SUPPLEMENTAL MATERIALS

Ube3a reinstatement mitigates epileptogenesis in Angelman syndrome model mice

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Supplemental Methods

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and were performed in accordance with the guidelines of the U.S. National Institutes of Health. Mice were group-housed on a 12:12 light/dark cycle with *ad libitum* access to food and water. Male and female mice were used for experiments in equal genotypic ratios. Mice were randomly assigned to experimental groups.

Our AS mouse colony has been maintained on a C57BL/6J background for over 10 generations (JAX: 016590). AS model mice (*Ube3a*$^{m-/p+}$) and their wildtype (WT) littermates were generated by breeding *Ube3a*$^{m+/p-}$ females with WT males.

Female *Ube3a*$^{m+/FLOX}$ mice with paternal inheritance of a conditional *Ube3a* allele were crossed with males heterozygous for either NEX-Cre (38) (generously provided by Dr. Klaus-Armin Nave, Max Planck Institute of Experimental Medicine, Göttingen, Germany) or Gad2-Cre (JAX: 010802) in order to generate *Ube3a*$^{FLOX/p+}$*::NEX-Cre* or *Ube3a*$^{FLOX/p+}$*::Gad2-Cre* conditional mutants, respectively. NEX-Cre mice were maintained on a congenic C57BL/6J background; Gad2-Cre mice were backcrossed at least 6 generations onto the C57BL/6J background prior to breeding for experiments.

The laboratory of Dr. Ype Elgersma, ENCORE Center for Neurodevelopmental Disorders, Erasmus Medical Center, Rotterdam, the Netherlands, originally generated and provided *Ube3a*^{STOP} mice on a congenic 129S2/SvPasCrl background (39). *Ube3a*$^{m+/STOP}$ females (backcrossed over 6 generations onto the C57BL/6J background) were crossed with C57BL/6J *Tg(CAG-cre/Esr1*)5Amc/J* males (JAX: 004682) in order to generate heterozygous *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$ mutants and littermate controls.

Tamoxifen (TAM) treatment

Both male and female P21 or adult (P60–P120) *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$ and their WT littermates were injected with TAM to induce Cre-mediated deletion of the stop cassette. TAM (Sigma-Aldrich) was freshly prepared before each round of treatment by dilution in sterilized corn oil to a concentration of 20 mg/ml. Vehicle (Veh, corn oil) or TAM was injected daily (100 mg/kg, i.p.) for 7 consecutive days.

Flurothyl induced seizure and flurothyl kindling

Each mouse was placed in a 2 liter glass chamber inside of a chemical fume hood and allowed to habituate for 1 min before the top of the chamber was closed. 10% flurothyl (bis-2,2,2-trifluoroethyl ether; Sigma-Aldrich) in 95% ethanol was then infused at a rate of 200 µL/min onto a disk of filter paper (Whatman, Grade 1) suspended at the top of the chamber. Mice exhibit various stages of increasing seizure severity in response to flurothyl exposure, including myoclonic seizure (sudden involuntary jerk/shock-like movements involving the face, trunk, and/or limbs) and generalized seizure (also known as clonic-forebrain seizures that are characterized by clonus of the face and limbs, loss of postural control, rearing, and falling).

Generalized seizures can immediately progress into brain stem seizures manifested by tonic extension of the limbs (40). Upon emergence of a generalized seizure, the lid of the chamber was immediately removed, allowing for rapid dissipation of the flurothyl vapors and exposure of the mouse to fresh air. Mice were then returned to their home cage following recovery from behavioral seizures. One mouse at a time was tested in the flurothyl chamber, which was recharged with fresh filter paper, cleaned using water, and thoroughly dried between subjects.

