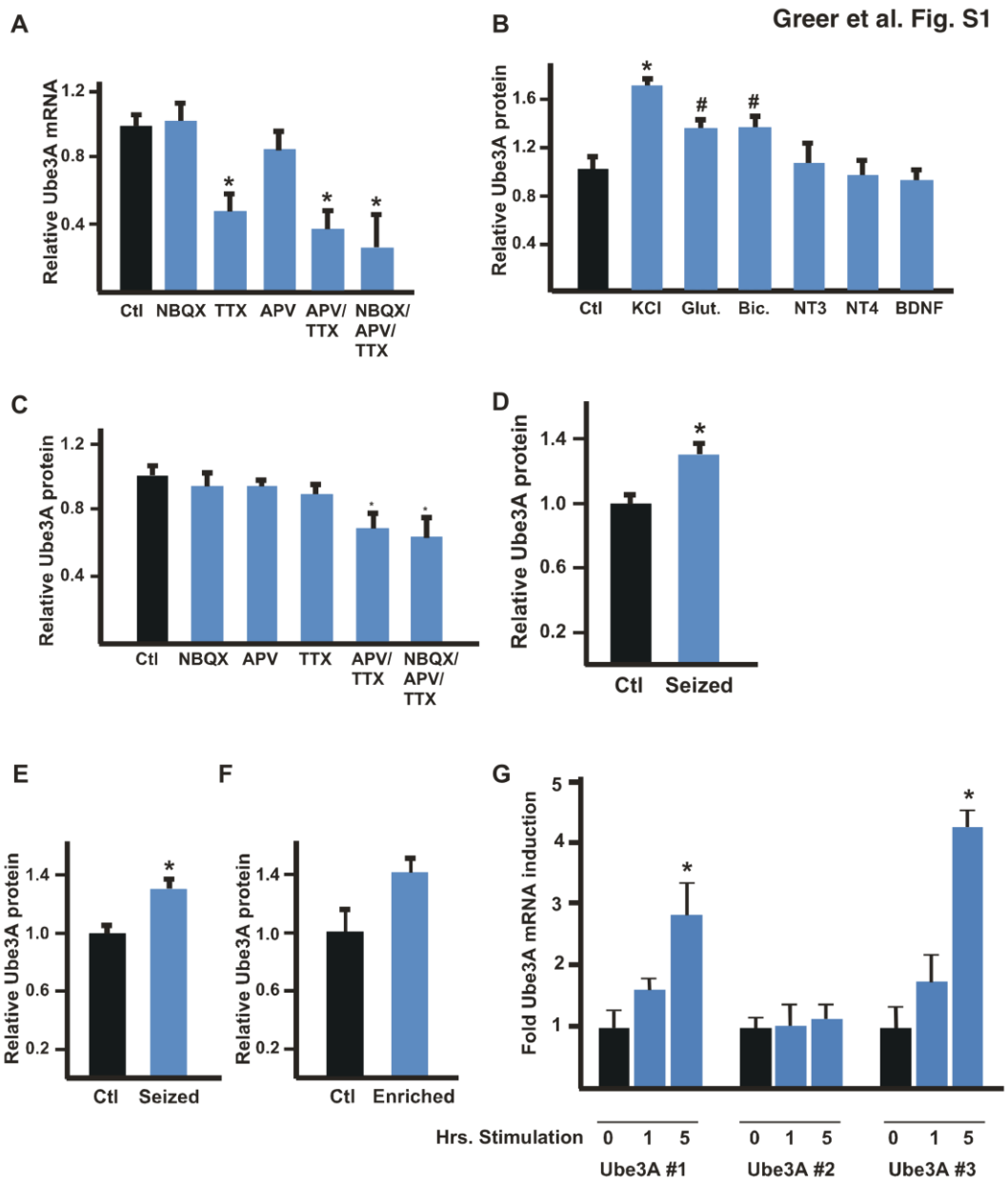
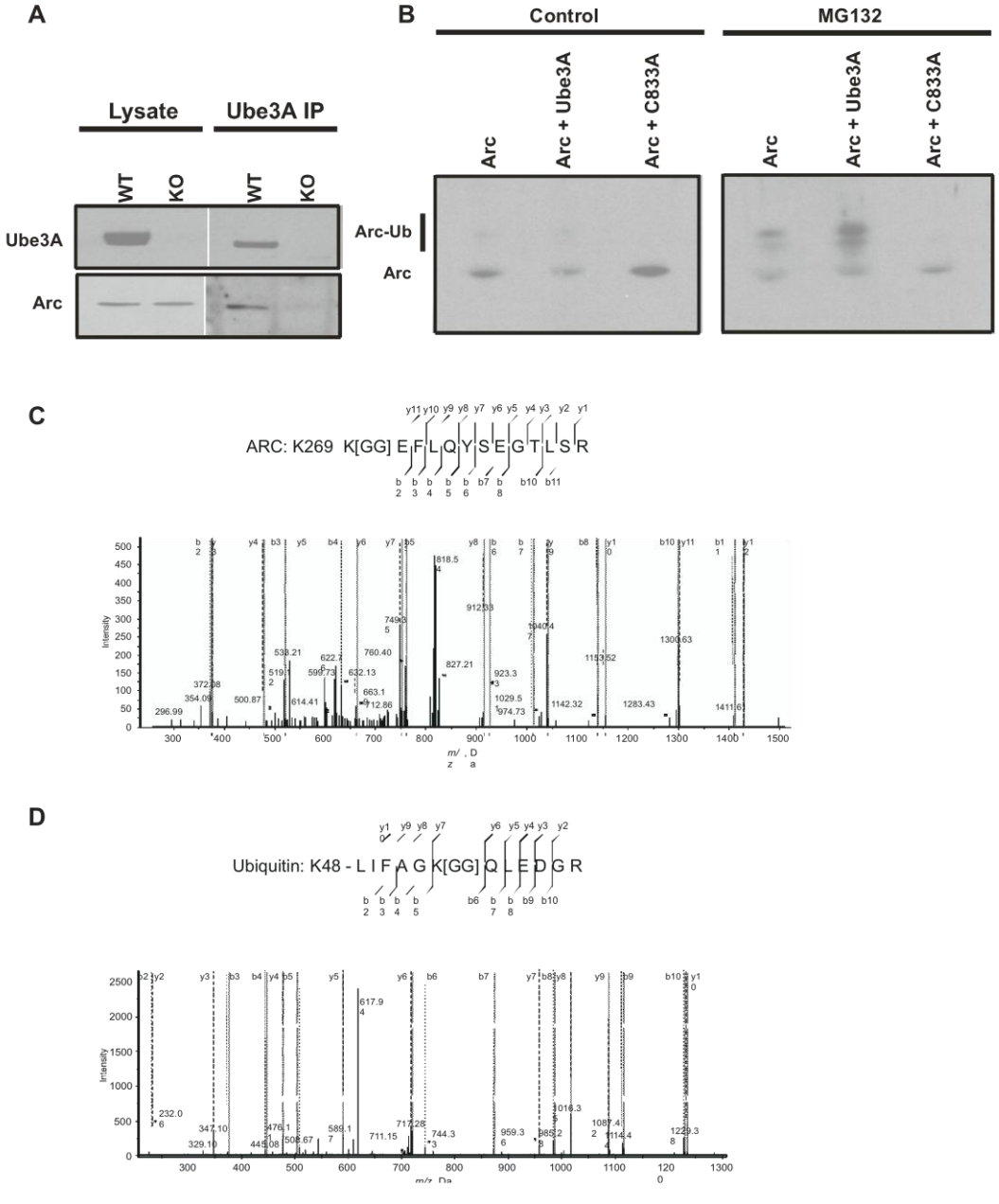
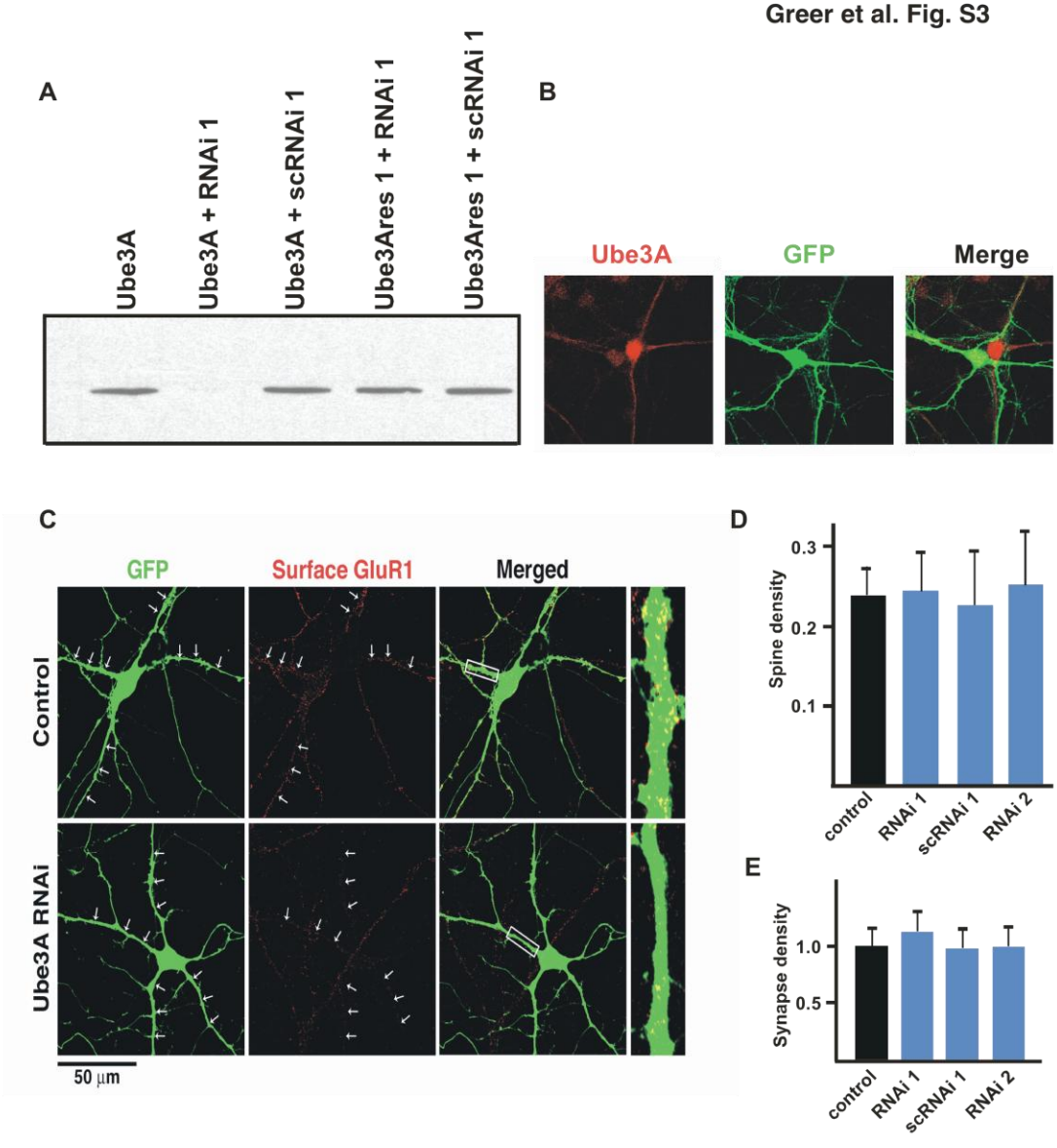

