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Supplementary Materials for

Increased Gene Dosage of *Ube3a* Results in Autism Traits and Decreased Glutamate Synaptic Transmission in Mice

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The PDF file includes:

Materials and Methods

To determine whether excess Ube3a gene copies are sufficient to produce the autism behavioral traits, we used BAC recombineering techniques (*S1*, *S2*), to insert a 162 kb segment of mouse chromosome 7, containing the entire 78kb exon-intron coding sequence of Ube3a as well as its 63 kb 5' and 21 kb 3'sequences, into FVB embryos to generate transgenic mice (Fig S1 and S2). Native and flag epitope-tagged transgenic Ube3a displayed matching patterns of expression across multiple brain areas (Figs. 1, E-G and S3). A FLAG epitope tag was added to the 3' end of exon 12. To control for site-of-insertion effects, two independent founder lines (1 and 2) were analyzed. The transgene construct lacks the transcription initiation site of the antisense transcript, which in the endogenous gene is responsible for paternal silencing in brain and is located over 500kb downstream of the BAC beyond the SNP/SNRPN. We confirmed that expression of FLAG-Ube3a is independent of parent-of-origin or sex of the animal (Fig. S2, C and D).

Generation of FLAG-Ube3a mice: We generated FLAG-tagged full length Ube3a using a BAC (RP24-178G7) construct following PCR-based methods in combination with the lambda red recombinase system, as previously described (*S1*, *S2*). The BAC DNA was prepared using double acetate precipitation and $CsCl_2$ gradient purification methods, and then linearized using the restriction enzyme PI-Sce (NEB) and microinjected into FVB embryos.

All procedures were performed in accordance with animal experimental protocols approved by the Beth Israel Deaconess Medical Center Animal Care & Use Committee, an agency accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Genotyping was performed as previously described (*S1*, *S2*).

Characterization of mice

Antibodies: Ube3a (BD Transduction labs and Santa Cruz), Actin (Santa Cruz), Flag M2 (Sigma), Arc (Santa Cruz H-300), PSD-95, (Neuromab), EAAT1, EAAT2, TSC2 (Cell Signaling), APP (Epitomics), GabrRα1, β1 and β3, GluR2, Kv1.1, Kv4.2, NR2B (Neuromab), GluR1, EACC1 (Millipore) NR2A (Santa Cruz), and PLIC/Ubiquilin (BD Transduction labs) were used.

Western Blots: Western blots were run using standard protocols. Protein concentrations of cortical lysates were measured by BCA assay (Pierce) and equal amounts of protein was loaded onto 8% gels, run at 120V, transferred to nitrocellulose, blocked with 4% milk in PBST, and incubated with the primary antibody at 1:1000 to 1:5000 overnight in 4% milk/PBST. Blots were then washed, incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at RT (Santa Cruz), washed, and developed with Femto luminol reagent (Pierce) and images were acquired with a digital camera in a gel dock system (BioRad). Arc protein in barrel cortex was assayed by Western blot of single housed male mice exposed to a novel object for three hours before sacrifice (as in *S3*).

In Vitro Ubiquitination Assay: Cortical lysates were prepared in PBS with 1% TritonX-100 and protease inhibitors, incubated with 4 μg of anti-FLAG antibodies overnight, and with

50μl of protein G magnetic beads (NEB) for immunoprecipitation (IP). Beads were washed 5x with PBS and Ube3a-FLAG was eluted with 3x FLAG peptide (Sigma) in 100 μl PBS, and IP success was confirmed by western blot. To ubiquitination buffer (in mMol: TRIS 20, NaCl 50, MgCl 10, DTT 0.1, MG132 10, ATP 4 pH 7.4) was added 1 μg recombinant Arc (Novus biologicals), 50ng E1, 100ng UbcH7 E2, 4 μg HA-Ubiquitin (all from Boston Biochem) and 10 μl of immunoprecipitate for a total reaction volume of 100 μl (adapted from *S3*). Reactions were incubated for 2 hours at 30° before the addition of SDS sample buffer and Western blotting.

