mTORC1-S6K1 Inhibition or mTORC2 Activation Improves Hippocampal Synaptic Plasticity and Learning in Angelman Syndrome Mice

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Jiandong Sun, Yan Liu, Jennifer Tran, Patrick O'Neal, Michel Baudry, Xiaoning Bi*

***Corresponding author:** Dr. Xiaoning Bi
Dept. of Basic Medical Sciences, COMP
Western University of Health Sciences
701 E. Second Street
Pomona, CA 91766-1854
Email: xbi@westernu.edu

Supplementary information

Supplementary materials and methods

Rapamycin treatment

Rapamycin (LC Laboratories) was dissolved in 100% DMSO and stored as a stock solution of 5 mg/ml at -20 ˚C. A working solution was prepared immediately before injection at a final concentration of 0.5 mg/ml rapamycin, 10% DMSO, 5% Tween-80, and 5% PEG400.

Fear conditioning

Mice were placed in the fear-conditioning chamber (H10-11M-TC, Coulbourn Instruments) 30 min after the last injection. The conditioning chamber was cleaned with 10% ethanol to provide a background odor. A ventilation fan provided a background noise at ~55 dB. After a 2 min exploration period, three tone-footshock pairings separated by 1 min intervals were delivered. The 85 dB 2 kHz tone lasted 30 s and co-terminated with a footshock of 0.75 mA and 2 s. Mice remained in the training chamber for another 30 s before being returned to home cages. Context test was performed one day after training in the original conditioning chamber with 5 min recording. On day three, animals were subjected to cue/tone test in a modified chamber with different texture and color, odor, background noise, and lighting. After 5 min recording, mice was exposed to a tone (85 dB, 2 kHz) for 1 min. Mouse behavior was recorded with the Freezeframe software and data were analyzed using the Freezeview software (Coulbourn Instruments). Motionless bouts lasting more than 1 s were considered as freezing. The percent of time animal froze was calculated and group means with S.E.M. of % freezing were analyzed.

P2/S2 fractionation and Western blot analysis

For P2/S2 fractionation, frozen hippocampus tissue was homogenized in ice-cold HEPES-buffered sucrose solution (0.32 M sucrose, 4 mM HEPES, pH 7.4) with protease inhibitors. Homogenates were centrifuged at 900 g for 10 min to remove large debris (P1). The supernatant (S1) was then centrifuged at 11,000 g for 20 min to obtain crude synaptosomal (P2) and cytosolic (S2) fractions. P2 pellets were sonicated in RIPA buffer (10 mM Tris, pH 8, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Protein concentrations were determined with a BCA protein assay kit (Pierce).

Western blots were performed according to published protocols (Sun et al., 2015a). Briefly, 10 ~ 25 μg of total proteins were separated on 8% SDS-PAGE gels and transferred to PVDF membrane (Millipore). After blocking with 3% bovine serum albumin (BSA) for 1 h, membranes were incubated with specific antibodies overnight at 4 ℃. Primary antibodies against the following proteins were used: UBE3A (1:2000, Sigma E8655, Saint Louis, MO), phospho-mTOR Ser2448 (1:1000, Cell Signaling Technology 2971, Beverly, MA), phospho-mTOR Ser2481 (1:1000, Cell Signaling Technology 2974, Beverly, MA), mTOR (1:1000, Cell Signaling Technology 2972, Beverly, MA), phospho-TSC2 Thr1462 (1:1000, Cell Signaling Technology 3611, Beverly, MA), TSC2 (1:1000, Cell Signaling Technology 4308, Beverly, MA), phospho-S6K1 Thr389 (1:1000, Cell Signaling Technology 9205, Beverly, MA), S6K1 (1:1000, Cell Signaling Technology 9202, Beverly, MA), phospho-S6 Ser235/236 (1:1000, Cell Signaling Technology 2211, Beverly, MA), phospho-S6 Ser240/244 (1:1000, Cell Signaling Technology 2215, Beverly, MA), S6 (1:1000, Cell Signaling Technology 2217,

Beverly, MA), PKCα (1:1000, Cell Signaling Technology 2056, Beverly, MA), phospho-Rictor Thr1135 (1:1000, Cell Signaling Technology 3806, Beverly, MA), Rictor (1:1000, Bethyl A300-459A, Montgomery, TX), Arc (1:200, Santa Cruz sc-15325, Dallas, TX), GluA1 (1:1000, Millipore AB1504, Temecula, CA) and β-actin (used as loading control; 1:10,000, Sigma A5541, Saint Louis, MO). Following incubation in primary antibodies, membranes were incubated with IRDye secondary antibodies for 2 h at room temperature. Antibody binding was detected with the Odyssey® imaging system. Images were analyzed for differences in levels using LI-COR Image Studio Software.