Greer et al. Fig. S1
A
Relative Ube3A mRNA
1.2
0.8
0.4
*
*
*
Ctl
NBQX
TTX
APV
APV/ TTX
NBQX/ APV/ TTX
B
Relative Ube3A protein
1.6
1.2
0.8
0.4
*
#
#
Ctl
KCl
Glut.
Bic.
NT3
NT4
BDNF
C
Relative Ube3A protein
1.2
0.8
0.4
Ctl
NBQX
APV
TTX
APV/ TTX
NBQX/ APV/ TTX
D
Relative Ube3A protein
1.4
1.0
0.6
0.2
*
Ctl
Seized
E
Relative Ube3A protein
1.4
1.0
0.6
0.2
*
Ctl
Seized
F
Relative Ube3A protein
1.4
1.0
0.6
0.2
Ctl
Enriched
G
Fold Ube3A mRNA induction
5
4
3
2
1
*
*
Hrs. Stimulation
0
1
5
0
1
5
0
1
5
Ube3A #1
Ube3A #2
Ube3A #3


A
Lysate
Ube3A IP
WT
KO
WT
KO
Ube3A
Arc
B
Control
MG132
Arc
Arc + Ube3A
Arc + C833A
Arc
Arc + Ube3A
Arc + C833A
Arc-Ub
Arc
C
ARC: K269 K[GG] E F L Q Y S E G T L S R
Intensity
m/z, Da
D
Ubiquitin: K48 - L I F A G K[GG] Q L E D G R
Intensity
m/z, Da

Greer et al. Fig. S3



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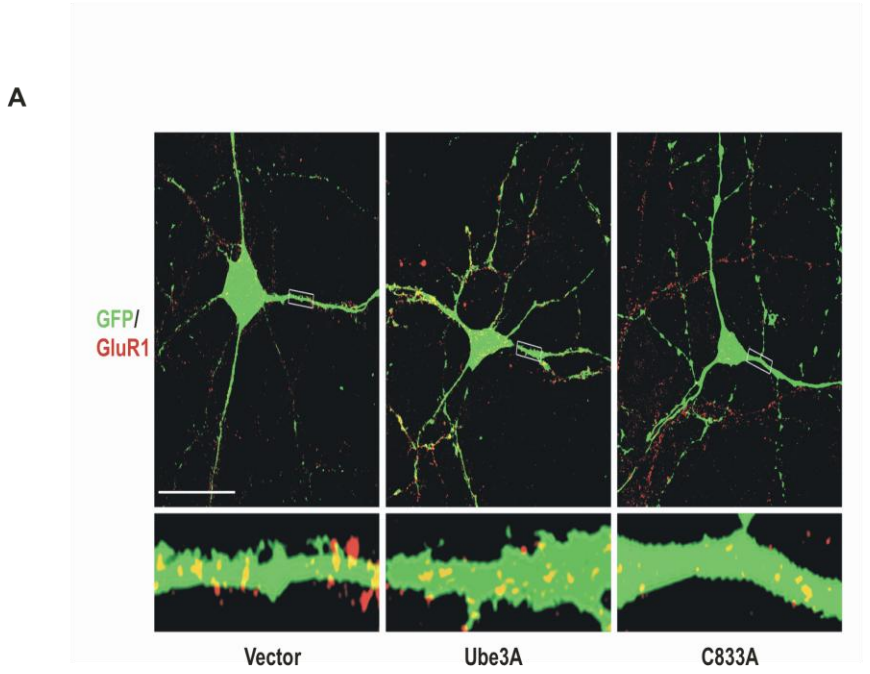

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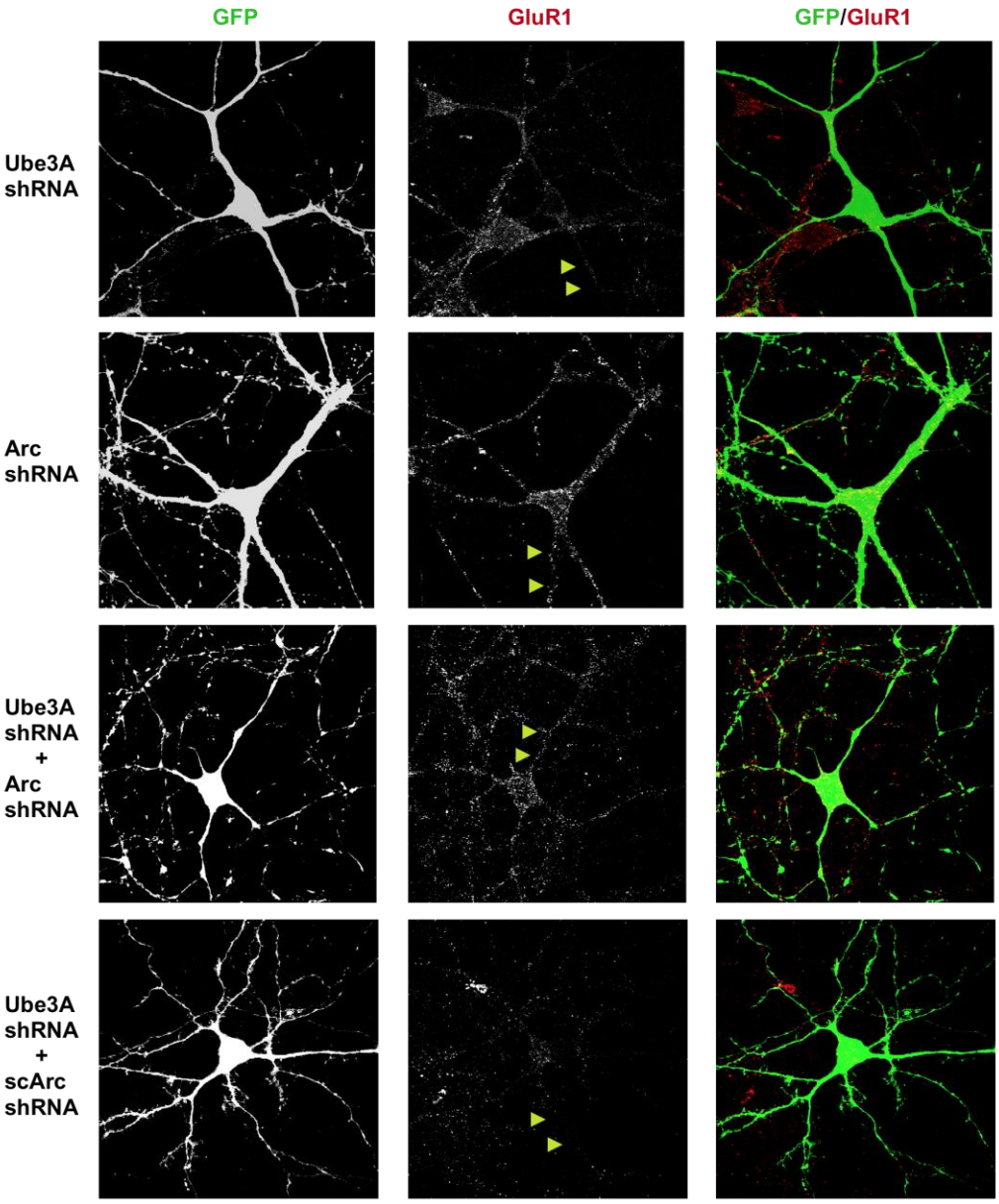

