Activity-dependent endocytosis of Wingless regulates synaptic plasticity in the Drosophila visual system

Hinata Kawamura, Satoko Hakeda-Suzuki and Takashi Suzuki

Graduate School of Life Science and Technology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8501, Japan

(Received 10 June 2020, accepted 21 July 2020; J-STAGE Advance published date: 10 December 2020)

Neural activity contributes to synaptic regulation in sensory systems, which allows organisms to adjust to changing environments. However, little is known about how synaptic molecular components are regulated to achieve activity-dependent plasticity at central synapses. Previous studies have shown that following prolonged exposure to natural ambient light, the presynaptic active zone (AZ), an area associated with presynaptic neurotransmitter release in Drosophila photoreceptors, undergoes reversible remodeling. Other studies suggest that the secretory protein Wingless (Wg; an ortholog of Wnt-1) can mediate communication between synaptic cells to achieve synaptic remodeling. However, the source of Wg and the mechanism of Wg signal modulation by neuronal activity remained unclear. Here, we found that Wg secreted from glial cells regulates synaptic remodeling in photoreceptors. In addition, antibody staining revealed that Wg changes its localization depending on light conditions. Although Wg is secreted from glial cells, Wg appeared inside photoreceptor axons when flies were kept under light conditions, suggesting that an increase in neuronal activity causes Wg internalization into photoreceptors by endocytosis. Indeed, by blocking endocytosis in photoreceptors, the localization of Wg in photoreceptors disappeared. Interestingly, Wg accumulation was higher in axons with disassembled AZ structure than in axons whose AZ structure was stabilized at the single-cell level, indicating that Wg endocytosis may trigger AZ disassembly. Furthermore, when we genetically activated Wg signaling, Wg accumulation in photoreceptors decreased. Conversely, when we suppressed Wg signaling there was an increase in Wg accumulation. Through RNAi screening of Ca$^{2+}$-binding proteins in photoreceptors, we found that Calcineurin is a key molecule that triggers Wg endocytosis. Overall, we propose that Wg signaling is regulated by a negative feedback loop driven by Wg endocytosis. The increase in neuronal activity is transmitted via calcium signaling, which leads to a decrease in Wg signaling and thereby promotes presynaptic remodeling.

Key words: Drosophila visual system, synaptic remodeling, Wingless

INTRODUCTION

Activity-dependent neuronal plasticity is a versatile ability of the nervous system preprogrammed to adapt to changing environmental stimuli. One major way to control synaptic strength is to modulate the probability of presynaptic vesicle release (Alabi and Tsien, 2012), which occurs at the highly specialized presynaptic active zone (AZ) (Kittel and Heckmann, 2016). The cytomatrix of the AZ is formed by a set of specialized proteins (Schoch and Gundelfinger, 2006; Südhof, 2012) which are conserved in worms, insects and mammals (Owald et al., 2010; Südhof, 2012). It is broadly accepted that the major molecular mechanism involved in the adjustment of synaptic vesicle release is molecular reorganization of AZ components in response to external stimuli (Owald et al., 2010; Lazarevic et al., 2011; Davydova et al., 2014).

Using the Drosophila visual system as a model nervous system, we have previously shown that activity-dependent...
presynaptic AZ remodeling does indeed occur (Sugie et al., 2015). The visual system of adult Drosophila consists of the retina and optic lobe, including a ganglion called the medulla. Among the eight types of photoreceptor cells (R1–R8), R7 and R8 project axons to the medulla to form synapses with secondary neurons. The molecular remodeling of photoreceptors in the AZ occurs after a mild but prolonged exposure to light. In our previous study (Sugie et al., 2015), we quantified AZ number in Drosophila photoreceptor cells using the Brp fusion protein as a marker. A 72-h dark (DD), 72-h light (LL) and 72-h light–dark cycle (LD; normal condition) exposure showed that the AZ number in R8 decreased by 10–20% under LL conditions compared to LD or DD (Sugie et al., 2015). This synaptic number change was reversible: when LL flies were transferred to DD, the AZ number recovered to DD levels (Sugie et al., 2015). We showed that within photoreceptors the signal that triggers AZ remodeling involved the divergent canonical Wnt pathway, possibly driven by Wingless (Wg; the Drosophila ortholog of vertebrate Wnt-1) (Sugie et al., 2015). However, the source of Wg and the mechanism by which the Wg signal is modulated in an activity-dependent manner remained unclear.

Wnt proteins are known to be involved in the cell patterning required for embryonic development of all animal species studied to date. Not only do Wnt proteins regulate cell fate, but a recent study also suggests an important role of the Wnt signaling pathway in synapse formation and maintenance at the neuromuscular junction (Koles and Budnik, 2012). To ensure that the Wnt signal is appropriately transduced and interpreted, multiple strategies restrict pathway activation. For instance, endocytic trafficking of developmental signals to the lysosome has been implicated in restricting the range of morphogen gradients and downregulating active ligand–receptor complexes (Seto et al., 2002). In the absence of such controls, deregulated Wnt signaling can lead to developmental abnormalities or disease. Thus, we considered that a similar type of regulatory mechanism might also be observed in the communicating neurons and glia that regulate synaptic remodeling.

We therefore aimed to elucidate the regulatory mechanism of Wg signaling that occurs during synaptic remodeling in the Drosophila visual system. First, we sought to determine the source of Wg. We found that Wg secreted from glial cells regulates synaptic remodeling in photoreceptors. In addition, Wg immunostaining showed that Wg secreted from glia cells was internalized into photoreceptors by endocytosis only in the LL condition. Surprisingly, Wg endocytosis and Wg signaling negatively regulate each other. Through a series of genetic and histological experiments, we determined that neuronal activity is transmitted via calcium signaling to induce Wg endocytosis, which results in a decrease of the Wg signal, and thereby promotes presynaptic remodeling. Our data suggest that Wg signaling is regulated by a negative feedback loop driven by Wg endocytosis.