For flurothyl kindling, flurothyl exposures were repeated once daily over eight consecutive days (induction phase). Mice were then given a 28-day rest period (incubation phase), during which they remained in their home cages with no flurothyl exposure. At the end of the incubation phase, mice were re-exposed to flurothyl once more on day 36 (retest). For *Ube3a*$^{FLOX/p+}$*::Gad2-Cre* and control mice, flurothyl exposures were repeated once daily over four consecutive days and once more on day 32. Mouse behavior during each flurothyl exposure was video-recorded and reviewed by investigators (blind to both genotype and treatment) who determined latency to the onset of both myoclonic and generalized seizures.

Hyperthermia-induced seizures

A rectal temperature probe was placed in the mouse's rectum using lubricant containing 0.5% bupivacaine and held in place by a small piece of sterile bandaging tape around the tail. Core body temperature was maintained at a command temperature ± 0.1°C using a rodent temperature controller (TCAT-2DF, Physitemp Inc.) and heat lamp positioned above the mouse. Mice were first held at 37.4 °C for 10 min before the body temperature was increased incrementally by 0.3°C per min until the emergence of behavioral seizures — characterized by

whole body clonus, wild running and jumping, and loss of postural control — or until a core body temperature of 42.5°C was reached. A video camera was used to record the experiments.

Repeated-low dose kainic acid induced seizures

A repeated-low dose kainic acid model of epilepsy was adopted in this study to evaluate seizure susceptibility involving limbic circuits. Kainic acid (Sigma-Aldrich) was prepared fresh in sterile distilled water at a concentration of 2 mg/ml, and injected (5 mg/kg, i.p.) once every 30 min until the onset of Class 4–5 seizures. Behavioral seizures were classified according to a modified Racine scale: Class 1, absence-like immobility; Class 2, hunching with facial or manual automatisms; Class 3, rearing with facial or manual automatisms and forelimb clonus; Class 4, repeated rearing with continuous forelimb clonus and falling; and Class 5, generalized tonic clonic convulsions with lateral recumbence or jumping and wild running followed by generalized convulsions. In order to identify behavioral and electrographic seizures, time-locked video-electroencephalography (EEG) recording in freely moving mice was performed and analyzed by investigators who were blind to genotype.

EEG recording in freely moving mice

For surgeries, adult (P60–P120) mice were anesthetized via intraperitoneal injections of ketamine (40 mg/kg) and xylazine (10 mg/kg), and 0.25% bupivacaine was applied topically for local analgesia. Stainless steel bipolar recording electrodes (Plastics One Inc.) were implanted in right dorsal hippocampus (coordinates from bregma: AP=-2.0 mm; L=1.6 mm; and D=-1.5 mm below dura), while ground electrodes were fastened to a stainless steel screw positioned on the skull above the cerebellum. Electrode positions were secured using dental cement.

Mice recovered for 7 days following surgery prior to the start of flurothyl kindling. A tethered system with a commutator (Plastics One Inc.) was used, allowing mice to roam freely within the home cage and enabling time-locked video-EEG recording of mouse brain function in conjunction with behavior. EEG recordings were amplified (1,000x) using single-channel amplifiers (Grass Technologies), sampled at a rate of 1000 Hz, and filtered with 0.3 Hz high-pass and 100 Hz low-pass filters. All electrical data were digitized with CED Micro1401 (Cambridge Electronic Design Ltd.) and analyzed with Spike2 software (Cambridge Electronic Design Ltd.). EEG spectrograms were generated in MATLAB (R2014b, the MathWorks Inc.) using a fast Fourier transform with bin sizes of 0.25 Hz.

Monitoring and detection of spontaneous recurrent seizures

To identify spontaneous recurrent seizures, mice were housed individually and monitored for 24 hr every other day during the 28-day incubation phase of the flurothyl kindling protocol. Video-EEG recordings were reviewed by investigators who were blind to genotype. Spontaneous recurrent seizures were defined electrographically as high frequency (> 5 Hz), high amplitude (> 2 X baseline) rhythmic epileptiform activity with clear initiation and extinction for a minimum duration of 5 sec. Behavioral correlates (modified Racine's 1–5) of these electrographic episodes were confirmed by reviewing the time-locked video recording.