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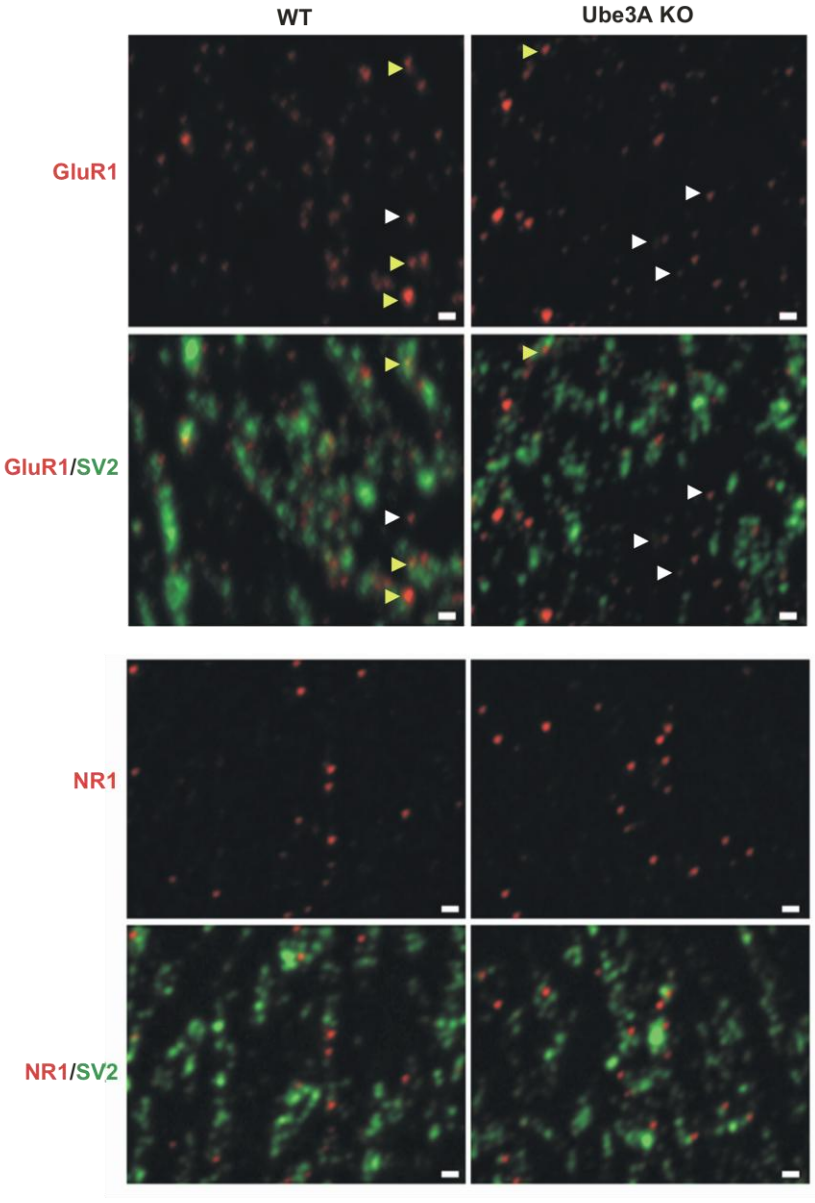
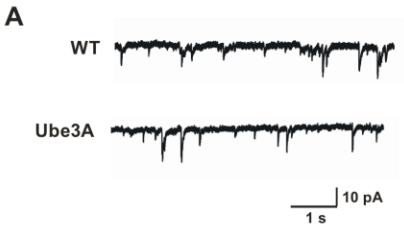
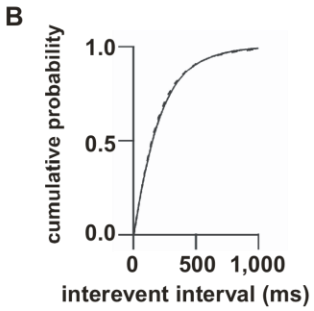
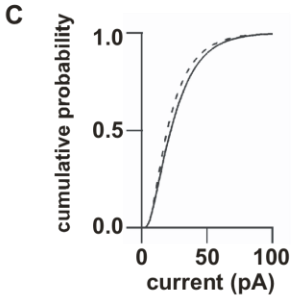


Greer et al. Fig.S7







Supplemental Figure Legends:

Figure. S1, related to Figure 1. Regulation of Ube3A mRNA and protein by neuronal activity. (A) Real-time PCR analysis of Ube3A mRNA extracted from hippocampal neurons at E18 + 10 DIV treated for six hours with the indicated agent. Data are means +/- SEM from three independent experiments. * indicates statistical significance in pairwise comparison to control: $P < 0.01$ T-test. (B) Quantitative Western blot analysis of Ube3A protein. Protein lysates were collected from hippocampal neurons at E18 + 8 DIV following treatment with the indicated agent for seven hours. This experiment was performed three times independently and the data were normalized to the control and are presented as means +/- SEM. * indicates $P < 0.01$, # indicates $P < 0.05$ in analysis of statistical significance in pairwise comparison to control by T-test. (C) Quantitative Western blot analysis of Ube3A protein. Protein lysates were collected from hippocampal neurons at E18 + 8 DIV following stimulation with the indicated agent for seven hours. This experiment was performed three times independently and the data were normalized to the control and are presented as means +/- SEM. * indicates $P < 0.05$ in analysis of

statistical significance in pairwise comparison to control by T-test. (D) Real-time PCR examining Ube3A and GAPDH mRNA levels in extracts from hippocampi of control mice injected with saline (ctl) or mice injected with kainic acid (kainate) to induce seizures. The expression of Ube3A and GAPDH is normalized to the expression of beta-tubulin which serves as an internal standard. Data are presented as mean +/- SEM from three independent experiments. * indicates statistical significance in pairwise comparison: $P < 0.01$ T-test. (E) Quantitative Western blot analysis of Ube3A protein from mice 2.5 hours after injection with saline (ctl) or kainic acid (seized) to induce seizures. Data are presented as mean +/- SEM from three independent experiments. * indicates statistical significance in pairwise comparison $P < 0.05$ T-test. (F) Quantitative Western blot analysis of Ube3A protein from mice housed in standard laboratory cages (control) or placed in cages with novel objects (enriched) for 2.5 hours. Data are presented as mean +/- SEM from three independent experiments. * indicates statistical significance in pairwise comparison $P < 0.05$ T-test. (G) Real-time PCR analysis of the three Ube3A transcripts from mRNA extracted from hippocampal neurons at E18 + 10 DIV stimulated for 0, 1, or 5

hours with 55 mM KCl. Data are presented as mean +/- SEM from three independent experiments. * indicates statistical significance in pairwise comparison: P< 0.01 T-test.