**MATERIALS AND METHODS**

**Fly strains** The lines used in this study are listed in Supplementary Table S1. The endogenous-wg-regulatory-region-FRT-wg(cDNA)-FRT-QF flies were kind gifts from Dr. Jean-Paul Vincent (Francis Crick Institute). The specific genotypes utilized in this study are listed in Supplementary Table S2. RNAi fly stocks for the screening were obtained from the Vienna Drosophila RNAi Center or the Bloomington Drosophila Stock Center. The stock numbers are listed in Supplementary Table S3.

**Light exposure** From the larval stage to hatchling, the flies were kept in columned vials or in bottles with media at room temperature (25 °C), 50–60% humidity, with a 12-h light–dark (LD) cycle. For the experimental part, the flies were exposed to DD or LL at 25 °C. The intensity of illuminance from LED panels (LEDXC170-W, MISUMI) was set at about 2,000 lux.

**Dissection and immunohistochemistry** The experimental procedures for adult brain dissection, fixation and immunostaining were performed as described previously (Hakeda-Suzuki et al., 2017). Briefly, adult fly brains were dissected in 0.15% PBT (PBS containing Triton X-100) and then fixed with 4% paraformaldehyde at room temperature for 90 min. Primary antibody was added and incubated for 48 h at 4 °C on a shaker; secondary antibody was added and incubated overnight at 4 °C. The following antibodies were used for immunohistochemistry: mouse anti-Wg (1:100, DSHB #4D4), mAb24B10 (1:50, DSHB), chicken anti-lacZ (1:1,000, abcam #9361), mouse anti-Repo (1:400, DSHB #8D12) and rabbit anti-Rab5 (1:1,000, abcam #31261). The secondary antibodies were Alexa488-, Alexa568- or Alexa633-conjugated (1:400, Life Technologies).

**Imaging and statistical analysis** A Nikon C2+ confocal microscope was used to acquire all confocal images. Each brain image was acquired between the depths of 30 and 40 μm from the surface with a Z step size of 1 μm; images were acquired twice and averaged. Images were processed with NIS-Elements AR. The proportion of Wg-positive R8 was quantified with NIS-Elements AR Analysis by dividing the number of axons with Wg accumulation by the total axon number. The proportion of Brp(short)-mCherry diffused signals was quantified with NIS-Elements AR Analysis by dividing the number of axons with diffused signals by the total axon number.
**RESULTS**

Wg secreted from glial cells regulates synaptic remodeling in R8 cells To investigate which cells secrete Wg, we utilized flies that have the QF expression cassette (a binary gene expression system: Riabinina and Potter (2016)) knocked in to the endogenous wg locus (endogenous-wg-regulatory-region-FRT-ug(cDNA)-FRT-QF: Baena-Lopez et al. (2013)). Combining with the hsFLP transgene, we heat-shocked the flies immediately before eclosion to induce recombinase expression only in adults, so that QF is switched on only in cells that express endogenous Wg in adults. QF-expressing cells were monitored using the QUAS-LacZ transgene, and lacZ staining was performed (Fig. 1A). LacZ staining overlapped with the glia-specific marker Repo, suggesting that glial cells are the source of Wg in adults (Fig. 1B). To determine if Wg secreted from glial cells was functionally relevant to synaptic plasticity in R8, we utilized a cell-specific Wg knockout fly (Baena-Lopez et al., 2013). We overexpressed Brp[short]-mCherry in R8 cells as a synapse marker (Sugie et al., 2015). Each image was acquired under the same conditions (laser power, depth, etc.) and the proportion of R8 axons with diffused signals of Brp that could not be recognized as clear individual puncta was quantified. In LD conditions, the proportion increased in glia-specific Wg knockout flies (either photoreceptor-specific or postsynaptic cell-specific) did not exhibit any phenotype (Fig. 1E–1G). These results strongly support the idea that glial cells are the source of Wg, which controls synaptic plasticity in R8 cells.

Localization of Wg changes depending on light stimulation To reveal how Wg secretion from glial cells is controlled by neural activity of R8, we conducted anti-Wg antibody staining under 24-h constant light (LL) or constant dark (DD) conditions. We found that in the DD group, Wg localization was observed near glial cells, and in LL, localization was confirmed to overlap with photoreceptors, especially in the M1 and M6 layers (Fig. 2A–2B). These localizations in LL were also found to be within cells (R8 or R7 neurons) when carefully assessed by double staining with cell membrane markers (Berger et al., 2008) (Supplementary Fig. S1A–S1B). Furthermore, cytoplasmic LacZ expressed in photoreceptors and Wg were localized in a mutually exclusive manner within photoreceptors (Supplementary Fig. S1C). Taking these observations together, we conclude that Wg accumulates inside photoreceptor cells in a compartment isolated from the cytoplasm under LL conditions.

Next, to analyze how Wg localization changes with a 12-h light/12-h dark cycle (LD), the proportion of R8 cells with Wg accumulation (Wg-positive R8) was quantified every 4 h (Fig. 2C). Surprisingly, nearly 20% of R8 already showed Wg localization only 4 h after switching from the dark state to the light state (Fig. 2D–2E). The level of Wg-positive R8 stayed constant between 4 and 12 h in the light state. When the flies were switched back to the dark state, the proportion of Wg-positive R8 significantly decreased after 4 h (Fig. 2D–2E); there was essentially no difference at 4, 8 or 12 h after switching (Fig. 2D–2E). These results indicate that Wg localization changes rapidly (in hours) in response to the light environment, and that this precedes synaptic remodeling, which can occur over days (around 72 h).