Immunohistochemistry

Mice were anesthetized with euthasol (100 mg/kg, i.p.) and perfused with phosphate-buffered saline (PBS), pH 7.4, followed by phosphate-buffered 4% paraformaldehyde, pH 7.4. Fixed brains were then removed from the skull, cryoprotected, and sectioned using a Microtome

(Leica Biosystems). Serial 40 µm coronal sections were cut through the forebrain spanning the entire hippocampus. Adjacent sections of dorsal hippocampus were taken for immunofluorescent staining.

Sections were incubated in biotin-conjugated WFA lectin (1:1000, Sigma Aldrich L1516) and anti-NeuN (1:500, Millipore MAB377) or anti-Znt3 (1:500, Synaptic systems #197 004) primary antibodies overnight at 4°C. Sections were then washed 3 times in 1X PBS prior to incubation in streptavidin Alexa-568 (Invitrogen S11226) and goat anti-mouse IgG Alexa-488 (Invitrogen A11001), or goat anti-guinea pig IgG Alexa-568 (Invitrogen A11075), each diluted 1:500, for 40 min at room temperature. Stained sections were mounted on slides with VECTASHIELD antifade mounting medium and images were acquired on an automated inverted epifluorescent microscope, Zeiss AxioObserver Z1 fluorescence microscope (Carl Zeiss Inc.) using a EP Plan-Neofluar 10x/0.3 objective, metal halide light source, and a Zeiss AxioCam MR R3 camera.

WFA staining intensity was quantified from one section per mouse using ImageJ software (NIH). Measures of pixel brightness values (arbitrary unit, a.u.) were obtained within regions of interest (ROI) contoured around the molecular layer of dentate gyrus and somatosensory cortex. Background fluorescence was measured within the same section as the ROI, from a fixed anatomical location in the fimbria. To correct for background fluorescence, the mean gray value of the background was subtracted from the mean gray value of the ROI. Imaging and quantification were performed by an experimenter blind to genotype and treatment condition.

Western blotting

Mice were anesthetized with euthasol (100 mg/kg, i.p.) prior to decapitation and brain removal. Neocortical hemispheres were then rapidly isolated in ice-cold PBS, snap-frozen with liquid nitrogen, and stored at -80°C. Frozen neocortices were homogenized with a glass tissue homogenizer in ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% triton x-100, 0.1% SDS, 0.5% Na Deoxycholate) supplemented with 5 mM EDTA and a protease inhibitor cocktail (Roche 11873580001). Homogenates were cleared via 15,000 x rpm centrifugation for 20 min at 4°C and protein concentrations were determined using the BCA assay (Thermo Scientific). Next, protein samples (20 µg per lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were subsequently blocked for 1 hr at room temperature in PBST (10 mM Phosphate, 150 mM Sodium chloride, 0.1% Tween 20) containing 5% nonfat milk. Blocked membranes were incubated for 1 hr at room temperature with mouse anti-UBE3A (1:1,000, Clone 330, Sigma-Aldrich E8655) or mouse anti-β-Actin (1:1,000, Sigma-Aldrich A1978) primary antibodies diluted in PBST containing 5% bovine serum albumin (Sigma-Aldrich A4503). Following 3 washes with PBST, the membranes were incubated for 1 hr at room temperature with goat anti-mouse IgG DyLight™ 680-conjugated secondary antibodies (Invitrogen 35519) diluted 1:5,000 in the same diluent as the primary antibodies. The membranes were washed 3 times with PBST, followed by 3 washes in PBS, prior to imaging with the Odyssey imaging system (LI-COR).

Statistics

All experiments and analyses were performed blind to genotype and treatment, and all statistical analyses were performed using GraphPad Prism 7.04 software (GraphPad Software

Inc.). Unless otherwise noted, comparisons between two groups were analyzed using unpaired t-tests (two-tailed), while multi-group comparisons were analyzed using 2-way ANOVA with Bonferroni's or Tukey's *post hoc* test. $P < 0.05$ was considered significant.