Figure S2, related to Figure3. Ube3A mediates the polyubiquitination and degradation of Arc. (A) Western blot analysis of protein lysates made from brains of wild type and Ube3A knockout mice two hours following kainate acid injection. Immunoprecipitations were performed with an anti-Ube3A antibody and blotted with an anti-Arc antibody to reveal co-immunoprecipitated Arc. Images presented are representative of experiments performed on four independent sets of wildtype and Ube3A knockout mice. (B) Protein lysates were prepared from HEK293T cells transfected with Myc-Arc and HA-tagged ubiquitin and the indicated constructs and then treated with either vehicle control or the proteasome inhibitor MG132 (10 μM, 8 hours). Arc was then immunoprecipitated using the anti-Myc antibody 9E10, and Western blot analysis was performed using an anti-Arc antibody to reveal both non-ubiquitinated and ubiquitinated forms of Arc. (C) Mass spectrometric peaks reveal

that Ube3A catalyzes the ubiquitination of Arc on lysine 269. Top panel reveals the peptide assigned to the spectra on the bottom. Note the presence of two glycine residues covalently linked to the first lysine of this peptide which is indicative of ubiquitin being attached to that specific residue. The spectra depicted in the bottom panel shows the intensity of peaks on the Y-axis and the mass:charge ratio on the X-axis. Additional data not pictured here reveal the presence of ubiquitinated lysine 268 as well. (D) Similar to (C) but this spectra reveals the presence of ubiquitin conjugates on ubiquitin isolated from Arc immunoprecipitates, suggesting that Arc is polyubiquitinated by Ube3A.

Figure S3, related to Figure 4. Ube3A RNAi reduces Ube3A protein expression. (A) Western blot analysis of Ube3A from protein lysates prepared from HEK293T cells transfected with the indicated construct(s). (B) Representative image of a hippocampal neuron transfected with Ube3A shRNA and GFP. An untransfected neuron in the same field shows Ube3A protein levels in the absence of Ube3A shRNA expression. (C) Representative images of E18 + 14 DIV hippocampal

neurons transfected with GFP and the indicated constructs at 10 DIV and then stained for plasma membrane-expressed GluR1. Arrows mark the dendrites of transfected neurons to make it easier to distinguish the dendrites of transfected from untransfected neurons. The inset on the right is a zoomed in image of the boxed region in the merged panel. Yellow staining is indicative of the presence of GluR1 puncta on the GFP-expressing dendrite of the transfected neuron. (D) Quantification of dendritic spine density from E18 + 14 DIV hippocampal neurons transfected at 10 DIV with GFP and vector control, either of two shRNAs targeting Ube3A (Ube3A RNAi 1 or 2) or scrambled control shRNA (Ube3A scRNAi 1). Data are presented as mean +/- SEM from three independent experiments. (E) Quantification of the overlap of PSD95 and synapsin1 puncta on E18 + 14 DIV hippocampal neurons transfected at 10 DIV with GFP and vector control, either of two shRNAs targeting Ube3A (Ube3A RNAi 1 or 2) or scrambled control shRNA (Ube3A scRNAi 1). Data are normalized to control and presented as mean +/- SEM from three independent experiments.

Figure S4, related to Figure 5. (A) Representative images of surface GluR1 expression from E18 + 17 DIV hippocampal neurons transfected with vector control, Ube3A, or Ube3A C833A. Transfected neurons are indicated in green, and GluR1 staining is depicted in red. Yellow staining is indicative of the presence of GluR1 puncta on the GFP-expressing dendrite of the transfected neuron. (B) Western blot analysis of extracts from HEK293T cells transfected with Arc alone, or in combination with either of two Arc shRNA constructs (RNAi 1 or 2), either of two control shRNAs (scRNAi 1 or 2), or either of two forms of Arc that are subtly mutated and thus resistant to the shRNAs (Arcres 1 or 2). Western blots were then performed on lysates from the transfected cells using an anti-Arc antibody. (C) Quantification of surface expression of GluR1 receptors from E18 + 19 DIV hippocampal neurons transfected with GFP and vector control, Ube3A RNAi, Ube3A scRNAi, Arc RNAi, or Arc scRNAi. Data are presented as mean +/- SEM from three independent experiments. * indicates statistical significance $P < 0.05$, ANOVA, with Bonferroni correction for multiple comparison.

Figure S5, related to Figure 5. Representative images of surface GluR1 expression from E18 + 16 DIV hippocampal neurons transfected at 10 DIV with Ube3A shRNA, Arc shRNA, Ube3A shRNA + Arc shRNA or Ube3A shRNA + Arc scRNA. Left panels indicate GFP, middle panels indicate surface GluR1 expression, and right panels are composite images of GFP and surface GluR1 staining. Arrows point to transfected dendrites facilitating visualization of surface GluR1 in transfected neurons.

Figure S6, related to Figure 6. Representative array tomography images obtained from hippocampal sections of P21 Ube3A knockout and wild type littermates stained with anti-GluR1 and anti-SV2 antibodies (top two panels) or anti-NR1 and anti-SV2 antibodies (bottom two panels). Scale bar represents 1 μm. From the images it can be seen that some GluR1 puncta are in close apposition to SV2 puncta and other GluR1 puncta are not proximal to SV2 puncta (white arrows point to representative GluR1 puncta not associated with SV2 puncta; yellow arrows point to representative GluR1 puncta that are associated with SV2 puncta). The percentage of GluR1 puncta associated with SV2 is significantly higher in wild type hippocampi compared

to Ube3A knockout hippocampi. We would like to note that SV2 is a synaptic vesicle associated protein and as synaptic vesicles are often fairly distant from post-synaptic components, there are a number of SV2 puncta that are not associated with any post-synaptic markers.

Figure S7, related to Figure 7. mIPSCs are unaltered in Ube3A knockout mice. (A) Representative mIPSC traces of hippocampal neurons from wild type (top) and Ube3A knockout neurons (bottom). (B) Quantification of mIPSC frequency from wild type (solid line) and Ube3A knockout (dashed line) mice. Data are presented as cumulative probability plots of interevent intervals and represent recordings from at least 15 neurons from at least three independent animals of each genotype. (C) Quantification of mIPSC amplitude from wild type (solid line) and Ube3A knockout (dashed line) mice. Data are presented as cumulative probability plots and represent recordings from at least 15 neurons from at least three independent animals of each genotype.

Supplemental Experimental Procedures:

Quantification of Synapse Density:

At 14-18 DIV, cultured hippocampal neurons were fixed in 2% formaldehyde/4% sucrose for 2 minutes at room temperature and then transferred to 100% methanol for 10 minutes at –20°C. Coverslips were washed three times with PBS and incubated 1 hour in GDB (0.1% gelatin, 0.3% TritonX-100, 4.2% 0.4 M phosphate buffer, 9% 5M NaCl). Primary antibodies were incubated for 1 hour in GDB at room temperature at the indicated concentrations: PSD-95 (mouse, 1:200; Affinity BioReagents), Synapsin I (rabbit, 1:200; Chemicon), Gad67 (mouse, 1:100; Chemicon), $GABA_A$ 2 (rabbit, 1:100, Chemicon). Coverslips were then washed three times with PBS for ten minutes each and then incubated with Cy3- and Cy5-conjugated secondary antibodies (1:300 each; Jackson ImmunoResearch Laboratories) in GDB for one hour at room temperature. Coverslips were then washed three times with PBS for ten minutes each, dipped briefly in water, and mounted on glass slides using Aquamount (Lerner Laboratories). Synapse density

was quantified as the overlap of GFP, pre-synaptic marker and post-synaptic marker using Metamorph software and custom macros as previously described (Paradis et al., 2007).

Mass Spectrometry:

The sample was separated by SDS-PAGE on a 4-12% NuPAGE gel (Novex/Invitrogen). The gel band was excised and in-gel digested using trypsin prior to mass spectrometric analysis. All LC/MS experiments were performed by using a LTQ-FT ICR mass spectrometer (Thermo Finnegan, San Jose, CA) coupled to a microscale capillary HPLC (Famos micro-autosampler (LC Packings, Sunnyvale, CA) driven by an Eksigent). Columns were packed in-house by using Magic C18 beads (5 μm particle size, 200 Å pore size; Michrom BioResources, Auburn, CA. Buffer A was 97.3% H_2O/ 2.5% acetonitrile/0.2% formic acid; buffer B was 97.3% acetonitrile /2.5% water/0.2% formic acid; and the loading buffer was buffer A plus 5% formic acid). Data were searched against the mouse IPI database v3.09.fasta using the Paragon and Mascot Algorithms. Mass additions for modifications such as

carbamidomethylated cysteine and ubiquitinated lysine were permitted to allow for the detection of these modifications. A confidence score of 99 was required for a peptide for the Paragon algorithm and for Mascot our cutoff score was 40. All modification sites were manually confirmed by interrogating the data.