**Activity-dependent accumulation of Wg in photoreceptor axons** To investigate how light stimulation affects Wg localization in R8, we measured the effects of mutations that block the response of photoreceptors to light. The proportion of Wg-positive R8 in LL decreased in norpA mutants, which are deficient in phospholipase C (required to elicit a photo-activated potential), indicating that Wg localization depends on photoreceptor activity (Fig. 2F, 2G and 2M) (Bloomquist et al., 1988). We also silenced photoreceptors by expressing UAS-KCNJ2-GFP (Hardie et al., 2001) in R8, which hyperpolarizes the resting membrane potential, and found that the proportion of Wg-positive R8 axons in LL also decreased (Fig. 2H, 2I and 2M). Conversely, sustained activation of photoreceptors induced by expression of UAS-TrpA1 at the restrictive temperature triggered an increase of Wg-positive R8 axons even in DD (Fig. 2J–2M). These results indicate that activation-induced depolarization is required and sufficient to induce Wg accumulation in R8. From previous studies, it is known that postsynaptic cell responses elicit activity-dependent AZ modifications in photoreceptors. Ort and Hise1 are histamine chloride channels and promote hyperpolarization of the
However, there was no phenotype related to Wg accumulation either in ort1hiscl134 histamine receptor double mutants (Iovchev et al., 2002; Hong et al., 2006; Gao et al., 2008) or in flies whose postsynaptic cells were depolarized by TrpA1 in Ort-positive cells (Supplementary Fig. S2A–S2E). Collectively, these results demonstrate that Wg accumulation inside photoreceptors depends on the neural activity of the photoreceptors, and is not dependent on feedback regulation from postsynaptic cells.

Photoreceptors internalize Wg by endocytosis

Given our results that glia cells secrete Wg and that it localizes inside photoreceptors in LL, we assumed that photoreceptors internalize Wg by endocytosis. To test this, we expressed shibire
ts1, which can block endocytosis at the restrictive temperature, and found no Wg localization in photoreceptors (Fig. 3A–3C). To determine whether Wg is localized in the endosome, we performed double staining under LL conditions with antibodies for Wg and Rab5 (McBride et al., 1999), a protein that controls the process of fusion of endocytic vesicles to early endosomes. We observed colocalization in the M1 layer and the M6 layer (Fig. 3D). Next, we investigated whether Arrow/Lrp5/6, an essential co-receptor in Wnt signaling (He et al., 2004), is required for Wg endocytosis. Arrow (arr) mediates the divergent canonical Wnt pathway that controls the AZ structure of photoreceptors. We generated arr mutant clones in approximately half of photoreceptors using ey3.5-FLP. Although R7 and R8 cells overlap in medulla columns, they can be distinguished from each other by their morphology. The R8 axon, which has no arr expression, showed Wg accumulation (Fig. 3E). The number of Wg-positive R8 cells was higher in arr mutant
Fig. 2. Wg localization changes depending on the light condition. (A–B) Localization of Wg in LL (A) and DD (B). (A’) Enlarged images of the boxed area in (A). Merged image in the left panel and green (anti-Wg) image in the right panel. Upper white arrows in (A) indicate Wg accumulation in the M1 layer of a photoreceptor; lower white arrows indicate Wg accumulation in the M6 layer. (B’) Enlarged images of the boxed area in (B). Merged image in the left panel and green (anti-Wg) image in the right panel. Arrows in (B) indicate Wg localization near a glial cell. Scale bar, 20 μm. (C) Schematic showing each time point at which flies raised in the LD incubator were dissected. Black arrow “1” indicates just before the light was switched on. Following arrows indicate time points every 4 h. (D) Localization of Wg at each time point. Scale bar, 20 μm. Arrows indicate Wg accumulation in individual photoreceptors. (E) Quantification of the proportion of Wg-positive R8 at each time point: 1 (n = 440 axons/6 brains); 2 (n = 340/5); 3 (n = 436/6); 4 (n = 413/6); 5 (n = 323/4); 6 (n = 378/5); 7 (n = 390/6). (F–L) Wg accumulation in photoreceptors of wild type flies (F) and norpA7 mutant flies (G) maintained in LL, upon expression of GMR-Gal4 in LL (H) or with UAS-KCNJ2-EGFP in LL (I), or upon expression of UAS-TrpA1 with GMR-Gal4 in DD at 18 °C (J), 29 °C (K) or control without UAS-TrpA1 in DD at 29 °C (L). White arrows in (F–L) indicate Wg accumulation in photoreceptors. Scale bar, 20 μm. (M) Quantification of the proportion of Wg-positive R8: control in LL (n = 2597 axons/25 brains) and norpA7 mutant in LL (n = 1268/13); control in LL (n = 521/5) and UAS-KCNJ2-EGFP in LL (n = 947/8); UAS-TrpA1 in DD at 18 °C (n = 376/3), 29 °C (n = 441/4), and in control without UAS-TrpA1 in DD at 29 °C (n = 441/4). *** P < 0.001, chi-squared test.
Fig. 3. Membrane trafficking genes regulate Wg accumulation.  (A–B) Wg accumulation in photoreceptors upon expression of GMR-Gal4 (A) or with UAS-shi
t in LL at 29 °C.  Scale bar, 20 μm.  (C) Quantification of the proportion of Wg-positive R8: UAS-shi
t in LL at 29 °C (n = 486 axons/4 brains), and in control without UAS-shi
t in LL at 29 °C (n = 539/5).  *** P < 0.001, chi-squared test.  (D) GMR-myr-RFP fly stained with anti-Wg antibody and anti-Rab5 antibody.  Arrows in (D') indicate overlapped localization of Wg and Rab5 in photoreceptors.  Scale bar, 20 μm.  (D') Enlarged images of the boxed area in (D).  Merged image in the leftmost panel, blue (photoreceptors) image in the middle left panel, green (anti-Wg) image in the middle right panel, and red (anti-Rab5) image in the rightmost panel.  Scale bar, 3 μm.  (E) arr mutant photoreceptor clones using ey3.5-FLP.  All axons are labeled by RFP, and arr-positive axons are labeled by GFP.  (E') Image showing arr-positive axons.  Bars indicate photoreceptors which do not express Arr.  (E'') Enlarged images of the boxed area in (E).  Merged image in the left panel, green (anti-Wg) and red (photoreceptors) image in the central panel, and green (anti-Wg) image in the right panel.  Arrows indicate Wg accumulation in an arr mutant cell.  Scale bar, 20 μm.  (F) Quantification of the proportion of Wg-positive R8 in cells having at least one copy of arr (WT) (n = 192 axons/6 brains) and in arr mutant cells (n = 178/6).  *** P < 0.001, chi-squared test.  (G–I) Wg accumulation in photoreceptors upon expression of GMR-Gal4 (G), with UAS-atg1RNAi (H) or with UAS-atg8aRNAi (I) in LL.  Arrows indicate Wg accumulation in photoreceptors.  Scale bar, 20 μm.  (J) Quantification of the proportion of Wg-positive R8: control (n = 521 axons/5 brains), UAS-atg1RNAi (n = 544/5) and UAS-atg8aRNAi (n = 669/6) in LL.  *** P < 0.001, chi-squared test.
Wg is degraded by autophagy following internalization into photoreceptors To elucidate the physiological importance of Wg endocytosis, we sought to identify the pathway that follows endocytosis. The main processes that occur after endocytosis are degradation and recycling pathways (Mellman, 1996; Maxfield and McGraw, 2004). Since autophagy reportedly acts in an optical stimulus-dependent manner in Drosophila (Midorikawa et al., 2010; Weiss and Minke, 2015), we investigated the involvement of autophagy. We suppressed autophagy by expressing RNAi for the autophagy-related genes \textit{atg1} and \textit{atg8a} (Nakatogawa et al., 2012), two of the more highly expressed genes in the eyes of adult Drosophila (Microarray data from FlyAtlas Anatomy Microarray). We found that the proportion of Wg-positive R8 increased both in \textit{atg1} and in \textit{atg8a} knockdown flies in the LL group (Fig. 3G–3J). These results indicate that a considerable portion of accumulated Wg is degraded and cleared by autophagy after it is internalized into photoreceptors.