Immunohistochemistry

In brief, tissue sections were blocked for 1 h at room temperature (RT) in 5% goat serum with 0.3% Triton X-100 in PBS. After blocking, sections were incubated in primary antibody diluted in a solution of 1% BSA with 0.3% Triton X-100 in PBS at 4˚C overnight. The following primary antibodies were used: Anti-Arc (1:50, Santa Cruz sc-15325, Dallas, TX), and anti-PSD95 (1:400, Thermo Scientific MA1-045, Waltham, MA). The following day, sections were briefly washed at RT 3 times in PBS followed by incubation with secondary antibodies (Alexa-488 anti-rabbit, 1:400; Alexa-594 anti-mouse, 1:200) diluted in a solution of 1% BSA with 0.3% Triton X-100 in PBS at RT for 2 h with gentle rocking. Sections were then washed 4 times at RT in PBS and mounted using Vectashield mounting media with DAPI (4’6’-diamindo-2-phenylindole) to stain cell nuclei. Brain sections were imaged using a Nikon confocal microscope with a 60x objective.

Acute hippocampal slice preparation and electrophysiology

Acute hippocampal transversal slices (350 µm-thick) were prepared from adult male mice as previously described (Baudry et al., 2012) and recording was done in an interface recording chamber; slices were continuously perfused with oxygenated (95 % O_2/5 % CO_2) and preheated (33 ± 0.5 ℃) artificial cerebrospinal fluid (aCSF) (in mM) [110 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 1.24 KH_2PO_4, 10 D-glucose, 27.4 $NaHCO_3$]. Field EPSPs (fEPSPs) were elicited by stimulation of the Schaffer collateral pathway in CA1 stratum radiatum. Before each experiment, the input/output (I/O) relation was examined by varying the intensity of the stimulation. Long-term potentiation was induced using theta burst stimulation (10 bursts at 5 Hz, each burst consisting of 4 pulses at 100 Hz). Data were collected and digitized by Clampex; the slope of fEPSP was analyzed.

Actin polymerization assay

Actin polymerization was quantified by measurement of "rhodamine-phalloidin fluorescent enhancement", as previously described with minor modifications (Briz et al., 2015). In brief, hippocampal slices (3-6 pooled slices) were fixed in PBS containing 4% PFA and 1% octyl-β-D-glucopyranoside for 15 min at room temperature. After two rinses with PBS, slices were homogenized and incubated with 15-30 nM phalloidin-TRITC (Invitrogen) for 30-45 min at room temperature. Protein concentrations of the homogenates were determined with a BCA protein assay kit (internal control). After 3 washes, lysates were collected in 200 µl/slice of PBS, and fluorescent intensity (excitation and emission wavelength were 546 and 590 nm, respectively) was determined using a POLARstar Omega fluorescence polarization microplate reader (BMG Laboratory).

Supplementary figures

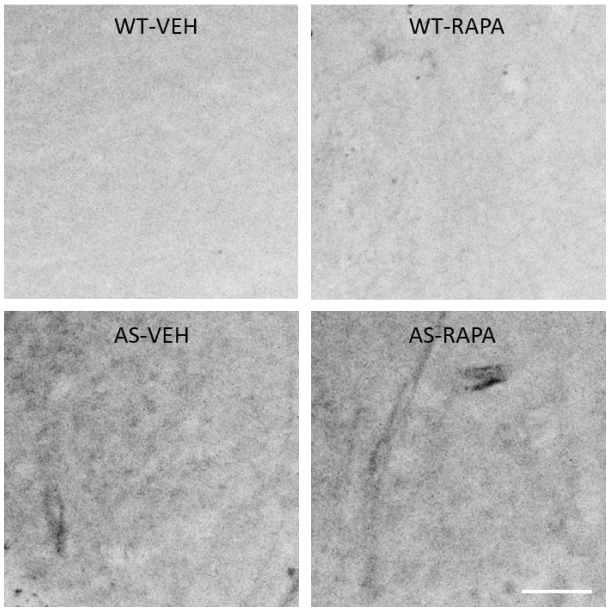

Figure S1 Representative images of *in situ* phalloidin staining after LFS in CA1 region of hippocampus from vehicle- or rapamycin-treated WT or AS mice. Scale bar = 20 μm. VEH, vehicle; RAPA, rapamycin