Staining: For tissue sections, mice were perfused with 4% PFA and brains removed and cut into 2mm pieces which were paraffin embedded. 15 μm sections were cut and mounted and deparaffinized in xylene, re-hydrated through an ethanol gradient, and boiled for 20 minutes in citrate buffer to unmask antigens. Alternately, sections were frozen in OCT and cut on a cryostat at 5, 20 or 100 μm for PSD/VGlut, Ube3a/FLAG, and external GluR1, respectively. Sections were blocked with MOM reagent in the case of anti-mouse secondary (Vector) and then with 10% normal goat serum/1% BSA/0.25-1% Triton X100 in PBS and incubated at room temperature overnight with antibody diluted 1:200 in blocking solution. Sections were then washed, incubated with Alexa-conjugated secondary antibodies (Invitrogen), and mounted in Vectashield with DAPI (Vector). Images were acquired on a LSM510 confocal microscope (Zeiss). For PSD95/Vglut1, confocal image stacks were taken at 63x magnification through the 5um slice from random positions in layer II/III. Co-localization of Vglut and PSD95 puncta were counted from 3 images for each section, at least 3 sections per animal, n=4 animals.

Tissue culture, P0 mice were euthanized and cortical neurons were prepared with a postnatal neuron isolation kit (Miltenyi Biotech) according to the manufactures instructions, and maintained in MACS neuronal culture media (Miltenyi Biotech) supplemented with B27 (Invitrogen). After 7 days, neurons were fixed in cold 4% PFA in PBS, blocked with blocking solution and stained as above.

Golgi Staining was performed using the FD rapid golgi stain kit (FD Neurotech). The number of spines were counted from the last branch point to the end on terminal dendrites of layer 2 pyramidal neurons which fulfilled the following requirements: 1) they were over 30 μm long ; 2) terminated within the slice; and 3) were traceable back to a cell body. The length of the terminal dendrites was measured and data were expressed as spines per μm. At least 10 dendrites were counted per mouse and averaged to give the measure for that mouse. Statistics were based on number of mice.

Electron Microscopy: Brains were removed for staining and ~1 mm cubes of barrel cortex containing the pial surface were cut and post-fixed in 3% formaldehyde, 3% gluteraldehyde and 0.1M Na-Cacodylate. Ultrathin sections (70-80 nm) were cut and observed on a transmission EM (JEOL, Co. JEM 1011). Glutamatergic (asymmetrical) synapses were counted at 10,000x magnification based on the appearance of a prominent post-synaptic density. 30 fields were counted from each animal and averaged to obtain the value for the animal. The area of synaptic vesicles was traced using ImageJ and the diameter was derived. 8 synapses imaged at 100,000x magnification, each with between 7

and 17 vesicles, were counted per animal and averaged to obtain the value for each animal.

Behavior testing

Single-transgenic mice on a pure FVB/NJ background were bred together to produce litters containing wild-type, single and double transgenic littermates that were used for all experiments, except those shown in Supplementary Figure 2, in which either male or female transgenic mice that were bred with a wild-type FVB. Mice were housed in same-sex groups of 3-5 under standard laboratory conditions, lights on from 7am to 7pm, ad libitum food and water. Testing was performed between 10am and 5pm. Each test was separated by at least three days to prevent one test from interfering with the others. All equipment was cleaned with mild detergent in between each mouse to eliminate residual orders. Wild-type, and single- or double-transgenic littermates were always examined.

Pup tests: On P3, pups were removed from the nest one at a time and placed in a clean plastic container at room temperature (23 ± 1) with the bat detector from the Ultravox systems (Noldus) mounted in a hole in the lid. Vocalizations were monitored for five minutes using the Ultravox system, which recorded the number of vocalizations and the time spent vocalizing. The pup was then placed on its back and the time to roll over onto all four paws was measured. The pup was then placed head-down on a wire screen inclined at 30 degrees, and the time the pup took to turn itself so that its head was above horizontal was recorded. The skin temperature of the pup was then monitored with a digital thermometer to ensure a lack of hypothermia. The pup was then weighted, tattooed on the foot for identification, and placed in a holding cage on a 37° heat pad until all pups were tested, at which time the litter was placed back in the nest. The tests were repeated every other day until P11 (inclusive).

All of the following tests were performed on adults (4-16 weeks old).

Open Field: Mice were placed in a clear acrylic box measuring 50 x 100 cm on a black surface. An overhead camera recorded activity and Ethovision (Noldus) was used to measure total distance traveled, time spent in the center (defined as the area formed by lines extending from 1/3 and 2/3 of the length of each side) and total entries into the center.

Social interaction Test #1: (As in *S4*) The same three-chambered arena was used. Mice were allowed to explore the empty arena for ten minutes. They were then placed in a holding cage. The small metal enclosures were then placed in the arena, and a same-sex, age-matched, non-littermate wild-type stranger mouse was placed in one of the two small cages, which were alternated to control for any innate side preference. These probe mice had been habituated to the small enclosures in 1 hour sessions for three days prior to testing. Mice were recorded with an overhead camera and the time spent in each third of the enclosure was automatically scored with Ethovision. An observer blinded to genotype of the mouse also scored the time spent interacting with the probe mouse or the empty cage.