Axons with disassembled AZs show Wg accumulation Next, we investigated the relationship between Wg accumulation and AZ structure. Since Wg accumulation and AZ remodeling in photoreceptors occur under LL conditions, we investigated whether there is a correlation between the two. We analyzed the correlation at the single-cell level; to do this, we overexpressed Brp[short]-mCherry in R8 to easily and clearly detect axons that have disassembled AZs (Fig. 4A–4B). Using these flies, we found that the proportion of Wg-positive R8 in axons showing a punctate signal was 5%; however, the proportion in axons showing a diffused signal was 50% (Fig. 4C). Our data demonstrate that the accumulation of Wg and AZ remodeling are strongly correlated at the single-cell level in photoreceptors.

The divergent canonical Wnt pathway regulates Wg accumulation in photoreceptors To better understand the relationship between Wg accumulation and the state of the AZ structure in more detail, we next examined the relationship between the divergent canonical Wnt pathway and the accumulation of Wg. Specifically, we were interested in determining whether the accumulation of Wg is regulated by the strength of the Wg signal. Since the proportion of Wg-positive R8 cells was increased in \textit{arr2} mutant cells (Fig. 3E–3F), we considered that there is a correlation between the divergent canonical Wnt pathway and the accumulation of Wg. Therefore, we manipulated the divergent canonical Wnt pathway using genetic techniques, and examined how this affected the localization of Wg. First, we inhibited the Wnt pathway. Dishevelled (Dsh) is a cytosolic phosphoprotein that is required after Wg signaling through the Frizzled 2 and Arr co-receptors (Gordon and Nusse, 2006) (Fig. 4D). In hemizygous \textit{dsh1} flies, the proportion of Wg-positive R8 increased in the LL group (Fig. 4E–4F, and 4K). Shaggy (Sgg), the Drosophila homolog of GSK3-\(\beta\), is negatively regulated by Wnt signaling (Fig. 4D). Sgg overexpression in R8 increased Wg-positive R8 cells in the LL group, which mimics the phenotype of \textit{arr2} or \textit{dsh1} mutants (Fig. 4G and 4K). Sgg modulates microtubule organization through MAP1B/Futsch phosphorylation, leading to microtubule destabilization (Fig. 4D) (Gögel et al., 2006). A \textit{futsch} mutant phenocopied the accumulation of Wg in LL observed in \textit{arr2} and \textit{dsh1} mutants and upon overexpression of Sgg (Fig. 4H and 4K). Next, we activated the Wnt pathway. Overexpression of Arr and downregulation of Sgg suppressed Wg accumulation in R8 (Fig. 4I–4K). Collectively, these results suggest that Wg accumulation in R8 is promoted when the divergent canonical Wnt signaling pathway is inhibited.

It is important to note that in the DD group, \textit{arr2} or \textit{dsh1} mutants or Sgg overexpression showed no significant difference in the level of Wg-positive R8 cells (Supplementary Fig. S3A–S3F). This is in contrast to the observations under LL conditions. Thus, light exposure was also required for Wg accumulation, and suppression of the divergent canonical Wnt pathway by itself did not simply trigger the accumulation of Wg. Taken together, our results demonstrate that the Wg signal negatively regulates Wg accumulation under light conditions.

