Title: Nicotinamide Mononucleotide Adenylyltransferase (NMNAT) Maintains Active Zone Structure by Stabilizing Bruchpilot

Shaoyun Zang[1], Yousuf O. Ali[1,2], Kai Ruan, and R. Grace Zhai

Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL 33137.

Correspondence should be addressed to R. Grace Zhai, Department of Molecular and Cellular Pharmacology, Miller School of Medicine, University of Miami, 1600 NW 10th Ave., RMSB 6068, Miami, FL 33136. Phone: 305-243-6316, Fax: 305-243-4555; gzhai@med.miami.edu

[1]These authors contributed equally to this work.

[2]Current address: Baylor College of Medicine, Jan and Dan Duncan Neurology Institute, 1250 Moursund, Houston TX 77025

Running title: NMNAT maintains active zone structure

Supplementary Methods

Fly Stocks and Culture. Flies were reared at room temperature in ambient light under a normal 12-h light/dark cycle. For dark-rearing experiments, flies were kept in complete darkness from the first instar larval stage onward. The following fly strains used in this experiment were obtained from the Bloomington Stock Center (Indiana): {w; OK371-GAL4}, {w, UAS-Dicer; bbg-C96-GAL4}, {y'v';; P{TRIP-JF03337}attp$_2$}, {w, C155-GAL4; UAS-Dicer}, {y'v';; P{TRIPJF01932}attp$_2$}; FRT82Biso.

MG132 feeding. A semi-circle of filter paper soaked in 50 uM of MG-132 (Sigma, C2211) or DMSO was placed at the bottom of the vial. A group of 40 flies were put into each vial and kept for 24 hours and then collected for protein analysis.

Mosaic Analysis. The MARCM technique was applied to analyze the level of synaptic proteins in nmnat mutant photoreceptor cells compared to their wild-type neighbors. All laminae shown with 50% mutant photoreceptor terminals are from animals of the following genotype: {y w eyFLP GMR-lacZ; Pfwþ1⁄4UAS- mCD8::GFP.LgLL4 / GMR-GAL4; FRT82B nmnat/ FRT82B Pfwþ1⁄4tub- GAL80g}.

Electron Microscopy. Fly heads were dissected and fixed at 4 °C in 2% paraformaldehyde, 2% glutaraldehyde, 0.1 M sodium cacodylate, 0.005% $CaCl_2$ (pH 7.2), and postfixed in 2% OsO_4. The 50-nm thin sections were stained with 4% uranyl acetate and 2.5% lead nitrate. Synaptic features of laminae were scored double-blinded by several observers.

Immunocytochemistry Third instar larval fillets and adult brains were fixed in phosphate-buffered saline (PBS) with 4% formaldehyde for 20 min and washed in PBS with 0.4% Triton X-100 for brain, or with 0.3% Tween 20 for larval neuromuscular junction preparations. Antibody dilutions used: anti-NMNAT 1:1000; anti-HRP 1:1000 (Rockland, Gilbertsville, PA); mAb nc82 1:200 (adult), 1:1000 (larvae) (DSHB, University of Iowa, IA); anti-DLAR 1:200 (DSHB, University of Iowa, IA). Secondary antibodies conjugated to Alexa-488, -546 and -647, Cy3, or Cy5 (Jackson ImmunoResearch, West Grove, PA; and Molecular Probes) were used at 1:250.

DSIM Super Resolution imaging DSIM Super Resolution imaging was performed on a DeltaVison OMX version 3 (Applied Precision, Inc.) equipped with 405, 488, and 561 nm solid-state lasers. Images were acquired using an UPlanS Apochromat 100× 1.4 NA oil immersion objective lens (Olympus) and Cascade II EMCCD camera (Photometrics). The light path was directed through a diffraction grating, generating an interference pattern of light resulting in a 3D sinusoidal pattern with lateral stripes in the image plane. Images were taken shifting the pattern to 5 phase positions along the sinusoidal pattern. These five phase images were repeated with the grating pattern rotated at a total of three angles separated by roughly 60 degrees of rotation around the optical axis for each z-section. Optical sections were spaced by 125 nm. Laser power and exposure times were adjusted to give a maximum intensity between ~15,000 and ~30,000 at a 16-bit depth dynamic range. Images for each channel were acquired sequentially to separate cameras. Raw data sets were reconstructed using softWoRx version 5.0 (Applied Precision, Inc.) to generate final high-resolution images. Channel registration was achieved using measured alignment parameters determined using a calibrated target objective.