Acute slice preparation:

Animals were handled in accordance with Federal guidelines and protocols approved by Children's Hospital, Boston. Hippocampal slices were prepared from wild type or Ube3A knockout mice between postnatal days 15 and18 (P15–P18). Animals were deeply anesthetized by inhalation of isoflurane. The cerebral hemispheres were quickly removed and placed into ice cold choline-based artificial cerebrospinal fluid (choline ACSF) containing (in mM): 110 choline chloride, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 7 $MgCl_2$, 25 glucose, 1 $CaCl_2$, 11.6 ascorbic acid, and 3.1 pyruvic acid, and equilibrated with 95% O_2/5% CO_2. Tissue was blocked and transferred into a slicing chamber containing choline-ACSF. Transverse hippocampal slices (300 μm) were cut with a Leica VT1000s (Leica Instruments,

Nussloch, Germany) and transferred into a holding chamber containing ACSF consisting of 127 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 25 mM glucose and were equilibrated with 95% O_2/5% CO_2. Slices were incubated at 31°C for 30–45 min and then left at room temperature until recordings were performed.

Electrophysiology:

Whole-cell recordings were obtained from CA1 pyramidal cells visualized under IR-DIC. mEPSC and mIPSC recordings were performed and analyzed as described previously (Lin et al., 2008). Whole-cell recordings were obtained from CA1 pyramidal cells visualized under IR-DIC. Recording pipettes were pulled from borosilicate glass capillary tubing with filaments to yield tips of 2.5-4.5 MΩ resistance. Spontaneous miniature inhibitory postsynaptic potentials (mIPSC) were recorded with pipettes filled with (in mM): 147 CsCl, 5 Na_2- phosphocreatine, 10 HEPES, 2 MgATP, 0.3 Na_2GTP, and 1 EGTA. Spontaneous miniature excitatory synaptic potentials (mEPSC) and AMPA/NMDA current ratios were recorded with

pipettes filled with (in mM): 120 Cesium Methanesulfonate, 10 HEPES, 4 $MgCl_2$, 4 Na_2ATP, 0.4 Na_2GTP, 10 Na_2-phosphocreatine, and 1 EGTA. Intracellular solutions were adjusted to pH 7.3 with CsOH and were 290-300 mOSM. Inhibitory events were pharmacologically isolated by bath application of tetrototoxin (0.5 μM, Tocris Bioscience, Ellisville, Missouri), (R)-CPP (10 μM, Tocris Bioscience, Ellisville, Missouri), and NBQX disodium salt (10 μM, Tocris Bioscience, Ellisville, Missouri), to antagonize voltage-gate sodium channels (VGSC), NMDA receptors, and AMPA receptors, respectively. Excitatory events were isolated with tetrodotoxin, and picrotoxin (50 μM, Tocris Bioscience, Ellisville, Missouri) to antagonize VGSC and $GABA_A$ receptors, respectively. Additionally, cyclothiazide (10 μM, Tocris Bioscience, Ellisville, Missouri) was added to the bath to reduce AMPAR desensitization and facilitate measurement and quantification of mEPSCs. AMPA/NMDA ratios were measured in the presence of picrotoxin. For mIPSC and mEPSC recordings, cells were held at -70 mV; AMPA/NMDA current ratios were measured holding the cell at -70 and +40 mV to assess AMPAR and NMDAR mediated currents, respectively. Data were acquired using Clampex10 software and an Axopatch 200B amplifier.

Current traces were filtered at 5 kHz, digitized at 10 kHz, and acquired in 10 second intervals. The cell capacitance, input resistance and series resistance were monitored with a 5mV hyperpolarizing step delivered at the beginning of each sweep. Cells were discarded if the series resistance was greater than 25 MΩ. Data were analyzed in IgorPro 5.05 using custom software modified from Shankar et al., 2007. For mIPSC and mEPSC analyses, the root mean square (RMS) was calculated for the first 150 ms of each trace and the event threshold set to be 1.5 times the RMS. Currents were counted as events if they had a rapid rise time (1.5 pA/ms), an exponential decay ($2 < \tau < 200$ ms, $1 < \tau < 50$ ms for mIPSC and mEPSC, respectively), and crossed the event threshold. Data are displayed as the cumulative distribution of all events recorded from a given genotype. Statistical significance was determined by randomly selecting 50 events from each cell, pooling events from cells of the same genotype and running a Kolmogorov-Smirnov test on the pooled data. $p < 0.05$ was considered statistically significant. Furthermore, data were randomly resampled and the analysis was repeated > 10 times. For each resampling, $p > 0.05$ for all parameters. For AMPA/NMDA current ratios, an

extracellular stimulating electrode was placed in stratum radiatum, approximately 200-300 μm from the patched cell in the direction of CA3. Brief current pulses were delivered (0.2 ms) and the evoked response was measured while holding the cell at -70 and +40 mV. The peak current measured at -70 mV was used in the numerator to represent the AMPAR-mediated response. The current amplitude 50-70 ms after the current peak measured at +40 was used in the denominator to represent the NMDAR-mediated response. Data are displayed as the geometric mean ± SEM. Significance was determined by students t-test of the log ratio measured from each cell.; $p<0.05$ was considered significant.

Western Blotting and Antibodies:

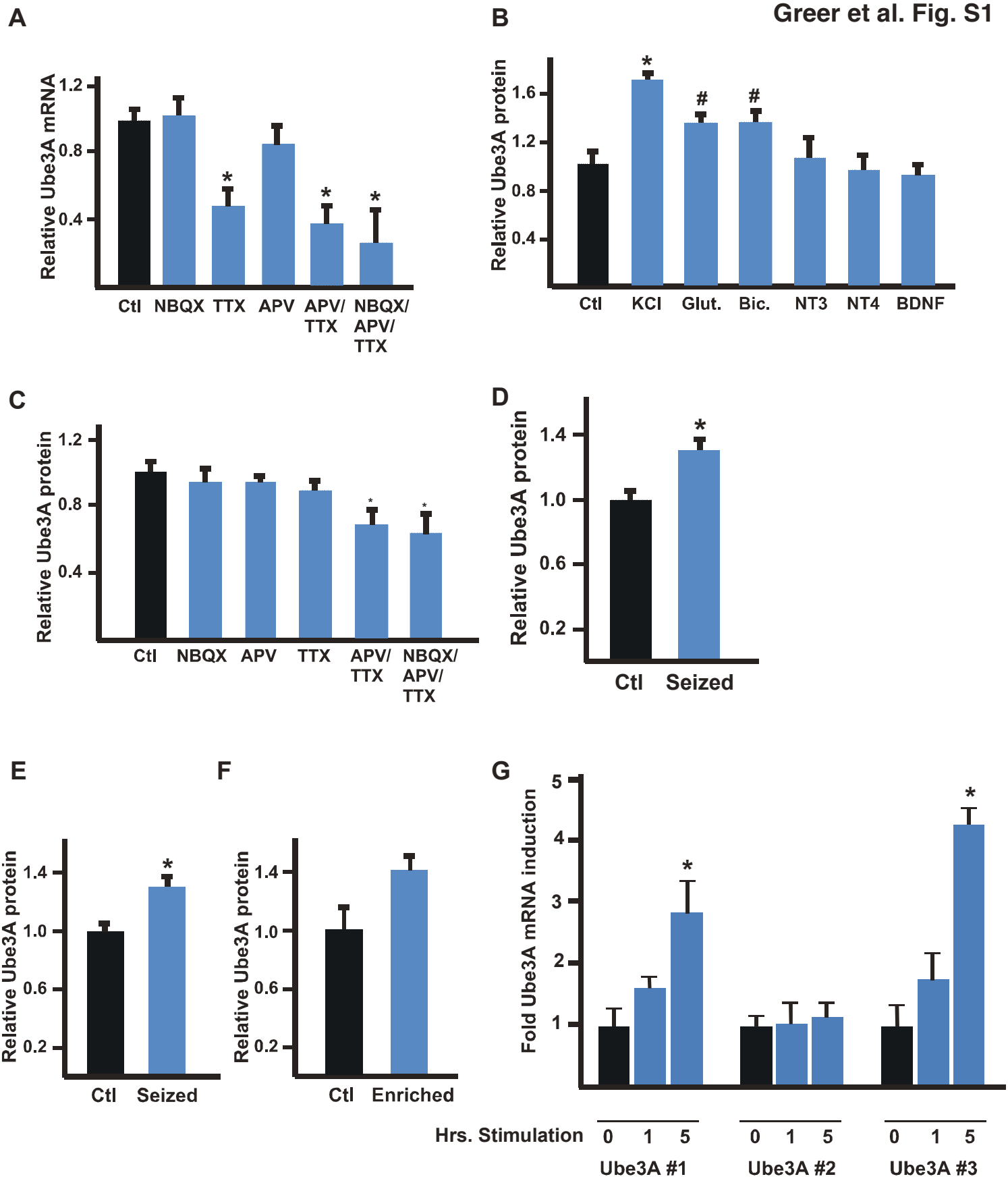
For Western blotting, whole rat or mouse brains or cultured cells were collected and homogenized in RIPA buffer (50 mM Tris pH 7.5-8.0, 150 mM NaCl, 1% TritonX-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF supplemented with complete protease inhibitor cocktail tablet (Roche)). Samples were boiled for 3-5 minutes in SDS sample buffer, resolved by SDS PAGE, transferred to