RNAi screening for calcium ion-binding proteins required to trigger Wg endocytosis It is not known how neuronal activity is interpreted within cells prior to endocytosis. Next, we sought to identify components that may bridge the gap between neuronal activity and endocytosis. Calcium ion influx at axon terminals has been shown to trigger various neural activity-dependent events, including fusion and recycling of synaptic vesicles to the membrane (Dolphin and Lee, 2020). This indicates that Wg endocytosis might also be calcium ion influx-dependent. Therefore, we focused on proteins that bind to calcium ions. There are 206 Ca\textsuperscript{2+}-interacting genes in \textit{Drosophila melanogaster}. Among them, RNAi lines for 85 genes whose expression was confirmed in adult eyes (Microarray data from FlyAtlas and RNA-sequence data from modENCODE Anatomy RNA-seq) were specifically expressed in photoreceptors, and their effect on Wg accumulation in LL was analyzed. Knockdown of five genes, namely \textit{no receptor potential A} (\textit{norpA}), \textit{retinal degeneration C} (\textit{rdgC}), \textit{Calbindin 53E} (\textit{Cbp53E}), \textit{Calmodulin} (\textit{Cam}) and \textit{Calcineurin B2} (\textit{CanB2}), was found to significantly reduce Wg accumulation compared to controls (Fig. 5A–5F and 5K). Conversely, knockdown of four genes, \textit{Myosin light chain cytoplasmic} (\textit{Mlc-c}),
Fig. 4. Correlation between Wg accumulation and Wg signaling. (A–B) Representative images of axons showing a punctate brp-short signal (A) and a diffused brp-short signal (B). Arrows in (B) indicate Wg accumulation in the axon. Scale bar, 2 μm. (C) Quantification of the proportion of Wg-positive R8: axons showing a punctate brp-short signal (n = 138 axons/6 brains), and axons showing a diffuse brp-short signal (n = 222/6). *** P < 0.001, chi-squared test. (D) Scheme of the divergent canonical Wnt pathway. (E–J) Wg accumulation in photoreceptors in LL of wild type flies (E), dsh1 mutant flies (F), GMR-Gal4 UAS-sgg (G), futschN94 mutant flies (H), GMR-Gal4 UAS-arr (I) and GMR-Gal4 UAS-sggRNAi (J). Arrows in (E–J) indicate Wg accumulation in photoreceptors. Scale bar, 20 μm. (K) Quantification of the proportion of Wg-positive R8 in LL: control (n = 2597 axons/25 brains), dsh1 mutant (n = 663/6) and futschN94 mutant (n = 805/7); control in LL (n = 521/5) and UAS-sgg (n = 699/7); control in LL (n = 521/5) and UAS-arr (n = 831/8); control in LL (n = 521/5) and UAS-sggRNAi (n = 561/6). *** P < 0.001, chi-squared test.
Activity-dependent endocytosis of Wingless spaghetti squash (sqh), short stop (shot) and Calsyntenin-1 (Cals), was found to increase Wg accumulation compared to controls (Fig. 5G–5K). From this RNAi screen, we conclude that a calcium ion-dependent membrane trafficking signaling pathway is involved in neuronal activity-dependent endocytosis in photoreceptors.

DISCUSSION

In this study, we sought to reveal how synaptic remodeling occurs in a neuronal activity-dependent manner. Specifically, we focused on elucidating how the Wg signal is regulated by neuronal activity. Through our analysis, we identified a molecular mechanism involved
in neuronal activity-related regulation of Wg signaling and a source of Wg that regulates synaptic remodeling in photoreceptors. Surprisingly, we found that neuronal firing not only suppresses Wg expression, but also triggers endocytosis of Wg, which dampens the Wg signal. Taken together, these data suggest that neuronal firing triggers two mechanisms that reduce the amount of extracellular Wg, resulting in decreased Wg signaling and thereby promoting presynaptic remodeling.

Regulation of synaptic remodeling by Wg secreted from glial cells Our data show that the Wg secreted from glial cells regulates synaptic remodeling in R8 photoreceptor cells (Fig. 1). In addition, immunostaining with an anti-Wg antibody showed more glial cell-like staining in DD compared to LL, indicating that the LL condition somehow suppressed Wg expression (Fig. 2A–2B). This indicates the existence of a mechanism in which glial cells recognize neuronal firing of photoreceptors to suppress Wg expression. Indeed, previous studies have shown that glial cells can recognize neuronal firing of photoreceptors by expressing a receptor for histamine, a neurotransmitter of photoreceptors (Gavin et al., 2007; Edwards and Meinertzhagen, 2010). These data suggest that neuronal firing of photoreceptors reduces Wg expression in glial cells, resulting in a decrease of the Wg signal.

Physiological significance of Wg internalization into photoreceptors by endocytosis After being secreted from glial cells, Wg is internalized into photoreceptors by endocytosis only in LL (Fig. 2A–2B). We wanted to determine whether or not Wg internalized by endocytosis carries a Wg signal. Wg has been shown to transmit a Wg signal in endosomes upon binding to a Wg receptor, Frizzled, and the co-receptor, Arrow (Rives et al., 2006). Specifically, Arr is known to be required for the divergent canonical Wnt pathway (Sugie et al., 2015). Since our data show that Arr is not required for Wg endocytosis (Fig. 3E) and that AZ remodeling mainly occurs in Wg-positive R8 (Fig. 4C), it is likely that Wg endocytosis does not transmit the Wg signal. Furthermore, knockdown of autophagy-related genes increased Wg accumulation (Fig. 3J), indicating that Wg follows the autophagic degradation pathway after endocytosis. In the Drosophila embryonic epidermis, endocytosis and degradation of Wg is required to limit Wg distribution. Inhibition of this pathway has been shown to cause extensive developmental impairment due to excessive Wg signaling (Dubois et al., 2001). This suggests that this type of regulatory mechanism could also be involved in the visual system. That is, Wg could be internalized by endocytosis and degraded, reducing the amount of extracellular Wg and thereby reducing the Wg signal and causing presynaptic remodeling. Consistent with this idea, decreasing the Wg signal by genetic manipulation was found to increase Wg accumulation in the LL group (Fig. 4K). Given that increased Wg accumulation might reflect either decreased Wg clearance or increased Wg endocytosis, Wg signaling should negatively regulate one or both of these processes. In the former case, an endocytosis-related decrease in Wg signaling suppresses Wg clearance; thus, Wg accumulates in the cell without being degraded. In the latter case, an endocytosis-related decrease in Wg signaling promotes Wg endocytosis, so the Wg signal is further decreased. This would cause a negative feedback loop that enables cells to tip the balance of Wg signaling toward “decrease” once the activation of Wg endocytosis and Wg signaling exceeds a certain threshold. Although these two scenarios are possible, we favor the latter, because presynaptic remodeling, which requires at least 24 h (Sugie et al., 2015), is a phenomenon that requires a threshold. Thus, it is likely that there is a bistable loop due to the feedback loop. This feedback loop may explain why only 20% of R8 cells have Wg accumulation in wild type LL condition, i.e., only 20% of R8 cells exceed the postulated threshold. Collectively, our data demonstrate a novel model of synaptic plasticity, in which regulators of synaptic plasticity are further regulated by neuronal activity-dependent endocytosis. Moreover, Wg signaling is likely regulated by a negative feedback loop driven by Wg endocytosis.