Supplemental References

38. Goebbels S, Bormuth I, Bode U, Hermanson O, Schwab MH, and Nave KA. Genetic targeting of principal neurons in neocortex and hippocampus of NEX-Cre mice. *Genesis (New York, NY : 2000).* 2006;44(12):611-21.

39. Silva-Santos S, van Woerden GM, Bruinsma CF, Mientjes E, Jolfaei MA, Distel B, et al. Ube3a reinstatement identifies distinct developmental windows in a murine Angelman syndrome model. *The Journal of clinical investigation.* 2015;125(5):2069-76.

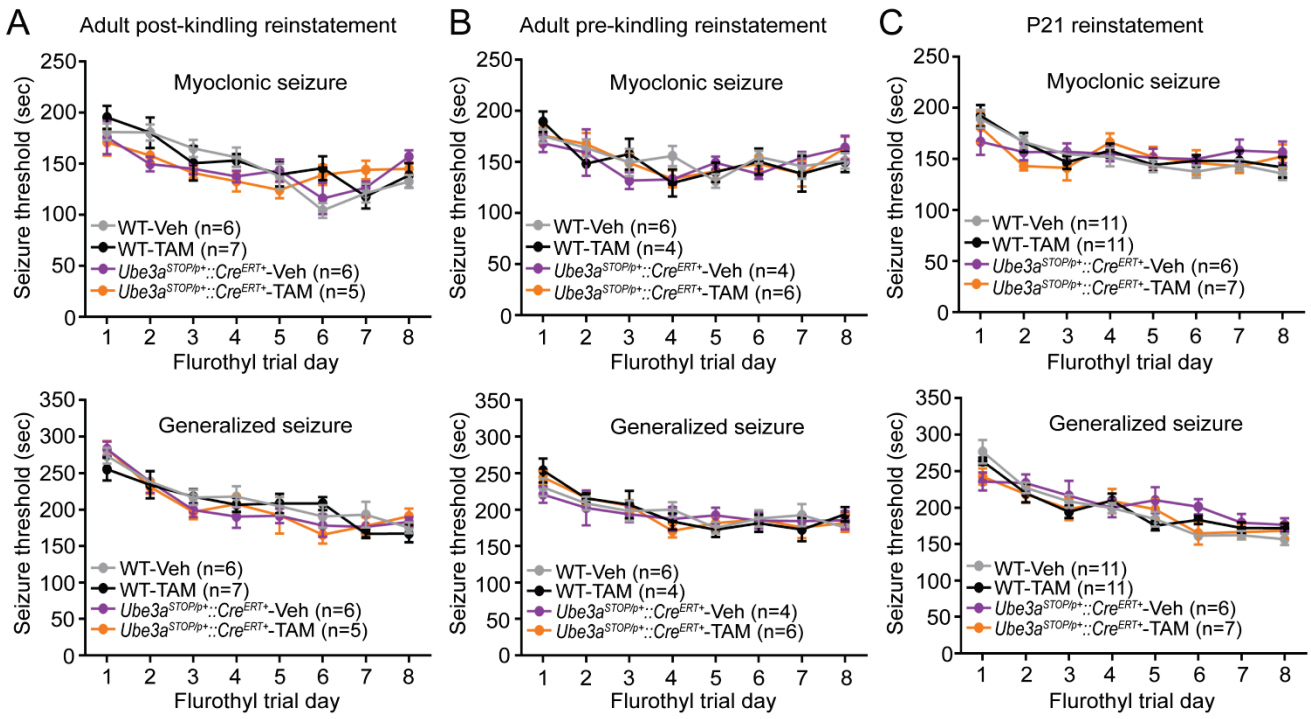
40. Samoriski GM, and Applegate CD. Repeated generalized seizures induce time-dependent changes in the behavioral seizure response independent of continued seizure induction. *J Neurosci.* 1997;17(14):5581-90.

