Systematic identification of genes regulating synaptic remodeling in the *Drosophila* visual system

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In many animals, neural activity contributes to the adaptive refinement of synaptic properties, such as firing frequency and the number of synapses, for learning, memorizing and adapting for survival. However, the molecular mechanisms underlying such activity-dependent synaptic remodeling remain largely unknown. In the synapses of *Drosophila melanogaster*, the presynaptic active zone (AZ) forms a T-shaped presynaptic density comprising AZ proteins, including Bruchpilot (Brp). In a previous study, we found that the signal from a fusion protein molecular marker consisting of Brp and mCherry becomes diffuse under continuous light over three days (LL), reflecting disassembly of the AZ, while remaining punctate under continuous darkness. To identify the molecular players controlling this synaptic remodeling, we used the fusion protein molecular marker and performed RNAi screening against 208 neuron-related transmembrane genes that are highly expressed in the *Drosophila* visual system. Second analyses using the STaR (synaptic tagging with recombination) technique, which showed a decrease in synapse number under the LL condition, and subsequent mutant and overexpression analysis confirmed that five genes are involved in the activity-dependent AZ disassembly. This work demonstrates the feasibility of identifying genes involved in activity-dependent synaptic remodeling in *Drosophila*, and also provides unexpected insight into the molecular mechanisms involved in cholesterol metabolism and biosynthesis of the insect molting hormone ecdysone.

Key words: active zone, *Drosophila* visual system, synaptic remodeling

INTRODUCTION

The nervous system can innately adapt to external stimuli, and activity-dependent neuronal plasticity is the key mechanism underlying this adaptation. Although activity-dependent neuronal plasticity is broadly accepted as far as mammals and other major vertebrates are concerned, few examples are known in the nervous system of *Drosophila*. One of the few recently discovered instances was found in the olfactory sensory system of *Drosophila*, where prolonged exposure to CO₂ induced a reversible change in the size of glomeruli to control CO₂ sensing and the subsequent behavioral response to CO₂ (Sachse et al., 2007). Such activity-dependent phenomena derive from physical and physiological synaptic changes based on neural activities, such as the physical increase or decrease in the size of synaptic connections and the physiological modulation of signal transduction efficiency through synapses. These types of synaptic regulation usually occur via both presynaptic and postsynaptic neurons. In presynaptic neurons, the frequency and amount of neurotransmitter release from the active zone (AZ) can be adjusted. The AZ is a protein complex composed of a set of evolutionarily conserved proteins (Südhof, 2012). In *Drosophila* the AZ consists of at least five proteins: Bruchpilot (Brp), RIM-binding protein (DRBP), DSyd-1, DLiprin-α, and Cacophony (Cac) (Wagh et al., 2006; Sugie et al., 2015). Each AZ component protein is closely involved in synaptic vesicle release; for example, Brp physically interacts with synaptic vesicles before their release (Fouquet et al., 2009). The major molecular mechanisms underlying the adjustment of synaptic vesicle release are broadly accepted to be the molecular reorganization of the AZ components upon 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 shown previously that activity-dependent presynaptic AZ remodeling does indeed occur (Sugie et al., 2015). The *Drosophila* visual system is composed of the retina and optic lobe, which includes 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. Molecular remodeling of photoreceptor AZs takes place after a mild but prolonged exposure to light. The AZ number in *Drosophila* photoreceptor cells was quantified using a Brp fusion protein as a marker. Since Brp is one of the indispensable components of AZ for synapse function, AZ number counted by the Brp marker corresponds to functional synapse number. A 72-h dark (DD), 72-h light (LL) and 72-h light–dark cycle (LD; normal condition) assessment shows that the AZ number in R8 decreases by 10–20% under LL compared with that under LD or DD. This shows that the AZ number in R8 decreases by 10–20% under LL compared with that under DD or LD. This synaptic number change is reversible: when flies conditioned to LL were transferred to DD, the AZ number recovered to the DD level. However, this phenomenon did not involve all the AZ components. Under LL, Brp, DLiprin-α and DRBP dissociated from the AZ and dispersed, while DSyd-1 and Cac remained associated with the AZ. Furthermore, suppression of postsynaptic histamine receptor-positive neuron activity controls the AZ component delocalization. Thus, AZ molecular composition can be modified in *vivo*, and activity-dependent feedback signaling mediates this modification (Sugie et al., 2015). In other nervous systems, such as in larval motorneuron development, Wingless–Frizzled2 and BMP–Gbb interactions function at the synaptic cleft in *trans* at the larval neuromuscular junction. Such interactions regulate synapse number and morphology, neurotransmitter quantity, and presynaptic AZ structure (Packard et al., 2002; Ataman et al., 2008). Considering the findings of such studies, we hypothesized that certain molecules must exist that mediate intercellular communication to transduce disassembly signals to AZs.

Here, we screened 208 cell surface and secreted molecules (Kurusu et al., 2008) using RNAi to identify proteins related to AZ disassembly in *Drosophila* photoreceptor axons. Our subsequent analyses confirmed that five genes are involved in activity-dependent AZ disassembly.