Protein Extraction, Immunoprecipitation and Western Blotting. Twenty female fly heads of each genotype were collected at specific ages and immediately flash-frozen before extraction. For protein extraction, brains were homogenized in lysis buffer consisting of 100 mM KCl, 20 mM HEPES, 10 mM EDTA, 1 mM DTT, 5% Glycerol, 0.1% Triton-X100, Protease Inhibitor Cocktail (Sigma), phosphatase inhibitor cocktail (Roche), and centrifuged for 5 min at 500 g to remove debris. For immunoprecipitation experiments, 40 2DAE female fly heads were homogenized in lysis buffer, pre-cleared with Protein-G beads for an hour and incubated with Protein-G beads conjugated with 10 μg antibody per sample overnight at 4°C. The bead pellets were collected and washed four times with lysis buffer before eluting with Lamelli buffer. Lysates were probed with anti-NMNAT (1:8000), mab nc82 (1:1000; DSHB, University of Iowa, IA), anti-DLAR (1:1000; DSHB, University of Iowa, IA), and anti-Actin antibodies (1:4000; Sigma), anti-DCSP (1:500; DSHB, University of Iowa, IA), and anti-synaptotagmin (1:500; DSHB, University of Iowa, IA). Western blot analysis was performed with infrared dye-conjugated secondary antibodies, IRDye 700DX and IRDye 800DX (LI-COR Biosciences); blots were imaged and processed on an Odyssey Infrared Imaging System. The wide linear range of the Odyssey system allows quantitative analysis of protein levels.

Densitometry, Quantification and Statistical Analysis. Densitometric analysis of blots was performed using ImageJ software (NIH). Data are represented as means ± SEM. Statistical analysis was performed using ANOVA. Values of $p < 0.05$ were considered statistically significant.

Image acquisition and processing. Images from fluorescently labeled specimens were taken on an Olympus IX81 confocal microscope and processed using FluoView10-ASW (Olympus) and Adobe Photoshop CS4 (Adobe Systems).

Real Time PCR. Fly heads were collected from two day-old female wild-type flies (genotype: yw; ; 82Biso), RNAi control flies (genotype: c155-Gal4/+;UAS-Dicer/+) and nmnat RNAi flies (genotype: c155-Gal4/+;UAS-Dicer/+;UAS-nmnatRNAi/+) and were used to extract RNA. Real time PCR (Bio-Rad) was then performed to measure the transcription level of *nmnat* and *brp*. A housekeeping gene, *rp49*, was used as an internal control to standardize mRNA expression. The *brp* probe (PE Applied Biosystems, Weiterstadt) specifically targets the c-terminal exons of *brp* gene.

