bar indicates knockdown. (F) Concurrency state 3 is significantly decreased in stumble mutant flies, indicating the loss of coordination in walking compared with wild-type flies. Left circle, control; right circle, knockdown. Quantitative analysis for the bar plots was performed using Student's *t* test. The bar plots represent mean  $\pm$  SEM (\*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ). n.s., not significant. Quantitative analysis for the pie chart was performed using two-way repeated measures (RM)-ANOVA and showed a significant difference in the concurrency state 3 of CS (Canton S) control flies and stumble mutant flies,  $F(3, 159) = 81.21$ ,  $P < 0.001$ . Post hoc testing using Sidak multicomparison test showed significant difference in state 3 of stumble mutant flies compared with wild-type controls (\*\* $P < 0.001$ ).



**Movie S1.** Accompanying Fig. 3, characterization of wild-type walking flies comparing fast and slower walking flies. The content was filmed at 200 fps and played at 25 fps. Left side indicates control and right knockdown.

[Movie S1](#)



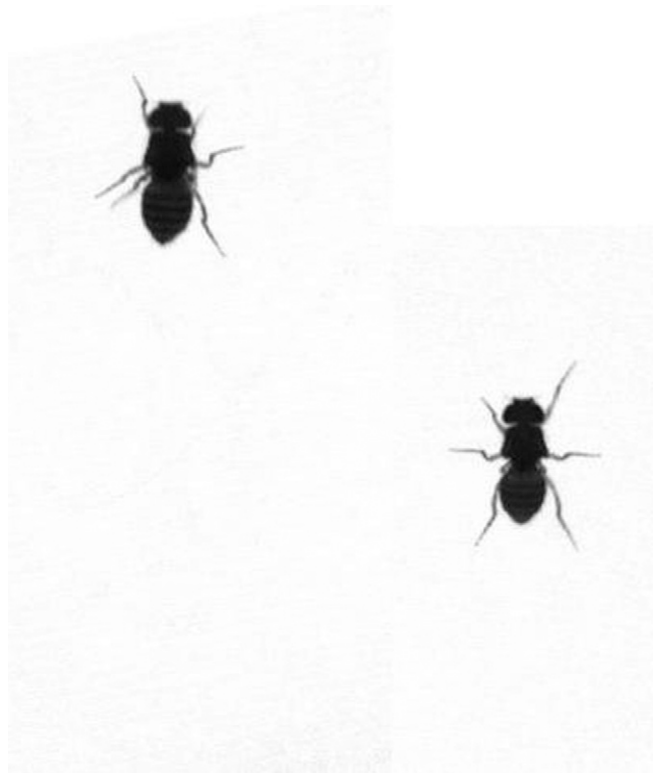
**Movie S2.** Accompanying Fig. 4C, OK371Gal4-targeted knockdown of Rdl showing slower walking speed compared with OK371 Gal4 controls. The content was filmed at 200 fps and played at 25 fps. Left side indicates control and right knockdown.

[Movie S2](#)



**Movie S3.** Accompanying Fig. 5C, adult-specific knockdown of Rdl using OK371Gal4; tubpgal80ts showing slower walking speed compared with OK371Gal4; tubpgal80ts controls. The content was filmed at 200 fps and played at 25 fps. Left indicates control and right knockdown.

[Movie S3](#)



**Movie S4.** Accompanying Fig. 6C, subset-specific knockdown of Rdl using VGN 1 Intron Reg-3 Gal4 driver showing slower walking speed compared with VGN 1 Intron Reg-3 Gal4 controls. The content was filmed at 200 fps and played at 25 fps. Left side indicates control and right knockdown.

[Movie S4](#)



**Movie S5.** Accompanying Fig. 7C, OK37Gal4-driven Rdl knockdown in a Tsh-Gal80 background showing no differences between OK371 Gal4; Tsh-Gal80 controls and OK371 Gal4; Tsh-Gal80 > UAS-Rdl RNAi experimental flies. The content was filmed at 200 fps and played at 25 fps. Left side indicates control and right knockdown.

[Movie S5](#)



**Movie S6.** Accompanying Fig. S2, uncoordinated walking as seen in stumble mutants compared with CS (Canton S) controls. The content was filmed at 200 fps and played at 25 fps. Left side indicates control and right knockdown.

[Movie S6](#)