**MATERIALS AND METHODS**

**RNAi screening** For RNAi screening, genes were initially selected using the *Drosophila* cell surface and secreted protein database (Kurusu et al., 2008). Subsequently, we verified their expression through FlyBase, using the following data set categories:

1. Central nervous system, pupae P8 (from RNA-seq)
2. Head, virgin one-day-old adult female (from RNA-seq)
3. Adult brain (from Microarray)

The 976 genes initially selected were sorted in the order of their expression level. The 205 genes with scores above 36 in the central nervous system at pupae P8 in RNA-seq data (modENCODE Anatomy RNA-seq) and 27 genes with scores above 500 in the adult brain in Microarray data (FlyAtlas Anatomy Microarray) were selected. After attempting to order UAS-RNAi lines of these 232 genes from the Bloomington *Drosophila* Stock Center at Indiana University and the Vienna *Drosophila* Resource Center, we were informed that 24 genes were unavailable. Therefore, 208 genes were selected for analyses using RNAi (Supplementary Table S1).

**Light exposure** From the larval stage to hatching, the flies were kept in columned vials or in bottles with medium at room temperature (RT; 25 °C), 50–60% humidity, with a 12-h LD cycle. For the experimental period, the flies were exposed to DD, LD or LL at RT. The intensity of illumination from LED panels (LEDXC170-W, MISUMI) was set at about 1,500 lux and 1,000 lux for R7 and R8 experiments, respectively.

**Dissection and immunohistochemistry** The experimental procedures for adult brain dissection, fixation and immunostaining were performed as described previously (Hakeda-Suzuki et al., 2011). Briefly, adult fly brains were dissected in 0.1% PBT (PBS containing Triton X-100) and then fixed with 4% paraformaldehyde at RT for 60 min. Primary antibody was added and incubated overnight or for up to 1 week at 4 °C on a shaker; secondary antibody was added and incubated for 1 h at RT. The following antibodies were used for immunohistochemistry: anti-V5 antibody (1:400, Invitrogen), GFP Tag Polyclonal Antibody Alexa Fluor 488 (Life Technologies 1:400, 1 h). The secondary antibodies were Alexa488 or Alexa568 (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 0.5 μm. Each image was acquired twice and averaged. Images were processed with NIS-elements AR. The rate of diffuse Brp[short]-mCherry signals was quantified with NIS-Elements AR Analysis by dividing the number of axons with diffuse signals by the total axon number. The number of synapses was counted with Imaris (Bitplane) by adding spots to signals with a diameter larger than 0.35 μm.

**Drosophila strains and genetics** Mutant alleles were generated by injecting a plasmid expressing a designed gRNA. A targetable 20-bp sequence was identified and
two complementary 24-bp oligonucleotides were designed and annealed to generate a double-strand DNA (Supplementary Fig. S1). These were cloned into BbsI-digested pBFv-U6.2 (Kondo and Ueda, 2013). Plasmid injection into fly eggs with Cas9 was done by BestGene or Well-Genetics. The candidates for knock-out alleles were verified by PCR (Supplementary Fig. S1). NPF mutants were kind gifts from Dr. Shu Kondo (National Institute of Genetics). RNAi fly stocks for the screening were obtained from Vienna Drosophila Resource Center or Bloomington Drosophila Stock Center. The stock numbers are listed in Supplementary Table S1. The following stocks used in this study are available at the Bloomington Drosophila Stock Center: Brp>stop>GFp (Brp-FSF-GFP) (#55753), ey3.5FLP (#33542), AyGal4 (#4413), UAS-mCD8GFp (#5136), Npc2a[376] (#41760), Df(2L)Exel6006 (#8000), CG34370[Ml09268] (#52089), Df(2R)B5CS62 (#27374), ob-st-b[EY09474] (#19774), UAS-Npc2a (#50764), CG7607[EP3548] (#19629). CG34370[d00934] was obtained from Harvard Medical School (d00934) and UAS-beat-VI was obtained from FlyORF (F002906). The detailed genotypes are listed in Supplementary Table S2.

RESULTS

Genetic screening of genes required for synaptic plasticity To identify molecules required for stabilization of the presynaptic AZ, we performed genetic screening. We expressed LexAop-Brp[short]-mCherry specifically in R7 axons (Rh4-LexA) or R8 axons (Rh6-LexA). Under LL for three days, Brp[short]-mCherry puncta diffused in both R7 and R8 axons, while the marker localized at synapses as puncta under DD (Fig. 1A–1C). This behavior was inferred as activity-dependent remodeling of AZ component proteins (Sugie et al., 2015). Because the localization pattern of Brp[short]-mCherry in LL and DD was easy to distinguish, we designated it as an indicator of synaptic disassembly for the screening. Under LL, white LED light (wavelength, 300–780 nm) was used so that both R7 (absorption wavelength, 300–400 nm) and R8 (absorption wavelength, 450–600 nm) could be activated properly.

