Figure S1, related to Figure 1. Proteolytic cleavage of DNlg2. (A) Immunoprecipitation of DNlg2 in $dnlg2^{KO70}$ and WT fly heads with anti-DNlg2$^{CTF}$ antibodies. The precipitates, as well as a portion (5% of the input) of the extracts, were subjected to immunoblotting with anti-DNlg2$^{CTF}$ or anti-tub (for the negative control) antibodies. (B) Schematic illustration of the Gal4/Tub-Gal80$^{ts}$ expression system. At 25 °C, binding of Gal80$^{ts}$ to Gal4 prevents the transcription. After shifting to 30 °C, Gal80$^{ts}$ was inactivated, allowing Gal4 to activate the transcription of $dnlg2$, which was then translated into DNlg2 protein. (C) Immunoblotting analysis of human SH-SY5Y and mouse Neuro-2a cells transfected with 1 μg pCDNA3.1-$dnlg2$ plasmid. The rabbit anti-DNlg2$^{CTF}$ antibodies were used. (D) DNlg2 proteins were overexpressed with Gal4/Tub-Gal80$^{ts}$ expression system for 37 hours, and were then immunoprecipitated purified. Purified proteins were then incubated with cleavage buffer, incubated time (hour) are shown at the top.
Figure S2, related to Figure 2. Cleavage of KDEL tagged DNlg2. (A) Schematic representation of truncated DNlg2 and the same truncated fragment with a KDEL tag. The truncated versions of DNlg2 are shown as: ① deletion of cytoplasmic region and transmembrane domain; ② deletion of cytoplasmic region and transmembrane domain, and tagged with KDEL sequences; ③ deletion of cytoplasmic region; and ④ deletion of cytoplasmic region and tagged with KDEL sequences. (B) Immunoblotting analysis of S2 cells transfected with truncated and KDEL tagged DNlg2 in accordance with (C). An extracellular monoclonal antibody 3D10 against DNlg2 was used.
Figure S3, related with Figure 6. Bip is required for the ER retention of the 130 kDa DNlg2. (A) Immunoblotting analysis of da-Gal4>WT (Control) and da-Gal4>UAS-BiP-RNAi (BiP RNAi) muscle cells with rat anti-BiP and mouse anti-α-tubulin antibodies. (B) Quantification of relative BiP expression level in da-Gal4>WT (Control, 100% ± 4.9%) and da-Gal4>UAS-BiP-RNAi (BiP RNAi, 70.6% ± 4.3%) muscle cells. (C and D) Immunoblotting analysis of da-Gal4>UAS-dnlg2 and da-Gal4> UAS-dnlg2, UAS-BiP-RNAi with rabbit anti-DNlg2CTF. Samples were purified with plasma-membrane extraction kit. Fmr1 and α-tub were used as negative control.
Figure S4, related to Figure 7. Electrophysiology analysis of the rescue lines at the NMJs. (A) Representative traces of EJP recorded from muscle 6 in abdominal segment 3 in the presence of 0.8 mM calcium in WT, dnlg2<sup>KO70</sup>, and rescue lines with UAS-dnlg2, UAS-dnlg2<sup>R598C</sup>, UAS-dnlg2<sup>SF</sup>, UAS-dnlg2<sup>CTF</sup> derived by da-Gal4. (B) Quantification of EJP amplitude. dnlg2<sup>KO70</sup> (33.62 mV ± 1.482 mV) showed reduced EJP amplitude when compared with WT (46.94 mV ± 1.654 mV) controls. UAS-dnlg2 (47.09 mV ± 1.547 mV) and UAS-dnlg2<sup>CTF</sup> (42.54 mV ± 1.285 mV), but not UAS-dnlg2<sup>R598C</sup> (37.40 mV ± 1.628 mV) and UAS-dnlg2<sup>SF</sup> (31.88 mV ± 3.112 mV), was able to rescue the EJP amplitude defect. (C and D) Summary graph showing normal mEJPs amplitude and frequency in WT, dnlg2<sup>KO70</sup>, and rescue lines with UAS-dnlg2, UAS-dnlg2<sup>R598C</sup>, UAS-dnlg2<sup>SF</sup>, UAS-dnlg2<sup>CTF</sup> derived by da-Gal4.
Figure S5, related to Figure 7. NMJ growth analysis of the rescue lines while BiP knock down. (A) Confocal images of NMJ6/7 from abdominal segment 2 of third-instar larvae labeled with rabbit anti-HRP (green) and mouse anti-DLG (red), showing boutons phenotypes in WT (100% ± 8.581%), da-Gal4>UAS-BiP-RNAi (101.2% ± 10.34%), dnlg2<sup>KO70</sup> (67.03% ± 3.614%), dnlg2<sup>KO70</sup>;da-Gal4>BiP RNAi (65.92% ± 4.870%) and dnlg2<sup>KO70</sup>;da-Gal4> UAS-dnlg2<sup>SF</sup>, UAS-BiP-RNAi (70.09% ± 5.253%). Scale bar: 25 μm. (B) Quantifications of type Ib and Is bouton number at NMJ6/7 adjusted to WT bouton number.
Supplemental Experimental Procedures

Cloning and UAS transgenic flies’ generation

Full-length DNlg2 cDNA was purchased from the BDGC (RH63339). Proper primers were used to amplify cDNAs, which were then subcloned into pAc5.1/V5-His-A (Invitrogen), pcDNA3.1(+) or pUAST-attB (Bischof et al., 2007) vectors and transfected into S2 cells, HEK 293T Cells or injected into attP transgenic flies (35568) respectively. Transgenic fly strains were generated based on the φ3I-mediated integration system using the landing site at the cytological position 87B (Bischof et al., 2007). The transgenes were subsequently crossed into a w^{1118} background. Fragment deletion and site-directed mutagenesis were performed by the overlap PCR. Briefly, two pairs of primers were used to amplify the upstream and downstream fragments, and then the two PCR products were mixed as new template with proper primers to generate full-length products.