Supplementary Figure Legends

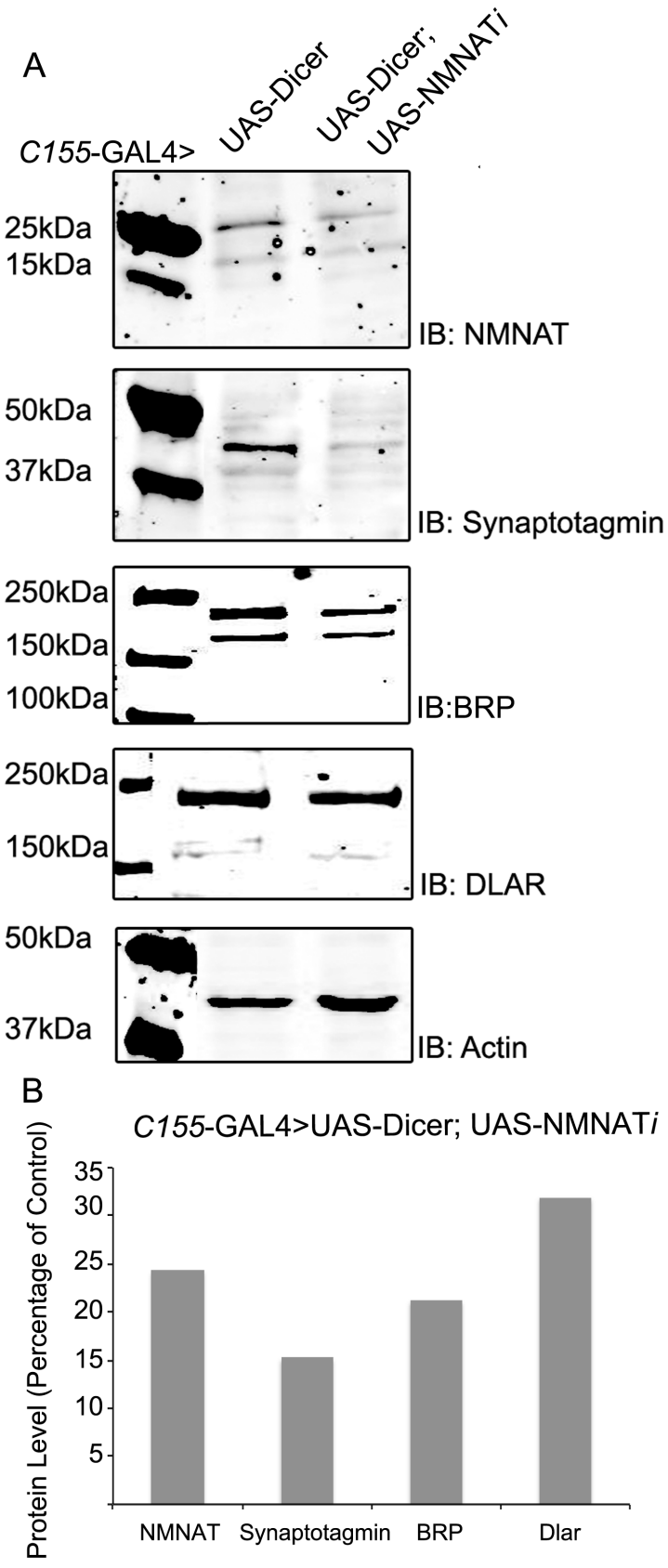
Figure S1 Western blot analysis of synaptic proteins in NMNAT RNAi brains shows a general reduction of synaptic proteins, including synaptotagmin, BRP and DLAR. (A) Brain lysates from 2 DAE flies were probed with different synaptic markers. (B) Quantification of protein levels in (A) using densitometry. Actin was used as a loading control.