Since proteins related to synaptic disassembly can be assumed to work through intercellular communication, we selected transmembrane or secreted molecules as first targets. Although there are more than 3,000 secreted or transmembrane gene products in Drosophila, the number of such proteins having putative neuron-related domains is limited to 976 (Kurusu et al., 2008). Among these, we analyzed 208 genes showing high expression in late-stage pupae or in adult heads based on information in FlyBase. Using RNAi lines to knock down these genes specifically in photoreceptors, we identified ten genes influencing synaptic stability. These genes were further tested by overexpressing them and generating mutant strains. Figure 1D shows the workflow of the screening procedure.

Twenty-seven genes selected from R7 screening The RNAI lines of 208 candidate genes were obtained from stock centers (Supplementary Table S1). Each UAS-RNAi line was crossed with ey3.5FLP, AyGal4, which induces high expression of RNAi specifically in photoreceptor cells from early developmental stages. Adult female virgins obtained from the cross were collected and exposed to LL for three days. First, we checked the synapses in R7 axons, whose localization is restricted to between M3 and M6 layers in the medulla (Shimozono et al., 2019), so that we could also analyze the change of synapse distribution simultaneously. To visualize only the synapses in R7, Rh4-LexA and LexAop-Brp[short]-mCherry (Berger-Müller et al., 2013) were used as synaptic markers. After three days of LL exposure, the heads were dissected and R7 synapses labeled by Brp[short]-mCherry in the medulla were imaged using confocal laser microscopy. Since six lines showed undesirable RNAi phenotypes, such as abnormal axon projection, compound eye disorientation and lethality, we did not analyze them further. For the remaining 202 lines, each axon from the collected images was manually classified into one of two groups: diffuse signals of Brp, which could not be recognized as clear individual puncta, or non-diffuse signals. The rate of axons with diffuse signals was then calculated as the number of axons with diffuse signals divided by the total number of axons (Fig. 2A). The rate of axons with diffuse signals for the control flies was 0.15 in DD and 0.85 in LL. For the screened genes, the rate of axons with diffuse signals ranged from 0.232 to 1.00, and 27 genes with values < 0.500 were selected for secondary screening (Fig. 2).

Ten genes are required for synaptic plasticity in R8 axons The 27 genes were analyzed in R8 using Rh6-LexA and LexAop-Brp[short]-mCherry to specifically label R8 synapses, distributed between M1 and M3 layers in the medulla. Although the diffuse signals of Brp[short]-mCherry in R8 were easier to define than those in R7, R8 synapses were more difficult to judge regarding the change in synapse distribution. The analyses were the same as for R7, except for the synaptic marker. The rate of R8 axons with diffuse signals in the control fly was 0.065 in DD and 0.65 in LL, whereas the rate for the screened genes spread from 0.24 to 0.96 (Fig. 3A). This indicates that around half of the genes did not show as strong a suppression of the diffuse signals in R8 as in R7.

Although Brp[short]-mCherry was convenient for initial screening, the diffuse signals may be a secondary consequence of synaptic remodeling, due for example to the lack of domains responsible for the degradation machinery. Therefore, we needed a more detailed and
direct synaptic marker to confirm that these genes indeed control synaptic numbers. Previously, it was shown that the diffuse signals of Brp[short]-mCherry coincide with the mild decrease in the Brp puncta numbers, which we regarded as AZ disassembly (Sugie et al., 2015). The STaR method is well established for visualizing presynaptic AZ structure in Drosophila neurons (Chen et al., 2014). This method visualizes the presynaptic AZ as a clear single punctum by labeling with semi-endogenous Brp-GFP fusion protein. Because the STaR construct

Fig. 1. Strategy of the screen. (A) Illustration of the optic lobe composed of the lamina, medulla and lobula complex. Among the eight photoreceptors, R7 and R8 project axons to the medulla and form synapses with secondary neurons. (B) Representative images of the control optic lobes. Brp[short]-mCherry was expressed in either R7 axons (Rh4-LexA) or R8 axons (Rh6-LexA). The flies were kept under 72-h dark (DD) or 72-h light conditions (LL) as indicated on each panel. Magnified images of a representative single axon are shown on the right side of each panel. (C) Schematic drawing of Brp[short]-mCherry patterns in the images in (B). In DD/LD conditions, Brp[short]-mCherry expression can be recognized as puncta, whereas in LL conditions the Brp[short]-mCherry signal is diffuse. (D) The flow of the screening, showing each step.
Genes required for synaptic remodeling

Genes insertion has a stop codon flanked by two FRT sites between Brp and GFP, it enables cell type-specific labeling of Brp proteins by expressing FLPase in a cell-specific manner. STaR puncta decrease in the light exposure condition (Sugie et al., 2015). By expressing FLPase specifically in photoreceptors using the eyeless-3.5 promoter, we visualized synapses in both photoreceptor R7 and R8 simultaneously. Photoreceptor R7 forms synapses on the M4–M6 layer in the medulla while R8 forms them on the M1–M3 layer; these characteristics enabled us to delineate R7 and R8 synapses and quantify them on their own. We conducted RNAi screening again for the selected 27 genes and quantified the number of synapses in R7 and R8 under LL. Since R7 synapses do not decrease in number upon prolonged light stimulation, we solely relied on the R8 phenotype. Twelve RNAi lines displayed a phenotype of suppressing the decrease of Brp puncta, judged by the overall quantification of the R8 puncta (Fig. 3B). Together with the results from Brp[short] diffuse signals and synaptic number decrease determined by the STaR system, we finally narrowed down the candidates to ten genes, which may be related to synapse disassembly (Fig. 3C–3E).