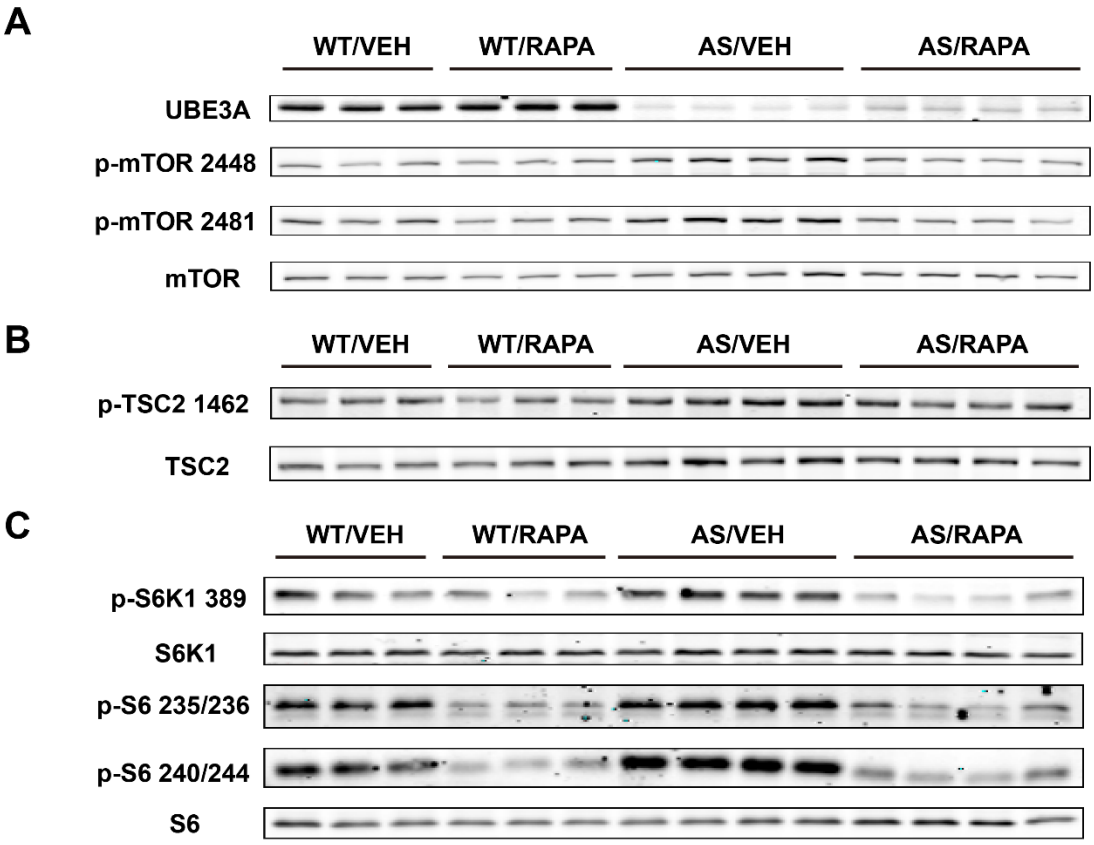

Figure S2 Representative images of western blots labeled with UBE3A, p-mTOR (2448), p-mTOR (2481), mTOR, p-TSC2 (1462), TSC2, p-S6K1 (389), S6K1, p-S6 (235/236 and 240/244), and S6 in P2 fractions. VEH, vehicle; RAPA, rapamycin