Supplemental Table and Figures

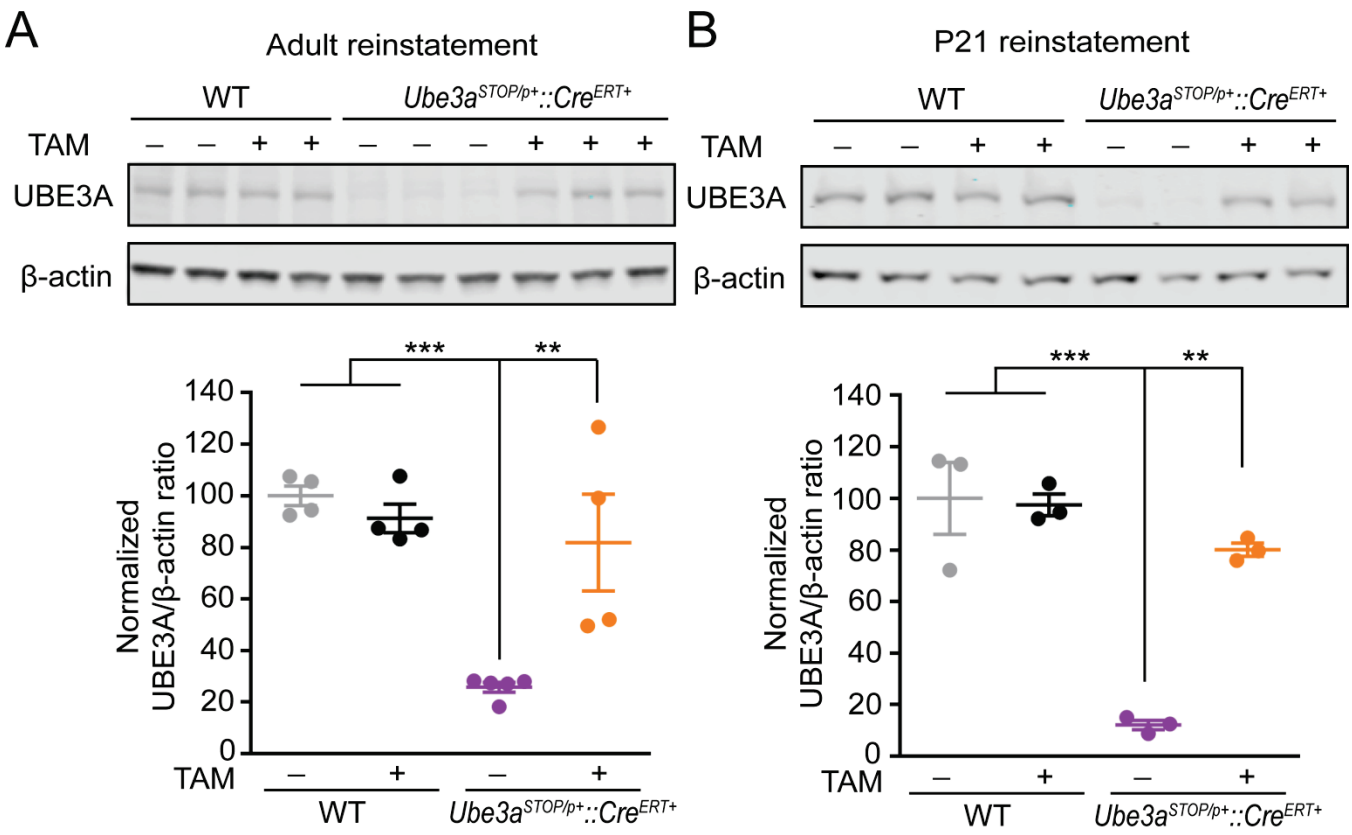
Supplemental Table1. Flurothyl kindling rate of (cell type-selective) *Ube3a* deletion mice