Synaptic phenotypes of knock-outs Utilizing RNAi lines, we selected 10 genes that may be involved in synaptic remodeling. However, RNAi is subject to off-target effects. To overcome this problem, we tried to knock out the genes to confirm their function accurately. By expressing guide RNAs (gRNAs) with Cas9, we deleted nucleotides around each gRNA's targeting area (Supplementary Fig. S1). These deletions often drive frame-shift mutations (Kondo and Ueda, 2013). Besides the three genes whose mutant alleles were available at the

Fig. 2. Suppression of diffuse signals by RNAi knock-down in R7 axons. (A) For 208 genes, the UAS-RNAi line was crossed with ey3.5FLP, AyGal4, which induces high expression in photoreceptor cells from an early developmental stage. Except for six genes that showed undesirable phenotypes, the synapses of R7 axons were visualized with Rh4-LexA and LexAop-Brp[short]-mCherry. After three days under LL, images were collected and each axon was manually classified into one of two groups, diffuse or non-diffuse. The rate of axons with diffuse signals was then calculated as the number of axons with diffuse signals/total number of axons. Black bars indicate the 27 genes whose rate was below 0.5. (B) R7 axons from wild type flies reared under DD or LL. (C–L) Representative images of R7 axons analyzed in (A) that showed marked suppression of the diffuse signals. The genes are identified under each panel. Scale bar, 5 μm.
stock centers, we newly generated mutant alleles for the remaining seven genes (Supplementary Fig. S1) and conducted mutant analysis with the STaR system. We utilized two STaR alleles, Brp-FSF-GFP and Brp-FSF-V5, separately, according to the chromosomal location of the gene of interest. As the number of synaptic puncta was slightly different for Brp-FSF-V5 and Brp-FSF-GFP in the controls, we compared synaptic number separately for each marker. We counted the synaptic number on R8 photoreceptors under DD and LL for each mutant. Six knock-out mutants showed the phenotype of suppressed synaptic remodeling (Fig. 4), indicating that these genes are necessary for the disassembly of AZ components. Since CG34370 showed a decrease in the number of synapses under DD, this gene may be involved in synaptic formation.

**Five genes are necessary and sufficient for synaptic remodeling** From the results of RNAi screening and mutant analyses, six genes were identified for their enhancing effects in synaptic remodeling. We next examined whether each gene is sufficient for synaptic remodeling by overexpression. We obtained the UAS lines from stock centers, except for ImpE1, which was not available. Thus, five genes were analyzed under DD and LD as overexpressing UAS lines using ey3.5-Gal4 (Hazelett et al., 1998) and GMR-Gal4, simultaneously. In the control animals, there was little difference in the number of puncta between DD and LD, while the number decreased significantly in flies overexpressing beat-VI, Niemann–Pick type C-2a (Npc2a), obstructor-B (obst-b), CG34370 and CG7607 (Fig. 5). Thus, each of these five genes is necessary and sufficient for synaptic remodeling and activity-dependent synaptic plasticity.
Genes required for synaptic remodeling

Fig. 4. Knock-out phenotype of the ten candidate genes. (A–B) Knock-out phenotypes of the ten candidate genes were analyzed using the STaR system in R8 axons. The synapse number was compared between DD and LL conditions in each mutant using Brp-FSF-GFP (A) or Brp-FSF-V5 (B) as a marker depending on the location of the gene of interest. Six mutants did not show a significant difference in synapse number between DD and LL (n.s. $P > 0.05$, *$P < 0.05$, Student’s $t$-test), indicating that these genes are necessary for disassembly of AZ components. (C) Representative images of R8 axons analyzed using Brp-FSF-GFP or Brp-FSF-V5 for the genes that showed the phenotype. Scale bar, 2 μm.

**DISCUSSION**

**Genes required for activity-dependent synaptic plasticity** In *Drosophila*, the AZ components of synapses in photoreceptor axons are remodeled by prolonged exposure to light. This remodeling occurs as a consequence of the cessation of the feedback signal from secondary neurons caused by continuous hyperpolarization via the histamine chloride channel (Sugie et al., 2015). This feedback signal is mediated by the divergent canonical Wnt pathway. However, other molecules seem also to be involved in this remodeling, since synaptic plasticity is regulated in highly sophisticated and redundant pathways.