Social Interaction Test #2: (As in *S5*) Dividers with small (10 x 10 cm) doors were placed into the open field box to create a three-chambered enclosure. Small cages (metal enclosures, inverted pencil holders, Office Depot) were placed in the upper corners of the outside chambers. Mice were allowed to explore the chambers and small cages for five minutes (during which time they showed no preference for one side over the other). They were then placed in a holding cage, and a same-sex, age-matched, non-littermate, stranger wild-type mouse was placed in one of the two small cages, which were alternated to control for any innate side preference. Mice were recorded with an overhead camera and the time spent in each third of the enclosure, and in the zone immediately next to the enclosure was automatically scored with Ethovision. The test was also performed with an object (a 10 cm high, 6 cm diameter plastic container, painted with alternating black and white lines) replacing the stranger mouse.

Elevated plus: Mice were placed, with their heads facing into a closed arm, onto an elevated plus maze 50 cm off of the ground, with 50 x 5 cm arms and were allowed to explore for five minutes. Mouse behavior was recorded with an overhead camera and the time spent in each arm and the number of entries into each arm was automatically scored with Ethovision.

Object Exploration/Memory: Mice were placed into the open field box with two of three objects placed in diagonally opposite corners. The mice were allowed to explore the objects for five minutes, after which time they were placed in a holding cage while the arena was cleaned and one of the two objects was replaced with the third "novel" object. After 10 minutes, the mouse was returned to the arena and allowed to explore both objects for a further five minutes. All sessions were recorded by an overhead camera, the video files were coded, and the number of exploratory sniffs to each target (defined as moving the nose to within 3cm of the object with the head facing the object) was counted by an experienced observer blinded to the genotype of each mouse. The order of object presentation and the location of the object in different diagonal corners were randomized to control for any innate object or location preference, but post-hoc analysis revealed no such preference.

Grooming: Mice were allowed to acclimate in a clean cage for ten minutes. The total amount of time spent grooming was then recorded with a stopwatch by an experienced observer blinded to the genotype of each mouse. As videotaped recordings were difficult to accurately score, scoring was done live.

Rotorod: The rotorod (Ugo Basile A-Rod for mice) was set to accelerate from 4 to 40 RPM over five minutes. Time to fall was recorded for each mouse, and if a mouse was still on the rod after 400 seconds, the session was ended and a score of 400 given. Each mouse was given four sessions a day, separated by approximately one hour, for three consecutive days.

Sexual Vocalizations: Male mice were single-housed for several days, and then exposed to brief (5 min) social interactions with both male and female mice for four days before the test. On the 5th day, mice were placed in a small plastic box inside a larger sound-proof container. A cotton swab dipped in freshly-collected urine pooled from at least 10 females from at least 5 different cages was suspended from the top of the smaller box, so that the

tip was approx 5 cm above the floor. An ultrasonic microphone recorded vocalizations and fed data into a computer running Avisoft-Recorder (Avisoft Bioacoustics) for five minutes. The program recorded the total number of vocalizations and time spent vocalizing. The WAV file was then analyzed using SASLab Pro (Avisoft Bioacoustics). A spectrogram was generated and an experienced observer classified each vocalization into one of ten categories. The categories were defined as: "2" a harmonic call where the higher frequency band was dominant; "d" a harmonic call where the lower frequency was dominant; "4" a characteristically shaped 4-part harmonic call; "s" a non-harmonic call with a sharp frequency step; "q" a call that first showed upward frequency modulation, then downward, then upward again in a sinusoidal waveform; "i" a call that showed upward then downward frequency modulation, like an inverted parabola; "p" a call that showed downward then upward frequency modulation, like a parabola; "e" a call that shows upward frequency modulation, then flattens; "f" a flat call; and "u" a call with consistent upwards frequency modulation. N = number of mice tested.

Social vocalizations: The same setup which was used for the pup vocalization testing was used for the social vocalization testing because it allowed direct visual monitoring of pairs of mice to ensure fighting did not occur. Procedure was loosely adapted from Scattoni et al. (*S6*). Age- and genotype-matched, non-littermate, female mice who had never encountered each other before were placed in the box simultaneously (to avoid resident-intruder aggression) and the number of vocalizations and time spent vocalizing were recorded automatically (Ultravox, Noldus) for five minutes. Data were not normally distributed, so non-parametric tests were used. N = pairs of mice tested.