Mice	Myoclonic seizure	Generalized seizure
WT (n=11)	-9.1 ± 1.4 (8 day)	-16.5 ± 1.4 (8 day)
Ube3a$^{m-/p+}$ (n=10)	-3.3 ± 1.5 (8 day)*	-8.2 ± 1.3 (8 day)***
Control (n=12)	-11.0 ± 2.3 (8 day)	-18.2 ± 2.1 (8 day)
Ube3a$^{FLOX/p+}$*::NEX-Cre* (n=7)	-10.2 ± 2.8 (8 day)	-12.6 ± 4.7 (8 day)
Control (n=10)	-26.6 ± 7.8 (4 day)	-57.1 ± 8.5 (4 day)
Ube3a$^{FLOX/p+}$*::Gad2-Cre* (n=8)	-1.1 ± 6.1 (4 day)**	-1.4 ± 7.2 (4 day)***

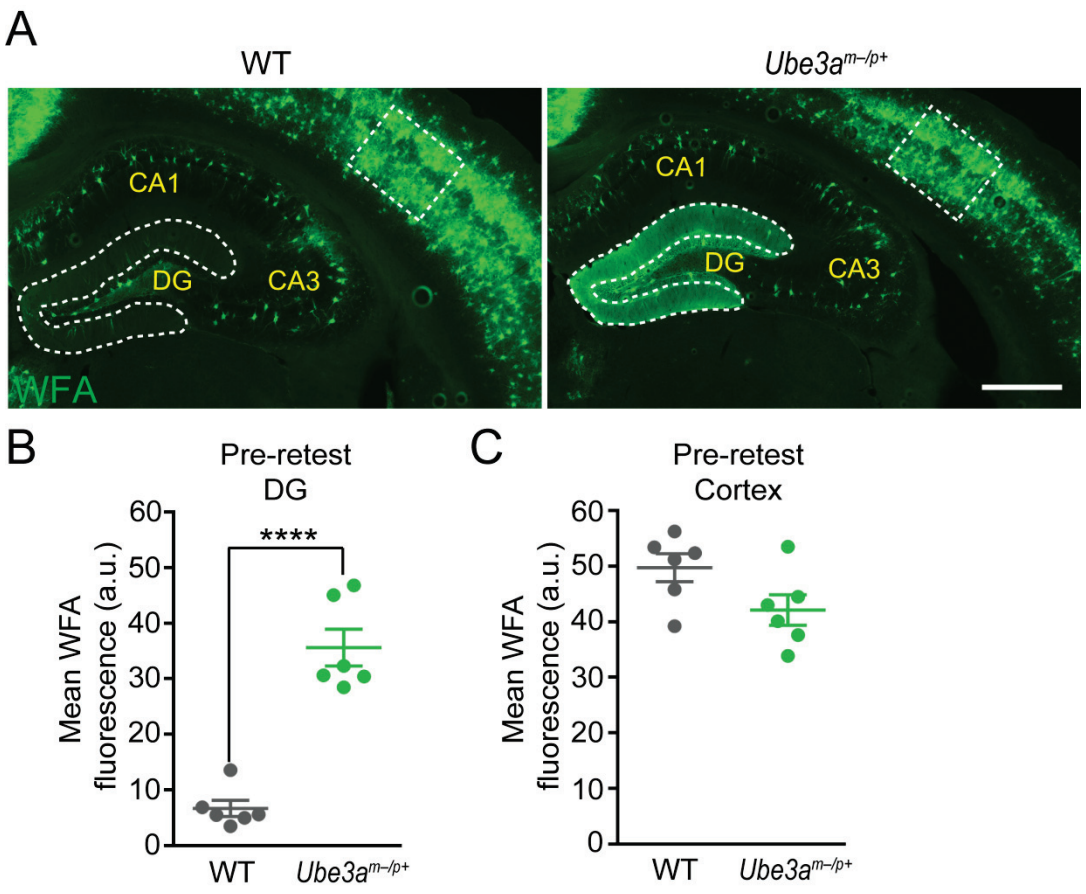
*P < 0.05, **P < 0.01 and ***P < 0.001, unpaired t-test