Although a powerful visualization system for synapses in *Drosophila* has been established (Chen et al., 2014), counting each synapse requires substantial work. Therefore, a simpler and faster analysis is needed to identify synaptic remodeling molecules expressed by various genes. We attempted to utilize the signals of Brp[short]-mCherry fusion protein, which became diffuse as a consequence of the molecule dissociating from the AZ (Sugie et al., 2015). Counting the number of axons with diffuse Brp-mCherry signals is easier and faster than counting the number of synapses on each axon. With screening using a combined method of STaR and Brp[short]-mCherry, we identified six genes involved in synaptic plasticity: beat-VI, Niemann–Pick type C-2a (Npc2a), obstructor-B (obst-b), CG7607, ecdysone-inducible gene E1 (ImpE1) and CG34370. To our knowledge, this is the first study to show that any of these six genes are linked to synaptic plasticity in *Drosophila*. Additionally, some genes
such as obst-b have not been predicted to be involved in synaptic remodeling because their known functions are unrelated to synapses. Our findings indicate that these genes play as-yet-unknown functions in synaptic remodeling. We first selected candidate genes from their phenotype of suppressing synaptic remodeling by RNAi knock-down, and followed this by mutant analyses to confirm the necessity of the gene function. The sufficiency of the gene function was further assessed by overexpression analysis, and finally five genes were selected. Those experiments ensure that the genes are involved in synaptic remodeling. The novelty of this screening may enable us to identify further synaptic genes that were undetectable before.

We attempted to detect genes required to detach synaptic components from the AZ, which can be referred to as a “scrap signal” for the synapse. Counteracting such a signal, there should be other genes working conversely to attach components to the AZ, which can be referred to as a “build signal”. Previously, we showed that microtubules and kinesins, which walk on the meshwork of the microtubules, were required for synaptic remodeling (Sugie et al., 2015). From these observations, we can speculate that AZ component proteins such as Brp are constantly shuttled and recycled by kinesins (and vesicles) moving on the microtubule meshwork. Through the inward and outward movement that underlies the supply and degradation of the components, the synaptic number is kept constant. Alteration in the scrap or build signal shifts the balance between these two movements and subse-

Fig. 5. Overexpression of five genes causes a decrease in the synapse number. (A) Five genes were overexpressed in photoreceptors using eye-specific ey3.5-Gal4 and GMR-Gal4 drivers, and synapses were labeled using the STaR system. In the control fly, there was little difference in the number of synapses between DD and LD conditions, while the number of synapses decreased significantly (*P < 0.05, Student’s t-test) for all of the five genes. (B) Representative images of R8 axons analyzed using Brp-FSF-GFP. Scale bar, 2 μm.
Genes required for synaptic remodeling

9

sequently changes the number of presynaptic AZs. Based on this viewpoint, it is clear that to broaden our understanding of synaptic remodeling a screening for “build signal” genes is needed in the future.

Contribution of cholesterol to synaptic plasticity

Cholesterol exists everywhere in animals and is needed in high quantities for the maintenance of cell membrane fluidity (Dawaliby et al., 2016). In neurons, the myelin sheath has a high amount of cholesterol, which works not only as an insulator but also as a great enhancer of neurotransmission speed. Cholesterol has been implicated in mammalian synaptic plasticity (Koudinov and Koudinova, 2001). However, the underlying mechanism remains unclear. It is notable that three cholesterol-related genes, CG34370, Npc2a and ImpE1, were identified from our screening as molecules related to synaptic plasticity.

To be stable in the blood, cholesterol exists as lipoproteins from insects to mammals. Lipoproteins are composed of cholesterol and apolipoprotein and they are categorized into five classes based on size and lipid composition. Low density lipoprotein (LDL) has a low composition rate of apoprotein and enters the cell by endocytosis together with LDL receptor. CG34370 is an LDL receptor with an LDL domain and 3 CUB domains and, as shown in the present study, has a significant effect on synaptic plasticity. This indicates that LDL receptors bind specific LDLs, and CG34370 may therefore interact with an LDL that is finally metabolized into a synapse-related molecule or that regulates transcription. When LDL receptors take up LDL into cells, an endosome is generated at the same time. The endosome isolates LDL and imports it to the lysosome, where LDL is metabolized to cholesterol; the cholesterol is then released to a variety of organelles, mainly to the endoplasmic reticulum. Npc2a has a cholesterol-binding domain (Friedland et al., 2003) and exists in lysosomes, where it carries cholesterol to Npc1 (Infante et al., 2008). This cholesterol relay is essential for proper localization of cholesterol after it is released from the lysosome via Npc1. Therefore, Npc2a mutants show dysfunctional cholesterol release from the lysosome and excessive accumulation of cholesterol (Vanier and Millat, 2003). In Drosophila, a double mutant of Npc2a and its homolog Npc2b is lethal, as is the Npc1 homozygous mutant. These mutants show a decreased amount of the insect hormone ecdysone, and their lethality can be rescued by adding the ecdysone precursor 7-dehydrocholesterol (Huang et al., 2007). The transcription of ImpE1 is induced by ecdysone, and our results indicate that its gene product is also linked to synaptic plasticity. Furthermore, a previous study reported that estrogen is abundant in the rat hippocampus and that estrogen supplementation increased the number of spines (Mukai et al., 2007). Ecdysone may play an important role for synaptic plasticity, similar to estrogen. Most notably, these three genes can cooperate. We propose that extra-cellular LDL enters the cell via endocytosis of CG34370 and is metabolized to cholesterol in the lysosome. This cholesterol is released from the lysosome by the action of Npc2a and is imported into cell organelles. Some of the imported cholesterol is metabolized into ecdysone, which induces the transcription of ImpE1. Moreover, Npc2a is one of the genes responsible for Niemann–Pick disease type C (NPC), a lysosomal disease characterized by a defect in cholesterol trafficking. The disease affects the nervous system, causing impairment of motor and intellectual function, which may be due to synaptic alterations (Toledano-Zaragoza and Ledesma, 2019). Our indication of a relationship between Npc2a and synaptic plasticity may shed new light on NPC research and therapy.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Supplementary Information for