Feedback signal from postsynaptic cells We suggested previously that feedback regulation from postsynaptic cells is necessary for presynaptic remodeling (Sugie et al., 2015). In that study, forced activation by TrpA1 in postsynaptic neurons resulted in the suppression of synaptic remodeling in the LL condition. These results suggested that a type of feedback signal emanates from the postsynaptic cells to effect synaptic remodeling in the presynaptic photoreceptor neuron. However, in the current study, we found that Wg accumulation did not depend on activity of postsynaptic cells (Supplementary Fig. S2), suggesting that Wg endocytosis is a cell-autonomous event. Given that Wg signaling affects Wg accumulation, the strength of the Wg signal should also be independent of activity of postsynaptic cells. This would indicate that the feedback signal from postsynaptic cells that regulates presynaptic remodeling is something other than Wg signaling in this particular case. The question then is: what are the signaling molecules, other than Wg, that come from the postsynaptic cells? Since multiple genes that control synaptic plasticity in Drosophila photoreceptors have been identified (Araki et al., 2020), it is highly suggestive that there are multiple signals that control presynaptic remodeling in addition to Wg signals. Thus, feedback regulation from postsynaptic cells is likely an independent signal that suppresses remodeling differently from Wg signaling.
The molecular machinery of Wg endocytosis

Through RNAi screening of Ca\(^{2+}\)-binding proteins in photoreceptors, we found five genes that were required for Wg accumulation: no receptor potential A (norpA), retinal degeneration C (rdgC), Calbindin 53E (Cbp53E), Calmodulin (Cam) and Calcineurin B2 (CanB2). One of the genes was norpA, which is consistent with results in norpA mutant flies (Fig. 2M) and supports the validity of the screening. RdgC is a Ser-Thr protein phosphatase, which mediates rhodopsin phosphorylation (Strauch et al., 2018). The loss of RdgC causes retinal degeneration (Vinos et al., 1997; Kiselev et al., 2000). Cbp53E is predicted to act as a calcium buffer and calcium sensor, and the loss of this protein results in abnormal axonal growth (Hagel et al., 2015). Since these two genes are involved in neuronal development, the suppression of Wg accumulation observed when these genes are knocked down in RNAl lines is likely due to developmental effects. CanB2 is a regulatory subunit of Calcineurin (CaN), a Ca\(^{2+}\) calmodulin (Cam: also a hit of the RNAi screen)-activated Ser-Thr protein phosphatase that is essential for the translation of Ca\(^{2+}\) signals into changes in cell function and development (Tarasova et al., 2018). Recent studies have revealed that the classic substrates of CaN are dephosphins (so named because they are dephosphorylated by CaN) that include dynamins 1 and 3, adaptor protein AP180, lipid phosphatase synaptojanin, and amphiphysin, among others (Sun et al., 2010; Liang et al., 2017). Dephosphorylation of these proteins by CaN promotes their assembly into multimolecular complexes involved in the multistage process of vesicle detachment from the presynaptic membrane. These data indicate that CaN activated by Ca\(^{2+}\) influx triggers Wg endocytosis.

In contrast, RNAi of four other genes resulted in increased Wg accumulation: Myosin light chain cytoplasmic (Mlc-c), spaghetti squash (sqh), short stop (shot) and Calsyntenin-1 (Cals). Mlc-c encodes two essential light chains of non-muscle myosin II (NMII), and sqh encodes two regulatory light chains of NMII (Franke et al., 2010). NMII has many functions including vesicle transportation (Vasquez et al., 2016). Recent studies have shown that NMII is localized in proximity to autophagosomal vesicles (Moura et al., 2018). The gene shot encodes a member of the spectraplakin family of large cytoskeletal linker molecules that are necessary for microtubule organization, extension of axons to target muscles, and vesicle trafficking (Kinoshita et al., 2017). Cals is known to dock vesicular cargo to kinesin-1 (Koncna et al., 2006). Since all of these genes are involved in vesicle transportation, it is likely that they are required for Wg clearance, indicating that Ca\(^{2+}\) influx triggers Wg uptake and clearance, which may cause a decrease in extracellular Wg.

There is also a possibility that the Wnt/Ca\(^{2+}\) signaling pathway affects the accumulation of Wg in R8. The Wnt/Ca\(^{2+}\) signaling pathway is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activate the kinase PKC (protein kinase C), CAMKII (Ca\(^{2+}\)/calmodulin-dependent protein kinase II) and CaN (Kohn and Moon, 2005). It is therefore conceivable that the Wnt/Ca\(^{2+}\) pathway triggers calcium elevation in R8, which induces the activation of CaN. However, from our observations that silenced photoreceptors showed lower Wg accumulation even in the LL condition (Fig. 2H–2I and 2M), and that the divergent canonical Wnt pathway, which regulates Futsch phosphorylation, affects Wg accumulation (Fig. 4D–4K), we favor the hypothesis that the trigger of CaN activation is due to calcium influx by neuronal firing.

In conclusion, our data demonstrate that neuronal activity is transmitted via calcium signaling to induce Wg endocytosis, which results in a decrease in the Wg signal, and thereby promotes presynaptic remodeling. Moreover, our data lead us to propose that Wg signaling is regulated by a negative feedback loop driven by Wg endocytosis.