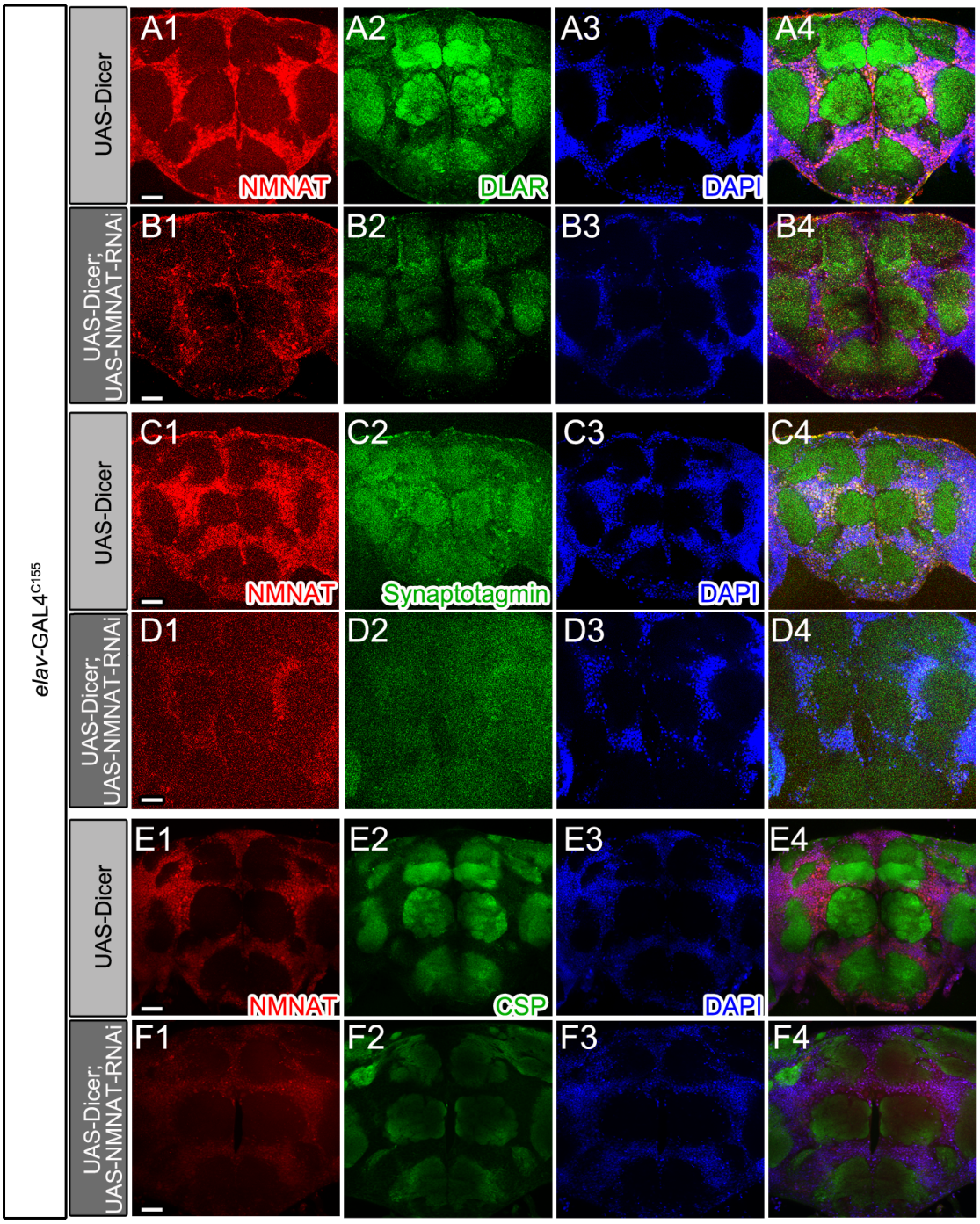
Figure S2 Immunostaining of adult fly (2 DEA) midbrain with synaptic protein, DLAR (A-B), synaptotagmin (C-D), and CSP (E-F) in NMNAT knockdown brains reveals a general reduction of synaptic in loss of NMNAT. Scale bars indicate 20 um.

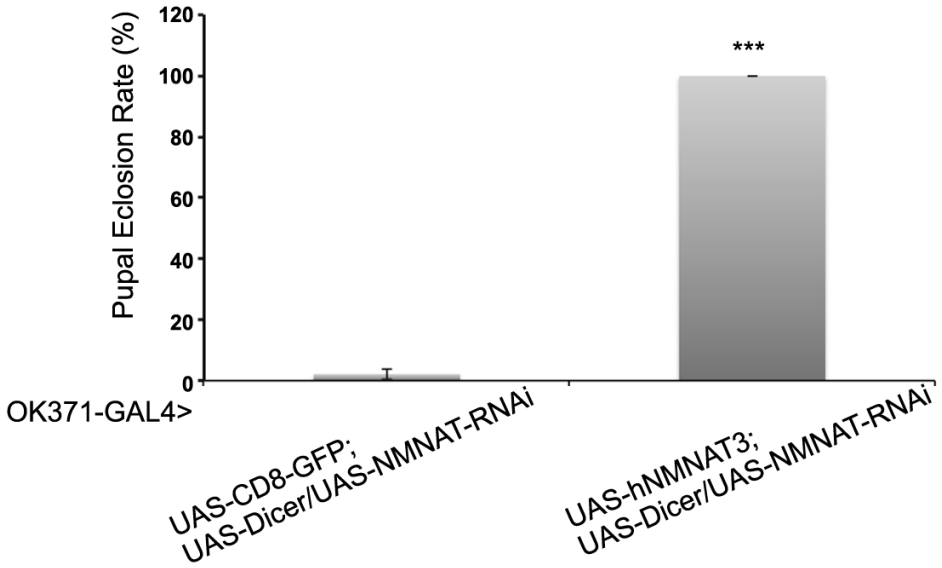
Figure S3 Pupal eclosion rate was determined for flies with motor neuron-specific knockdown of NMNAT with OK371 driver. Overexpression of human NMNAT3 rescues the low pupal eclosion rate associated with NMNAT knockdown, suggesting the specificity of RNAi knockdown in nmnat gene expression. All data were presented as mean ± S.E.M. Significance level was established by t-test. *$P<0.05$. n=3.