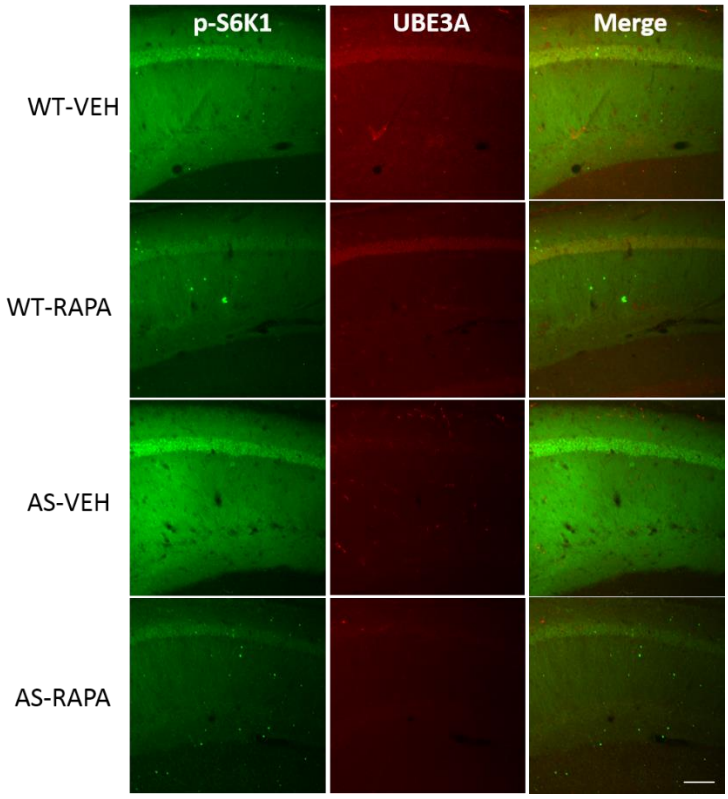

Figure S3 Representative immunostaining images of UBE3A (red) and p-S6K1 (green) in CA1 region of hippocampus from vehicle- or rapamycin- treated mice. Scale bar = 100 μm. VEH, vehicle; RAPA, rapamycin

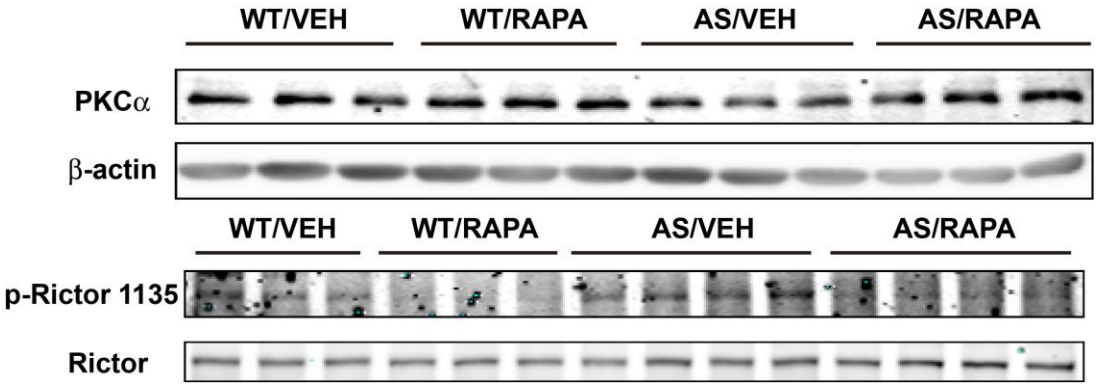

Figure S4 Representative images of western blots labeled with PKCα, β-actin, p-Rictor (1135) and Rictor in P2 fractions. VEH, vehicle; RAPA, rapamycin

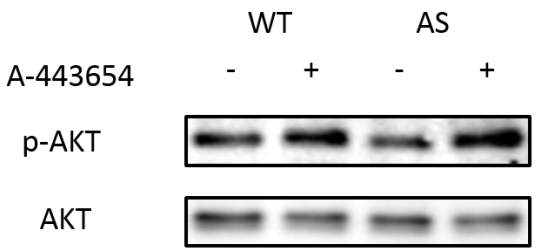

Figure S5 Representative images of Western blots labeled with p-AKT and AKT in P2 fractions of WT and AS hippocampal slices treated with vehicle or A-443654