nitrocellulose, and immunoblotted. Antibodies specific for Ube3A (Sigma), MEF2D (BD Biosciences), MEF2A (Santa Cruz Biotechnology), Arc (Santa Cruz Biotechnology), HA (Roche), and beta-actin (Abcam) are all commercially available. Antibodies for MeCP2 and phospho MeCP2 (Zhou et al., 2006) as well as Vav2 (Cowan et al., 2005) were previously described.

Supplemental References:

Cowan, C.W., Shao, Y.R., Sahin, M., Shamah, S.M., Lin, M.Z., Greer, P.L., Gao, S., Griffith, E.C., Brugge, J.S., and Greenberg, M.E. (2005). Vav family GEFs link activated Ephs to endocytosis and axon guidance. Neuron *46*, 205-217

Supplemental Figure



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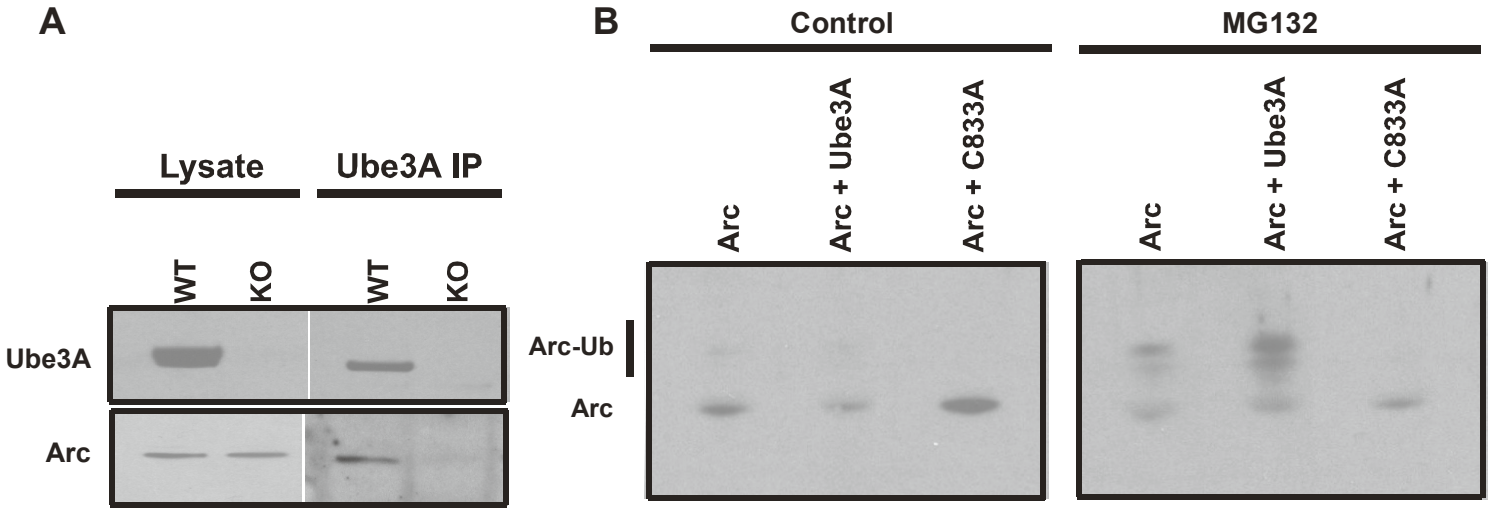