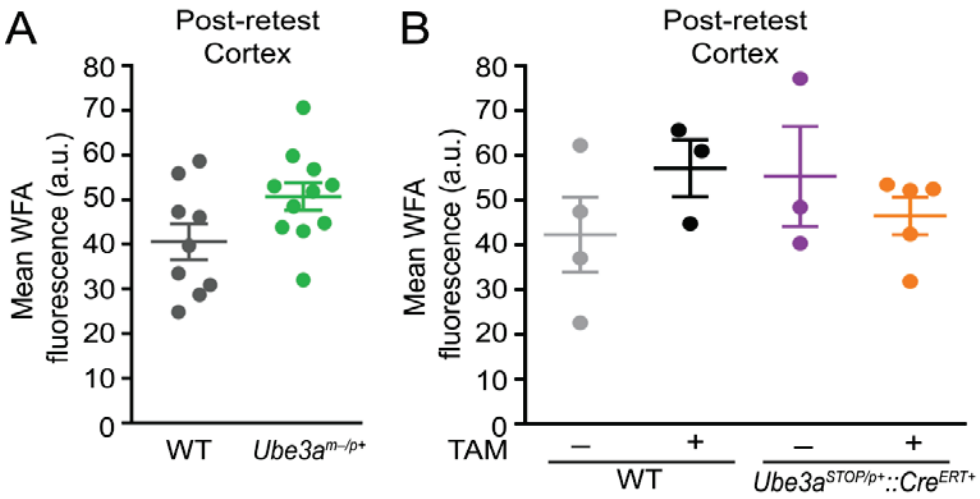
Supplemental Figure 1. Rates of flurothyl kindling are similar between *Ube3a*-reinstated mice and controls. *Ube3a*-reinstated mice (*Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$-TAM) and controls (*Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$-Veh, WT-Veh, and WT-TAM) were treated with Vehicle (Veh) or TAM (**A**) immediately following flurothyl kindling in adulthood, (**B**) 2 weeks prior to flurothyl kindling in adulthood, or (**C**) beginning at P21, with flurothyl kindling in adulthood.



Supplemental Figure 2. Tamoxifen (TAM) treatment beginning at adulthood or P21 restores UBE3A protein to similar levels in *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$ mice. Representative Western blots and quantification of UBE3A/β-actin ratios demonstrate successful UBE3A reinstatement by TAM in both (**A**) adult (WT-Veh, n=4; WT-TAM, n=4; *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$-Veh, n=5; *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$-TAM, n=4) and (**B**) juvenile *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$ mice (WT-Veh, n=3; WT-TAM, n=3; *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$-Veh, n=3; *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$-TAM, n=3). **P < 0.01 and ***P < 0.001, 2-way ANOVA with Bonferroni's *post hoc* test.



Supplemental Figure 3. Increased deposition of PNNs in dentate gyrus of flurothyl-kindled AS mice prior to flurothyl retest. (**A**) Representative staining for WFA (green) from WT and AS model mice that were euthanized on Day 36 in lieu of undergoing flurothyl retest. Scale bar = 400 µm. (**B and C**) Mean WFA immunofluorescence intensity (arbitrary unit, a.u.) within (B) dentate gyrus stratum moleculare and (C) somatosensory cortex of kindled WT (n=6) and AS (n=6) model mice. ****P < 0.0001, unpaired t-test.



Supplemental Figure 4. Similar levels of PNN staining in the somatosensory cortex of flurothyl-kindled WT and AS mice following flurothyl retest. Mean WFA immunofluorescence intensity (a.u.) within somatosensory cortex of adult (**A**) WT (n=9) and AS (n=11) model mice and (**B**) Control (Veh, n=4; TAM, n=3) and *Ube3a*$^{STOP/p+}$*::Cre*$^{ERT+}$ (Veh, n=3; TAM, n=5) mice treated with either Veh or TAM at P21.