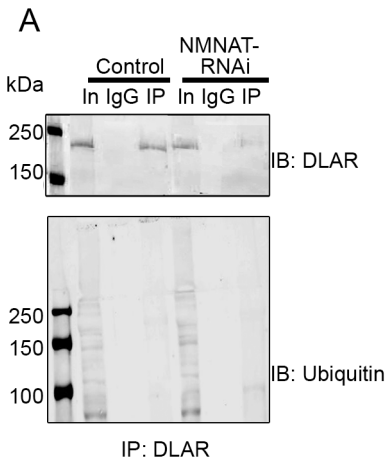
Figure S4 No ubiquitination of synaptic proteins, including DLAR, synaptotagmin, and CSP was observed in NMNAT knockdown brains. Immunoprecipitation of brain lysates from flies overexpressing UAS-Dicer or UAS-Dicer; UAS-NMNAT-RNAi with DLAR antibody (A), synaptotagmin antibody (B), and CSP antibody (C) reveals no ubiquitination of these proteins in NMNAT knockdown brains.

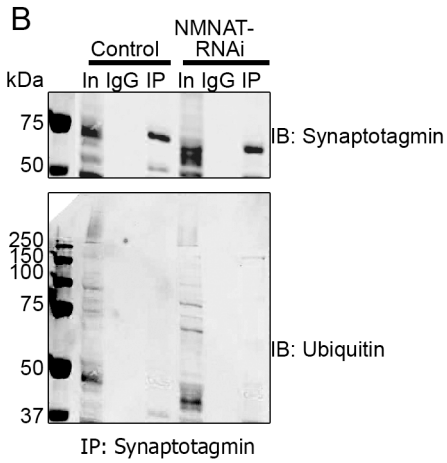
Figure S5 Multiplex western analysis of BRP ubiquitination. (A) Western blots were probed simultaneously with ubiquitin antibody (top panel) and BRP antibody (middle panel). The yellow band shown in the merge image (bottom panel) indicates ubiquitinated BRP that is recognized by both antibodies (arrows). (B) Quantification of the percentage of ubiquitinated BRP (arrows) over the total BRP (bracket in middle panel).

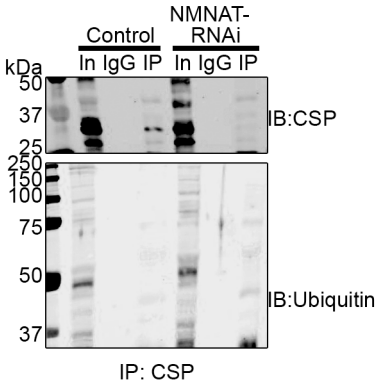

A
C155-GAL4>
UAS-Dicer
UAS-Dicer;
UAS-NMNATi
25kDa
15kDa
IB: NMNAT
50kDa
37kDa
IB: Synaptotagmin
250kDa
150kDa
100kDa
IB:BRP
250kDa
150kDa
IB: DLAR
50kDa
37kDa
IB: Actin
B
C155-GAL4>UAS-Dicer; UAS-NMNATi
Protein Level (Percentage of Control)
35
30
25
20
15
10
5
NMNAT
Synaptotagmin
BRP
Dlar