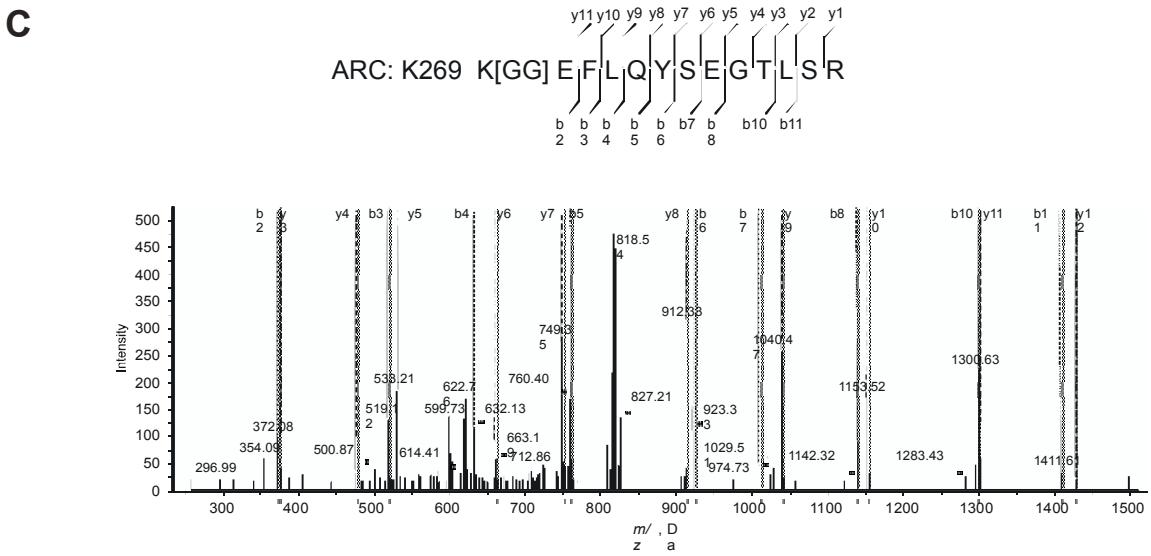

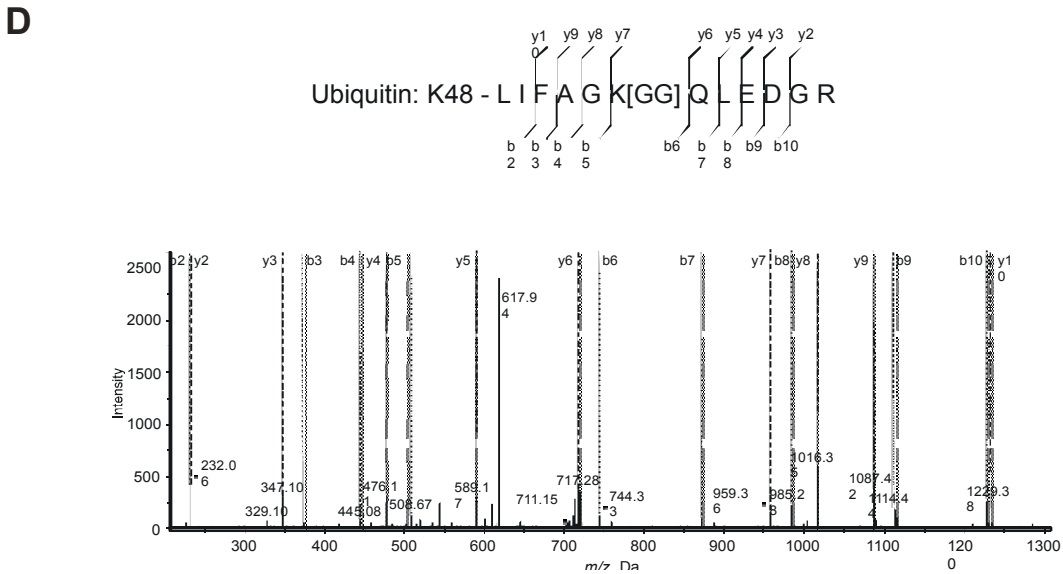

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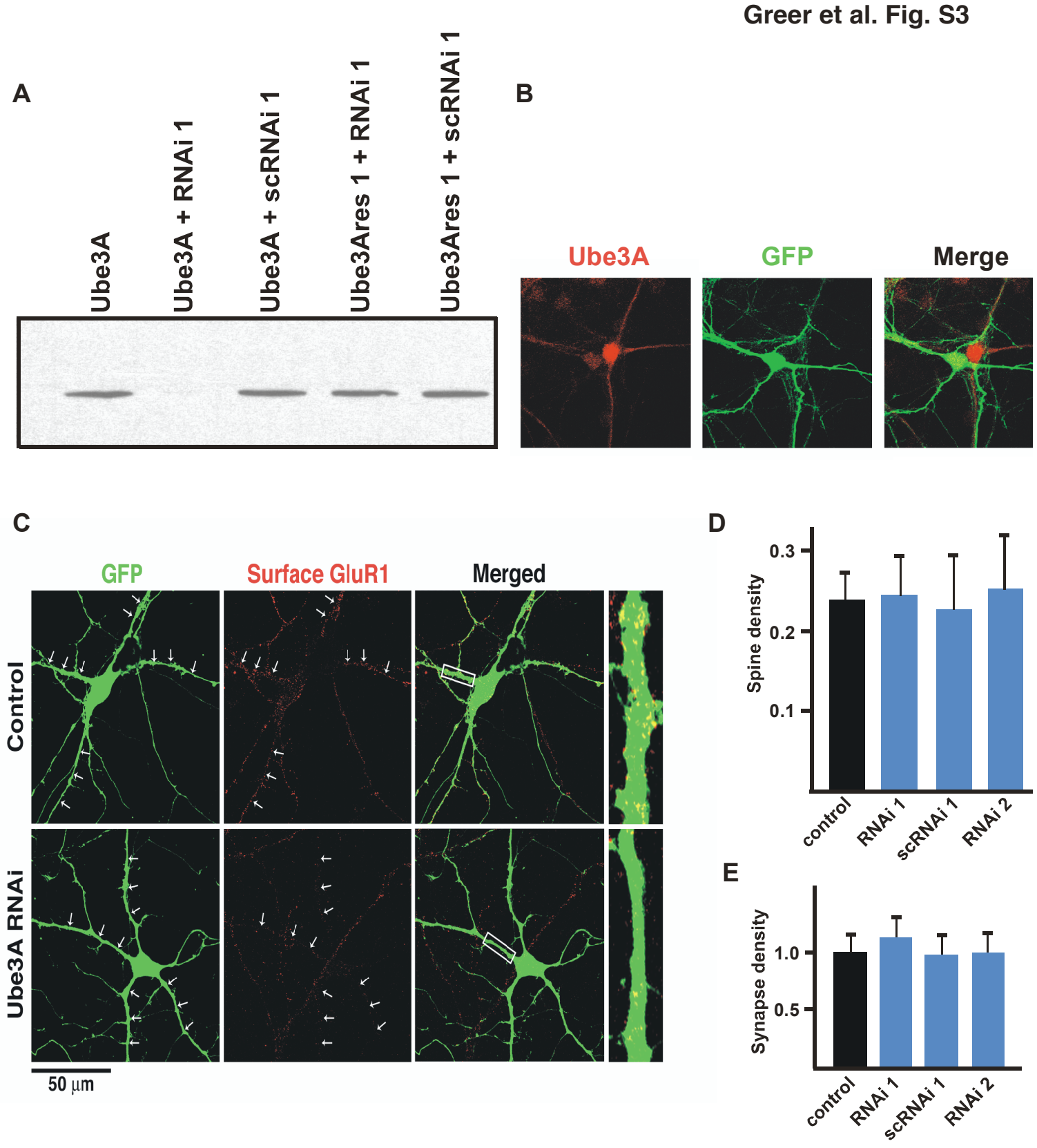

Supplemental Figure4

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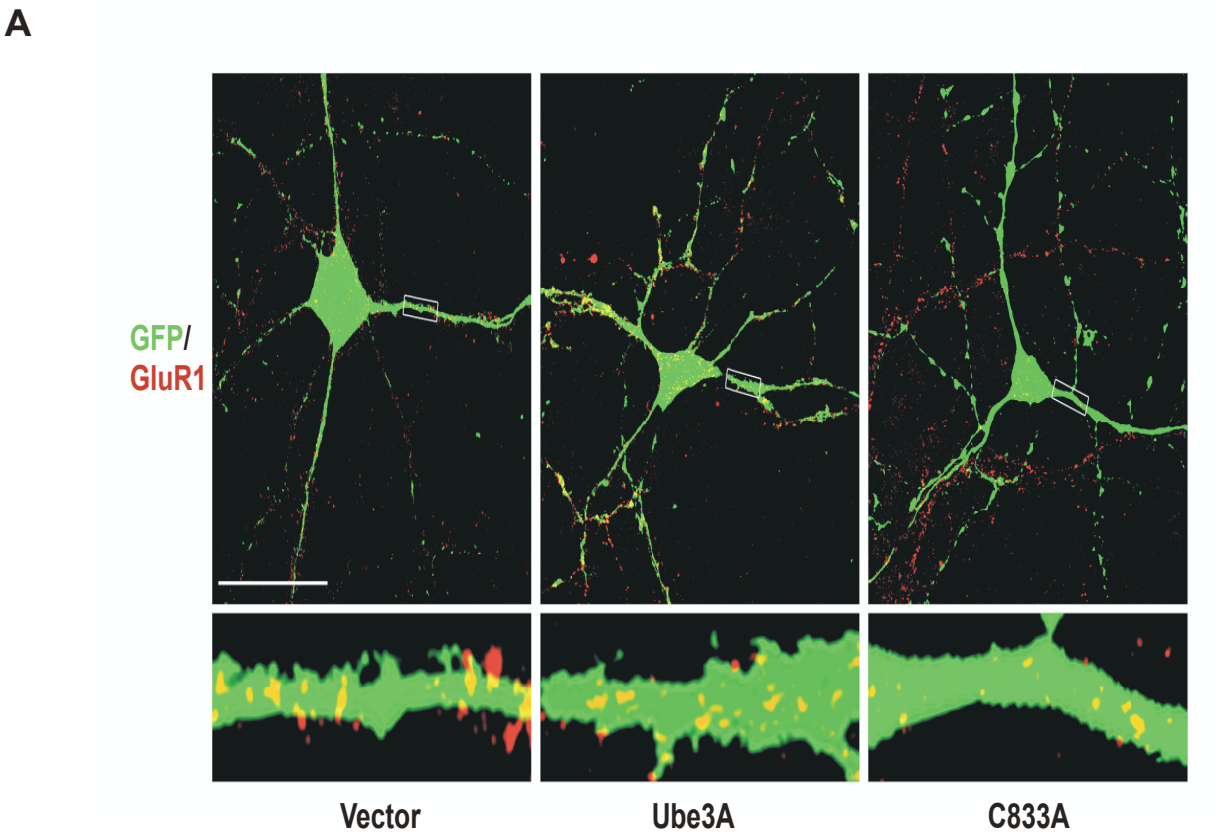
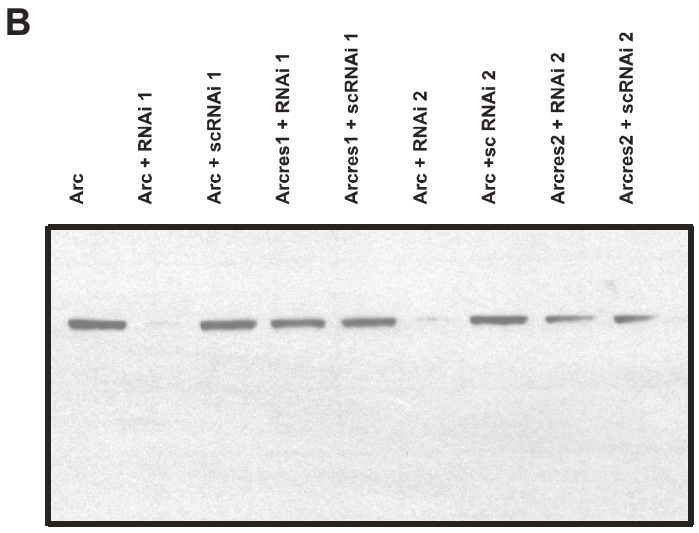
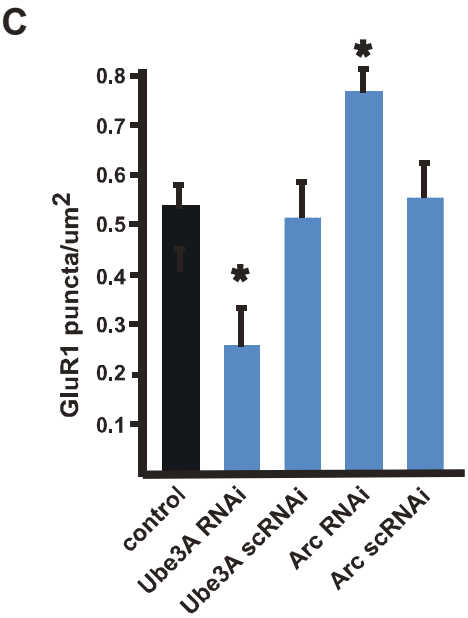
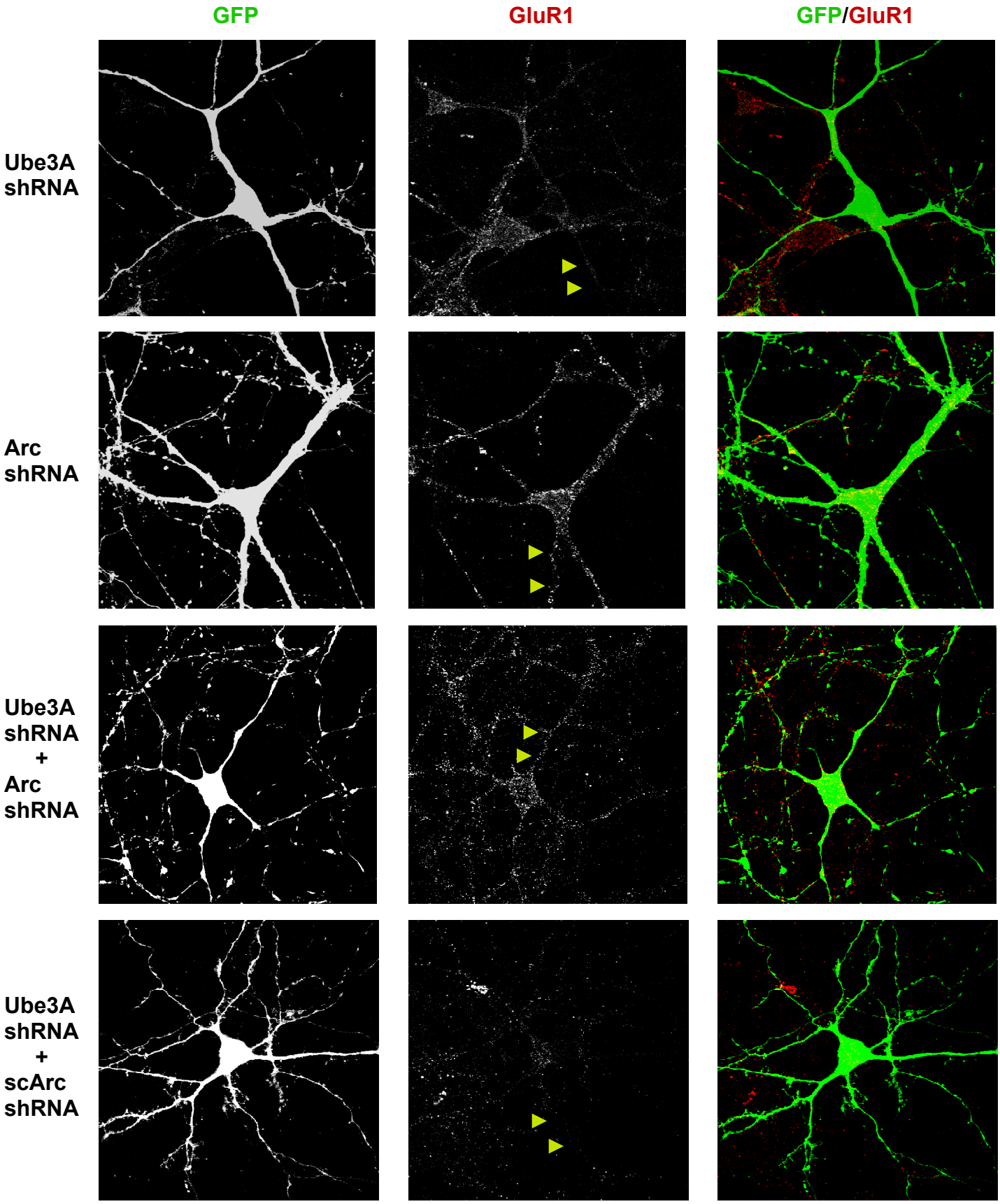