Systematic identification of genes regulating synaptic remodeling in the *Drosophila* visual system

by Araki et al.

Supplementary Fig. S1
Supplementary Tables S1 and S2
Supplementary Fig. S1. Schematic diagrams of the gene loci and the gDNA recognition sites (blue triangles) and sequences (underlined) used for mutagenesis, together with the modified region (red) of each mutant.
Supplementary Table S1. List of the 232 genes Selected for the Analyses
Scores of the first screening are also shown.

| selected   | analyzed   | RNAi lines | score |
|------------|------------|------------|-------|
| 1          | CG10005    | CG10005    | 0.691 |
| 2          | CG10106    | CG10106    | 0.898 |
| 3          | CG10145    | CG10145    | 1     |
| 4          | CG10152    | CG10152    | 0.758 |
| 5          | CG10186    | CG10186    | 0.764 |
| 6          | CG10287    | CG10287    | 0.643 |
| 7          | CG10334    | CG10334    | NA    |
| 8          | CG10342    | CG10342    | 0.303 |
| 9          | CG10363    | CG10363    | 0.836 |
| 10         | CG10443    | CG10443    | NA    |
| 11         | CG10497    |            |       |
| 12         | CG10521    | CG10521    | 0.756 |
| 13         | CG10593    | CG10593    | 0.893 |
| 14         | CG10717    | CG10717    | 0.929 |
| 15         | CG10823    | CG10823    | 0.701 |
| 16         | CG10946    | CG10946    | 0.956 |
| 17         | CG10975    | CG10975    | 0.792 |
| 18         | CG11051    | CG11051    | 0.648 |
| 19         | CG11059    | CG11059    | 0.232 |
| 20         | CG1106     | CG1106     | 0.844 |
| 21         | CG11064    | CG11064    | 0.593 |
| 22         | CG11081    | CG11081    | 0.69  |
| 23         | CG11099    | CG11099    | 0.778 |
| 24         | CG11280    | CG11280    | 0.784 |
| 25         | CG11282    | CG11282    | 0.617 |
| 26         | CG11326    | CG11326    | 0.737 |
| 27         | CG11372    | CG11372    | 0.562 |
| 28         | CG11440    | CG11440    | 0.861 |
| 29         | CG11527    | CG11527    | 0.604 |
| 30         | CG1168     | CG1168     | 0.8   |
| 31         | CG1171     | CG1171     | 0.848 |
| 32         | CG11895    | CG11895    | 0.964 |
| 33         | CG11910    | CG11910    | 0.796 |
| 34         | CG12002    | CG12002    | 0.556 |
| 35         | CG12070    |            |       |
| 36         | CG12143    | CG12143    | 0.63  |
| 37         | CG1221     | CG1221     | 0.781 |
| 38         | CG12274    | CG34353    | 0.681 |
| 39         | CG34353    | CG12369    | 0.805 |
| 40         | CG34353    | CG12369    | 0.612 |
| 41         | CG12369    | CG12369    | 0.63  |
| 42         | CG12484    | CG12484    | 0.731 |
| 43         | CG12484    | CG12484    | 0.595 |
| 44         | CG12789    | CG12789    | 0.411 |
| 45         | CG12950    | CG12950    | 0.686 |
| 46         | CG13061    | CG13061    | 0.756 |
| 47         | CG13094    |            |       |
| 48         | CG13140    | CG13140    | 0.798 |
| 49         | CG13349    | CG13349    | 0.687 |
| 50         | CG13439    | CG13439    | 0.662 |
| 51         | CG13499    |            |       |
| 52         | CG13506    | CG13506    | 0.736 |
| 53         | CG13521    | CG13521    | 0.395 |
| 54         | CG13532    | CG13532    | 0.889 |
| 55         | CG13566    | CG13566    | 0.573 |
| 56         | CG13633    | CG13633    | 0.85  |
| 57         | CG13643    | CG13643    | 0.684 |
| 58         | CG13648    |            |       |
| 59         | CG13772    | CG13772    | 0.867 |
| 60         | CG13830    | CG13830    | 0.342 |