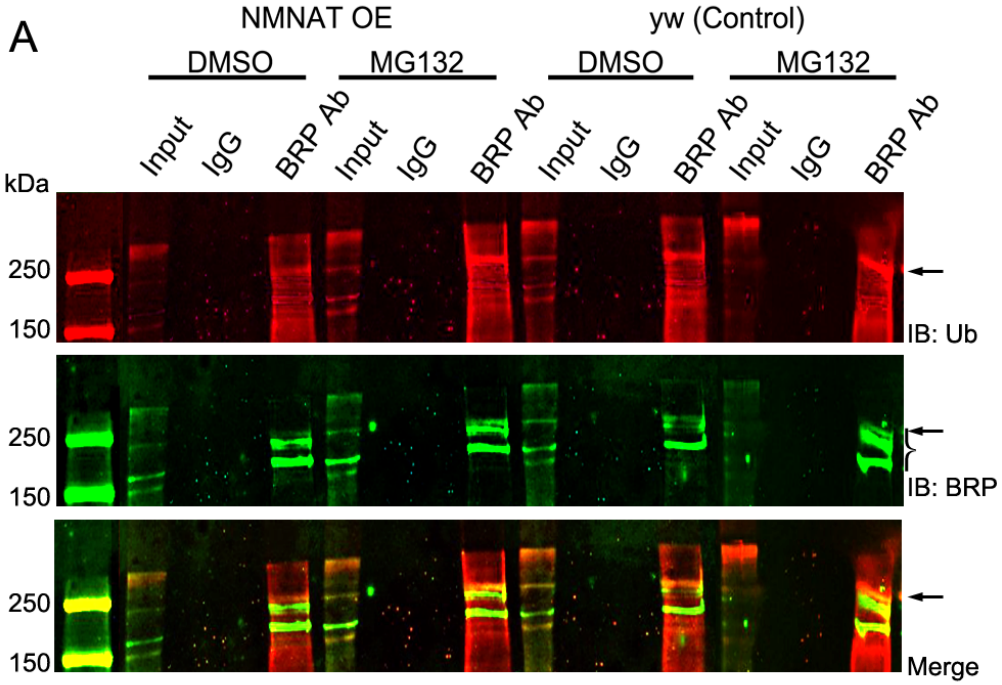

elav-GAL4C155
UAS-Dicer
UAS-Dicer; UAS-NMNAT-RNAi
A1
NMNAT
A2
DLAR
A3
DAPI
A4
B1
B2
B3
B4
C1
NMNAT
C2
Synaptotagmin
C3
DAPI
C4
D1
D2
D3
D4
E1
NMNAT
E2
CSP
E3
DAPI
E4
F1
F2
F3
F4

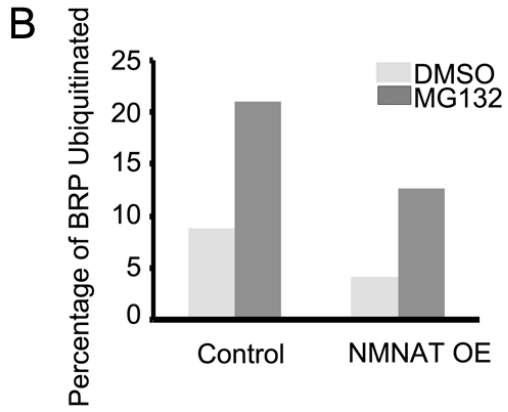

Pupal Eclosion Rate (%)
120
100
80
60
40
20
0
***
OK371-GAL4>
UAS-CD8-GFP;
UAS-Dicer/UAS-NMNAT-RNAi
UAS-hNMNAT3;
UAS-Dicer/UAS-NMNAT-RNAi


A
Control
NMNAT-RNAi
kDa
In IgG IP In IgG IP
250
150
IB: DLAR
250
150
100
IB: Ubiquitin
IP: DLAR


B
Control
NMNAT-RNAi
kDa
In IgG IP In IgG IP
75
50
IB: Synaptotagmin
250
150
100
75
50
37
IB: Ubiquitin
IP: Synaptotagmin


C
Control
NMNAT-RNAi
kDa
In IgG IP In IgG IP
50
37
25
IB:CSP
250
150
100
75
50
37
IB:Ubiquitin
IP: CSP


A
NMNAT OE
yw (Control)
DMSO
MG132
DMSO
MG132
Input
IgG
BRP Ab
Input
IgG
BRP Ab
Input
IgG
BRP Ab
Input
IgG
BRP Ab
kDa
250
150
IB: Ub
250
150
IB: BRP
250
150
Merge


B
Percentage of BRP Ubiquitinated
25
20
15
10
5
0
DMSO
MG132
Control
NMNAT OE