Antibody production

Rabbit polyclonal antibody (anti-DNlg2^{CTF}) and mouse monoclonal antibody (6D5) against DNlg2 intracellular domain were generated previously (Sun et al., 2011). Another mouse monoclonal antibody (3D10) against DNlg2 extracellular domain adjacent to transmembrane domain was also generated. To generate GluRIIB polyclonal antibody, a peptide (C-ASSAKKKKTRRIEK) representing amino acids 899-913 of GluRIIB was synthesized and injected into rabbit by Genscript (Nan Jing, China), and the resultant anti-serum was used for staining.

Subcellular fractions isolation

To extract plasma membrane proteins, the Plasma Membrane Protein Extraction Kit (Abcam, ab65400) was used according to the manufacture’s instruction. To isolate ER/Golgi apparatus, the Endoplasmic Reticulum Isolation Kit (Sigma-Aldrich, ER0100) was used according to the manufacture’s instruction.
**Immunoblotting**

Adult heads or larval body wall muscle were homogenized with 1×SDS loading buffer, then incubated with boiling water for 5 min, centrifuged at 13,000 rpm at 25°C for 5 min, and the supernatant was collected. The primary antibodies used for western blots analysis were anti-DNlg2 (CTF; 1:2500), anti-DNlg2 (3D10; 1:50), anti-Syn16 (ab32340; Abcam 1:2000), anti-BiP (MAC 143; Abcam 1:2000), anti-HA (C29F4; GST 1:2000), anti-β-Actin (EBA-007; ZOONBIO 1:10000) and anti-α-Tubulin (DM1A; Sigma 1:10000). The samples were then incubated with goat anti-mouse or anti-rabbit IgG (H+L) HRP-conjugated secondary antibodies (31430, 31460; Thermo Scientific; 1:10000) at room temperature for 1 hour. The targeted proteins were visualized with SuperSignal West Pico (34078; Thermo Scientific) or SuperSignal West Femto (34095; Thermo Scientific).

**Immunostaining**

Adult whole brains, larval VNC and NMJ staining were performed as previously described (Sun et al., 2011; Xing et al., 2014). Note that, for GluRIIB staining, samples were fixed for 5 min with methanol (precool to -20°C). After dissection and fixation, samples were stained with anti-DNlg2 (CTF 1:100; 6D5 1:20), anti-DLG (4F3; 1:100; DSHB), anti-BRP (nc82; 1:25; DSHB), anti-HRP (1:1000; Jackson Immuno Research, West Grove, PA), anti-GluRIIB (1:1000), anti-BiP (MAC 143; Abcam 1:200) and anti-DVGLUT (1:1000, gift from Dr. A. DiAntonio, Washington University, St. Louis, MO). The samples were then incubated with fluorophore-conjugated secondary antibodies (Invitrogen, 1:500) antibodies at room temperature for 2 hours. Following the antibody incubation, the samples were washed extensively and mounted in vectashield mounting medium (Vector Laboratory). Samples were imaged on an LSM 510 or 710 confocal microscopes (Zeiss). ImageJ 1.46 r (National Institutes of Health, USA) was used for intensity analyses.

**Cell culture and transfection**

S2 cells were purchased from lookbio (ShangHai SXBIO Biotechnology), grown
at 25 °C in HyClone SFX-Insect Cell Culture Media (ThermoFisher Scientific) and maintained at a density of 1×10^6 to 8×10^6 cells/ml in 6 ml per T75 plastic tissue culture flask to ensure exponential growth. Under these conditions, S2 cells were divided every 4 days. pAc5.1/V5-His-A (Invitrogen) plasmid containing a Actin 5C promoter was used as the expression vector. One day before transfection, 2×10^6 cells were plated in a 6 well culture plate with 2 ml medium each. After one day’s growth, transfections were performed using relative amount of each plasmid and X-treme GENE HP (6366546001; Roche) transfection reagent according to the manufacturer's instructions. HEK 293T, human SH-SY5Y, and mouse Neuro-2A cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen), and the transfection experiments were also performed with X-treme GENE HP (6366546001; Roche) according to the manufacturer's instructions.

**Immunoprecipitation**

Fly heads of desired genotypes were homogenized using a glass homogenizer in a weight/volume ratio of 1:3 in ice-cold cell lysis buffer (Beyotime Biotechnology, China;) containing 20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4 and leupeptin. The lysates were kept on ice for 30 min and centrifuged at 12,000 g for 30 min at 4 °C, and then diluted the supernatant 5 times with PBS. For each IP reaction 500 μl supernatant was incubated with 1 μg primary antibodies for 4 hours at 4°C. Then the supernatant-antibody mix was incubated with 20 μl Protein A/G beads (sc-2003, Santa Cruz Biotechnology) overnight at 4°C. After four time washes, the bound complexes were eluted with 2× SDS sample buffer and subjected to SDS-PAGE and immunoblotting.

**In vitro cleavage**

Immunoprecipitated DNIg2 proteins were incubated in cleavage buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 1 mM EDTA) 25 °C. At various time points, samples were withdrawn and analyzed by immunoblotting.
**Statistical analysis**

Data are presented as mean ± SEM. For statistical analysis, two-tailed Mann-Whitney test were used with 95% confidence intervals (performed in GraphPad Prism). p values between two bars refer to comparisons between those two genotypes. Error bars indicate SEM, and the numbers in the histograms indicate the number of samples. *p < 0.05; **p < 0.01; ***p < 0.001; and N. S, not significant, in all Figures.

**References**

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