Gray cells: 24 genes whose RNAi line stocks were not available. NA: not applicable.
Gray cells: 24 genes whose RNAi line stocks were not available. NA: not applicable.
| selected | analyzed | RNAi lines | score |
|----------|----------|------------|-------|
| 121      | CG31605  |            |       |
| 122      | CG31619  | CG31619    | 0.561 |
| 123      | CG31637  | CG31637    | 0.681 |
| 124      | CG31640  | GD15717    | 0.6   |
| 125      | CG31839  | CG31839    | 0.435 |
| 126      | CG32183  | CG32183    | 0.765 |
| 127      | CG32209  |            |       |
| 128      | CG32354  |            |       |
| 129      | CG32356  | CG32356    | 0.275 |
| 130      | CG32372  | CG32372    | 0.786 |
| 131      | CG32432  | JF03300    | 0.666 |
| 132      | CG32464  | GD4766     | 0.651 |
| 133      | CG32600  | JF03172    | 0.672 |
| 134      | CG32647  | CG32647    | 0.568 |
| 135      | CG32659  | JF03375    | 0.681 |
| 136      | CG32667  | GD17086    | 0.761 |
| 137      | CG32687  | CG32687    | 0.654 |
| 138      | CG32699  | CG32699    | 0.386 |
| 139      | CG3302   | JF02023    | 0.76  |
| 140      | CG33103  | CG33103    | 0.706 |
| 141      | CG33141  | GD65       | 0.699 |
| 142      | CG33147  | JF03033    | 0.777 |
| 143      | CG33202  | KK104767   | 0.906 |
| 144      | CG3322   | KK107803   | 0.701 |
| 145      | CG3359   | CG3359     | 0.72  |
| 146      | CG33955  | JF02708    | 0.573 |
| 147      | CG3413   | GD2152     | 0.564 |
| 148      | CG34370  | JF03156    | 0.354 |
| 149      | CG34378  | CG34378    | 0.805 |
| 150      | CG3441   | JF01911    | 0.695 |

| selected | analyzed | RNAi lines | score |
|----------|----------|------------|-------|
| 151      | CG3541   | CG3541     | 0.871 |
| 152      | CG3624   |            |       |
| 153      | CG3665   | CG1664     | JF02918 | 0.354 |
| 154      | CG3829   | CG3829     | KK111090 | 0.829 |
| 155      | CG3915   | CG3915     | JF01981 | 1 |
| 156      | CG4135   | CG4135     | KK112363 | 0.705 |
| 157      | CG4250   | CG4250     | KK110313 | 0.923 |
| 158      | CG4322   | CG4322     | GD709  | 0.564 |
| 159      | CG4383   |            |       |
| 160      | CG4451   | CG4451     | GD3078 | 0.761 |
| 161      | CG4551   | CG4551     | KK108832 | 0.736 |
| 162      | CG4690   |            |       |
| 163      | CG4698   | CG4698     | JF03378 | 0.695 |
| 164      | CG4700   | CG4700     | GD5476 | 0.47 |
| 165      | CG4778   | CG4778     | KK112751 | 0.454 |
| 166      | CG4821   | CG4821     | GD4757 | 0.445 |
| 167      | CG4926   |            |       |
| 168      | CG4999   | CG4999     | KK108729 | 0.974 |
| 169      | CG5008   | CG5008     | KK104210 | 0.735 |
| 170      | CG5407   | CG5407     | JF03236 | 0.582 |
| 171      | CG5490   | CG5490     | KK103505 | 1 |
| 172      | CG5597   | CG5597     | GD4822 | 0.643 |
| 173      | CG5634   | CG5634     | KK107964 | 0.48 |
| 174      | CG5723   | CG5723     | JF03323 | 0.929 |
| 175      | CG5758   | CG5758     | KK107223 | 0.789 |
| 176      | CG5784   | CG5784     | KK107456 | 0.671 |
| 177      | CG5803   | CG5803     | KK103117 | 0.648 |
| 178      | CG5820   | CG5820     | KK104931 | 0.632 |
| 179      | CG5851   | CG5851     | GD11788 | NA |
| 180      | CG5936   | CG5936     | JF03029 | 0.688 |