We gratefully acknowledge Dr. Jean-Paul Vincent for providing the endogenous-wg-regulatory-region-FRT-wg(cDNA)-FRT-QF flies. We thank the Bloomington Drosophila Stock Center and the Vienna Drosophila Resource Center for RNAi fly stocks, and the Developmental Studies Hybridoma Bank for providing antibody stocks. We thank Enago (www.enago.jp) for the English language review. This work was supported by JSPS KAKENHI Grant Number 18J22499 (H. K.), JSPS KAKENHI Grant Number 18K06250 (S. H.-S.), a Grant-in-Aid for Scientific Research on Innovation Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan 16H06457 “Dynamic regulation of Brain Function by Scrap & Build System” (T. S.) and a Takeda Visionary Research Grant from the Takeda Science Foundation (T. S.).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

Alabi, A. A., and Tsien, R. W. (2012) Synaptic vesicle pools and dynamics. Cold Spring Harb. Perspect. Biol. 4, a013680.

Araki, T., Osaka, J., Kato, Y., Shimozono, M., Kawamura, H., Iwanaga, R., Hakeda-Suzuki, S., and Suzuki, T. (2020) Systematic identification of genes regulating synaptic remodeling in the Drosophila visual system. Genes Genet. Syst. 95, 101–110.

Baena-Lopez, L. A., Alexandre, C., Mitchell, A., Pasakarnis, L., and Vincent, J.-P. (2013) Accelerated homologous recombination and subsequent genome modification in Drosophila. Development 140, 4818–4825.

Berger, J., Senti, K.-A., Senti, G., Newsome, T. P., Asling, B., Dickson, B. J., and Suzuki, T. (2008) Systematic identification of genes that regulate neuronal wiring in the Drosophila visual system. PLoS Genet. 4, e1000085.
Bloomquist, B. T., Shortridge, R. D., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin, G., and Pak, W. L. (1988) Isolation of a putative phospholipase C gene of Drosophila, norpA, and its role in phototransduction. Cell 54, 723–733.

Davydova, D., Marini, C., King, C., Klueva, J., Bischof, F., Romorini, S., Montenegro-Vanegas, C., Heine, M., Schneider, R., Schroder, M. S., et al. (2014) Bassoon specifically controls presynaptic P/Q-type Ca2+ channels via RIM-binding protein. Neuron 82, 181–194.

Dolphin, A., and Lee, A. (2020) Presynaptic calcium channels: specialized control of synaptic neurotransmitter release. Nat. Rev. Neurosci. 21, 213–229.

Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E., and Vincent, J. P. (2001) Regulated endocytic routing modulates wingless signaling in Drosophila embryos. Cell 105, 613–624.

Edwards, T. N., and Meinertzhagen, I. A. (2010) The functional organisation of glia in the adult brain of Drosophila and other insects. Prog. Neurobiol. 90, 471–497.

Franke, J. D., Montague, R. A., and Kiehart, D. P. (2010) Non-muscle myosin II is required for cell proliferation, cell sheet adhesion and wing hair morphology during wing morphogenesis. Dev. Biol. 345, 117–132.

Gao, S., Takemura, S.-y., Ting, C.-Y., Huang, S., Lu, Z., Luan, H., Rister, J., Thum, A. S., Yang, M., Hong, S.-T., et al. (2008) The neural substrate of spectral preference in Drosophila. Neuron 60, 328–342.

Gavin, B. A., Arruda, S. E., and Dolph, P. J. (2007) The role of carcinoine in signaling at the Drosophila photoreceptor synapse. PloS Genet. 3, e206.

Gisselmann, G., Pasch, H., Hovemann, B. T., and Hatt, H. (2002) Two cDNAs coding for histamine-gated ion channels in D. melanogaster. Nat. Neurosci. 5, 11–12.

Gögel, S., Wakefield, S., Tear, G., Klämbt, C., and Gordon-Weeks, R. (2017) Tpr is phosphorylated by Shaggy/Zeste-white 3 at an homologous GSK3β phosphorylation site in MAP1B. Mol. Cell. Neurosci. 33, 188–199.

Gordon, M. D., and Nusse, R. (2006) Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J. Biol. Chem. 281, 22429–22433.

Hagel, K. R., Beriont, J., and Tessier, C. R. (2015) Drosophila Cbp53E regulates axon growth at the neuromuscular junction. PloS One 10, e0132636.

Hakeda-Suzuki, S., Takechi, H., Kawamura, H., and Suzuki, T. (2017) Two receptor tyrosine phosphatases dictate the depth of axonal stabilizing layer in the visual system. eLife 6, e31812.

Hardie, R. C., Raghu, P., Moore, S., Juusola, M., Baines, R. A., and Sweeney, S. T. (2001) Calcium influx via TRP channels is required to maintain PIP2 levels in Drosophila photoreceptors. Neuron 30, 149–159.

He, X., Semenov, M., Tamai, K., and Zeng, X. (2004) LDL receptor-related proteins 5 and 6 in Wnt/b-catenin signaling: arrows point the way. Development 131, 1663–1677.

Hong, S.-T., Bang, S., Paik, D., Kang, J., Hwang, S., Jeon, K., Chun, B., Hyun, S., Lee, Y., and Kim, J. (2006) Histamine and its receptors modulate temperature-preference behaviors in Drosophila. J. Neurosci. 26, 7245–7256.

Iovchev, M., Kodrov, P., Wolstenholme, A. J., Pak, W. L., and Semenov, E. P. (2002) Altered drug resistance and recovery from paralysis in Drosophila melanogaster with a deficient histamine-gated chloride channel. J. Neurogenet. 16, 249–261.

Kinoshita, T., Sato, C., Fuwa, T. J., and Nishihara, S. (2017) Short stop mediates axonal compartmentalization of mucin-type core 1 glycans. Sci. Rep. 7, 41455.

Kiselev, A., Sooolich, M., Vinós, J., Hardy, R. W., Zuker, C. S., and Ranganathan, R. (2000) A molecular pathway for light-dependent photoreceptor apoptosis in Drosophila. Neuron 28, 139–152.