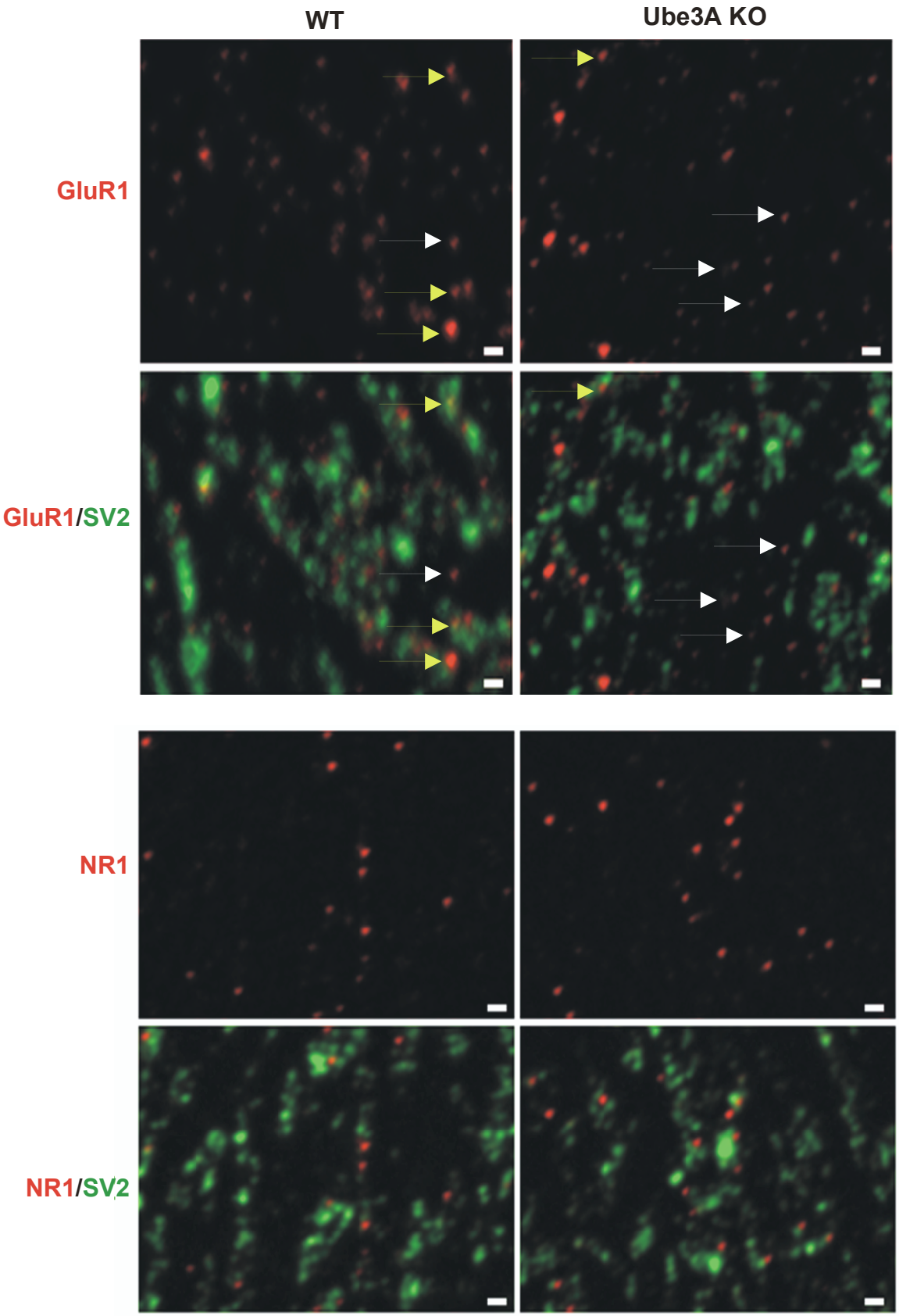
Supplemental Figure5

Greer et al. Fig.S5



Supplemental Figure6

Greer et al. Fig.S6



Supplemental Figure7

Greer et al. Fig.S7