Gray cells: 24 genes whose RNAi line stocks were not available. NA: not applicable.
| selected | analyzed | RNAi lines   | score |
|----------|----------|--------------|-------|
| CG6014   | CG6014   | GD5549       | 0.84  |
| CG6024   | CG6024   | JF03040      | 0.487 |
| CG6120   | CG6120   | JF03040      | 0.731 |
| CG6323   | CG6323   | GD310        | 0.49  |
| CG6371   | CG6371   | JF03122      | 0.847 |
| CG6378   | CG6378   | KK108185     | 0.586 |
| CG6438   |          |              |       |
| CG6440   | CG6440   | JF02144      | 0.9   |
| CG6456   | CG6456   | JF02145      | 0.772 |
| CG6496   | CG6496   | JF01820      | 0.909 |
| CG6575   | CG6575   | GD6216       | 0.91  |
| CG6588   | CG6588   | KK109572     | 0.722 |
| CG6669   | CG6669   | JF03174      | 0.626 |
| CG6822   | CG6822   | KK100137     | 0.964 |
| CG6827   | CG6827   | GL00631      | 0.721 |
| CG6863   | CG6863   | GD245        | 0.786 |
| CG6953   | CG6953   | KK110998     | 0.9   |
| CG7013   | CG7013   | KK108792     | 0.662 |
| CG7050   | CG7050   | JF02652      | 0.807 |
| CG7052   | CG7052   | KK101058     | 0.438 |
| CG7100   | CG7100   | JF02653      | NA    |
| CG7166   |          |              |       |
| CG7291   | CG7291   | KK108573     | 0.263 |
| CG7503   |          |              |       |
| CG7607   | CG7607   | GD3870       | 0.493 |
| CG7644   | CG7644   | KK105368     | 0.716 |
| CG7727   | CG7727   | JF02878      | 0.979 |
| CG7749   | CG7749   | GD14442      | 0.565 |
| CG7851   | CG7851   | KK101749     | 0.44  |
| CG7890   |          |              |       |

Gray cells: 24 genes whose RNAi line stocks were not available. NA: not applicable.
## Supplementary Table S2. List of all full genotypes used

| Fig. 1B | Rh6mCD8GFP, ey3.5FLP/w; Rh4LexA, LexAopBrp-short-mCherry, AyGal4/+ |
|---------|------------------------------------------------------------------|
| Fig. 2  | Rh6mCD8GFP, ey3.5FLP/w; Rh4LexA, LexAopBrp-short-mCherry, AyGal4/+ (control) |
|         | Rh6mCD8GFP, ey3.5FLP/w; Rh4LexA, LexAopBrp-short-mCherry, AyGal4/UAS-RNAi*(II) |
|         | Rh6mCD8GFP, ey3.5FLP/w; Rh4LexA, LexAopBrp-short-mCherry, AyGal4/+; UAS-RNAi*(III)/+ |
| Fig. 3D | Rh6mCD8GFP, ey3.5FLP/w; Rh6LexA, LexAopBrp-short-mCherry, AyGal4/+ (control) |
|         | Rh6mCD8GFP, ey3.5FLP/w; Rh6LexA, LexAopBrp-short-mCherry, AyGal4/UAS-RNAi*(II) |
|         | Rh6mCD8GFP, ey3.5FLP/w; Rh6LexA, LexAopBrp-short-mCherry, AyGal4/+; UAS-RNAi*(III)/+ |
| Fig. 3E | ey3.5FLP/w; BrpFSFGFP, AyGal4/+ (control) |
|         | ey3.5FLP/w; UAS-RNAi*(II)/+; BrpFSFGFP, AyGal4/+ |
|         | ey3.5FLP/w; BrpFSFGFP, AyGal4/UAS-RNAi*(III) |
| Fig. 4  | sensFLP/w; BrpFSFGFP |
|         | sensFLP/w; obst-b[120]/obst-b[108]; BrpFSFGFP |
|         | sensFLP/w; Npc2a[376]/Df(2L)Exel6006; BrpFSFGFP |
|         | sensFLP/w; CG7607[5A]/CG7607[11B]; BrpFSFGFP |
|         | sensFLP/w; CG34370[M109268]/Df(2R)BSC802; BrpFSFGFP |
|         | sensFLP/w; Swim[1C]/Swim[3A]; BrpFSFGFP |
|         | sensFLP/w; BrpFSFV5/++; beatVI[106]/beatVI[117] |
|         | sensFLP/w; BrpFSFV5/++; ImpE1[107]/ImpE1[115] |
|         | sensFLP/w; BrpFSFV5/++; NPF[SK1]/ NPF[SK2] |
|         | sensFLP/w; BrpFSFV5/++; Teq[21]/ Teq[82] |
|         | sensFLP/w; BrpFSFV5/++; Tsp97E[43]/Tsp97E[23] |
| Fig. 5  | sensFLP/w; eyGal4, GMRGal4/+; BrpFSFGFP/+ |
|         | sensFLP/w; eyGal4, GMRGal4/obst-b[EY09474]; BrpFSFGFP/+ |
|         | sensFLP/w; eyGal4, GMRGal4/+; BrpFSFGFP/UAS-beatVI |
|         | sensFLP/w; eyGal4, GMRGal4/UAS-Npc2a; BrpFSFGFP/+ |
|         | sensFLP/w; eyGal4, GMRGal4/+; BrpFSFGFP/CG7607[EP3548] |
|         | sensFLP/w; eyGal4, GMRGal4/CG34370[d00934]; BrpFSFGFP/+ |