Kittel, R. J., and Heckmann, M. (2016) Synaptic vesicle proteins and active zone plasticity. Front. Synaptic Neurosci. 8, 8.

Kohn, A. D., and Moon, R. T. (2005) Wnt and calcium signaling: β-catenin-independent pathways. Cell Calcium 38, 439–446.

Koles, K., and Budnik, V. (2012) Wnt signaling in neuromuscular junction development. Cold Spring Harb. Perspect. Biol. 4, a008045.

Koncina, A., Frischknecht, R., Kinter, J., Ludwig, A., Steuble, M., Meskenaite, V., Indermühle, M., Engel, M., Cen, C., Mateos, J. M., et al. (2006) Calsyntenin-1 docks vesicular cargo to kinesin-1. Mol. Biol. Cell 17, 3651–3663.

Lazarevic, V., Schöne, C., Heine, M., Gundelfinger, E. D., and Fejtova, A. (2011) Extensive remodeling of the presynaptic cytomatrix upon homeostatic adaptation to network activity silencing. J. Neurosci. 31, 10189–10200.

Li, X., Wei, L., and Chen, L. (2017) Exocytosis, endocytosis, and their coupling in excitable cells. Front. Mol. Neurosci. 10, 109.

Liu, W. W., and Wilson, R. I. (2013) Transient and specific inactivation of Drosophila neurons in vivo using a native ligand-gated ion channel. Curr. Biol. 23, 1202–1208.

Maxfield, F. R., and McGraw, T. E. (2004) Endocytic recycling. Nat. Rev. Mol. Cell Biol. 5, 121–132.

McBride, H. M., Rybin, V., Murphy, C., Giner, A., Teasdale, R., and Zerial, M. (1999) Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell 98, 377–386.

Mellman, I. (1996) Endocytosis and molecular sorting. Annu. Rev. Cell Dev Biol. 12, 575–625.

Midorikawa, R., Yamamoto-Hino, M., Awano, W., Hinohara, Y., Suzuki, E., Ueda, R., and Goto, S. (2010) Autophagy-dependent rhodopsin degradation prevents retinal degeneration in Drosophila. J. Neurosci. 30, 10703–10719.

Moura, P. L., Hawley, B. R., Manekelow, T. J., Griffiths, R. E., Dobbe, J. G. G., Striebeck, G. J. A., Anstee, D. J., Satchwell, T. J., and Toye, A. M. (2018) Non-muscle myosin II drives vesicle loss during human reticulocyte maturation. Haematologica 103, 1997–2007.

Nakatogawa, H., Ohbayashi, S., Sakoh-Nakatogawa, M., Kakuta, S., Suzuki, S. W., Kirisako, H., Kondo-Kakuta, C., Noda, N. N., Yamamoto, H., and Ohsumi, Y. (2012) The autophagy-related protein kinase Atg1 interacts with the ubiquitin-like protein Atg8 via the Atg8 family interacting motif to facilitate autophagosome formation. J. Biol. Chem. 287, 28503–28507.

Owald, D., Fouquet, W., Schmidt, M., Wichmann, C., Mertel, S., Depner, H., Christiansen, F., Zube, C., Quentin, C., Körner, J., et al. (2010) A Syd-1 homologue regulates pre- and postsynaptic maturation in Drosophila. J. Cell Biol. 188, 565–579.

Ribabinina, O., and Potter, C. J. (2016) The Q-system: a versatile expression system for Drosophila. Methods Mol. Biol. 1479, 59–78.

Rives, A. F., Rochlin, K. M., Wehrli, M., Schwartz, S. L., and DiNardo, S. (2006) Endocytic trafficking of Wingless and its receptors, Arrow and Dfzrizzled-2, in the Drosophila wing. Dev. Biol. 293, 268–283.

Schoe, S., and Gundelfinger, E. D. (2006) Molecular organization of the presynaptic active zone. Cell Tissue Res. 326,
Activity-dependent endocytosis of Wingless

379–391.
Seto, E. S., Bellen, H. J., and Lloyd, T. E. (2002) When cell biology meets development: endocytic regulation of signaling pathways. Genes Dev. 16, 1314–1336.
Strauch, L., Pfannstiel, J., Huber, A., and Voolstra, O. (2018) Solubility and subcellular localization of the three Drosophila RDGC phosphatase variants are determined by acylation. FEBS Lett. 592, 2403–2413.
Südhof, T. C. (2012) The presynaptic active zone. Neuron 75, 11–25.
Sugie, A., Hakeda-Suzuki, S., Suzuki, E., Silies, M., Shimozono, M., Möhl, C., Suzuki, T., and Tavosanis, G. (2015) Molecular remodeling of the presynaptic active zone of Drosophila photoreceptors via activity-dependent feedback. Neuron 86, 711–725.
Sun, T., Wu, X.-S., Xu, J., McNeil, B. D., Pang, Z. P., Yang, W., Bai, L., Qadri, S., Molkentin, J. D., Yue, D. T., et al. (2010) The role of calcium/calmodulin-activated calcineurin in rapid and slow endocytosis at central synapses. J. Neurosci. 30, 11838–11847.
Tarasova, E. O., Gaydukov, A. E., and Balezina, O. P. (2018) Calcineurin and its role in synaptic transmission. Biochemistry (Mosc) 83, 674–689.
Vasquez, C. G., Heissler, S. M., Billington, N., Sellers, J. R., and Martin, A. C. (2016) Drosophila non-muscle myosin II motor activity determines the rate of tissue folding. eLife 5, e20828.
Vinós, J., Jalink, K., Hardy, R. W., Britt, S. G., and Zuker, C. S. (1997) A G protein-coupled receptor phosphatase required for rhodopsin function. Science 277, 687–690.
Weiss, S., and Minke, B. (2015) A new genetic model for calcium induced autophagy and ER-stress in Drosophila photoreceptor cells. Channels (Austin) 9, 14–20.