Olfactory habituation/dishabituation: One hour before the test, the mouse was acclimated to the swab, suspended from the center of the top of a clean cage to 5cm above floor level. A fresh swab was then dipped in odorant solution and suspended as above for two minutes. Sessions were video-recorded and an observer blinded to the genotype of the mouse scored the amount of time the mouse spent sniffing the swab. After two minutes, the swab was replaced. Each odorant was presented three times to measure habituation, and four different odorants were presented to measure dis-habituation and the ability of the mice to smell different substances. Odorants were: 1) distilled water; 2) swab was wiped across the bottom of a dirty female cage; 3) 1:10 dilution of imitation banana extract (McCormick); and 4) 1:10 dilution of almond extract (McCormick).

Electrophysiology

Slice Preparation: Mice between 8 and 16 weeks old were used for mEPSC, mIPSC, and biophysical properties, and between 4-8 weeks old for all other tests. Cells from at least 3 mice were analyzed, and *n* was based on number of cells. Testing order was random with respect to genotype. Mice were anaesthetized with 2,2,2,tribromomethanol (0.25mg/g body weight) and transcardially-perfused with ice-cold sucrose-containing cutting solution (in mM: Sucrose 234, KCl 5, NaH2PO4 1.25, MgSO4 5, NaHCO3 26, Dextrose 25, CaCl2 1, balances with 95%O2/5%CO2). The brain was removed and coronal slices (approx 280 μm) were cut on a tissue slicer (Leica VT1200S) in cutting solution. Barrel cortex was identified as in (Paxinos Atlas). Slices were incubated at 35°C for 30 min in ACSF (in mM:

NaCl 125, KCl 3, NaH2PO4 1.25, MgCl2 1, NaHCO3 26, Dextrose 25, CaCl2 2) before being incubated at room temperature for at least 30 min before recording.

Electrophysiological recording: Whole-cell recordings of layer 2/3 pyramidal neurons (PNs) in the barrel cortex were performed on the coronal brain slices. PNs were identified under infrared differential interference contrast (IR-DIC) optics on an upright Olympus BX-51WI microscope (Olympus, Tokyo, Japan) based on their location and morphology. Recording pipettes were pulled from 1.5 mm OD capillary tubing (A-M Systems, Carlsborg, WA, USA) using a Flaming/Brown P-97 pipette puller (Sutter Instruments, Novato, CA, USA) and had tip resistances of 3–5 MΩ when filled with internal solution (see below). Pipettes were connected to the headstage of a Heka EPC 10 patch-clamp amplifier (Heka Elektronik) and Patchmaster 2 software (HEKA Instruments, Southboro, MA, USA) was used. Fast and slow capacitance and series resistance compensations were carefully adjusted. Liquid junction potentials were not corrected. Cells with a resting Vm between −60 and −82 mV and a series resistance < 20 MΩ were included for analysis. Recordings with series resistance change exceeding 20% were terminated and discarded. Recordings were filtered at 2.9 kHz and digitized at 50 kHz.

Basic biophysical and firing properties were recorded in current-clamp mode using the following intracellular solution (in mM: 135 KCH3SO3, 4 KCl, 2 NaCl, 10 HEPES, 4 MgATP, 0.3 Tris-GTP, 7 Tris-Phosphocreatine) and regular extracellular ACSF. Input resistance was estimated with a negative square pulse (–25 pA, 200 ms). Membrane time constant was obtained by fitting a single exponential equation to the voltage response to this small negative current pulse. Positive current steps (duration: 1 s or 5 ms) were used to acquire the firing properties.

Evoked postsynaptic currents were recorded in voltage-clamp mode using cesium-based artificial intracellular fluid (in mM: 100 $CsCH_3SO_3$, 20 KCl, 10 HEPES, 4 Mg-ATP, 0.3 Tris-GTP, 7 $Tris_2$-Phosphocreatine, 3 QX-314) and regular ACSF. A bipolar platinum/iridium electrode (CE2C55, FHC Inc., Bowdoin, ME) was placed at layer 2/3 of the barrel cortex 200 μm away from the recording site. Presynaptic axons were stimulated using current pulse stimuli (duration = 180 μs, amplitude = 10–500 μA, and frequency = 0.1 Hz for baseline condition) delivered *via* a constant-current stimulator. Excitatory postsynaptic currents (EPSCs) were recorded at a holding potential of –50 mV. A glass pipette filled with 0.5 mM bicuculline methiodide (BMI) in ACSF was placed above the soma of the cell being recorded. A small positive pressure was applied to the pipette to establish a stable flow of BMI that locally inhibited GABAergic transmission. Inhibitory postsynaptic currents (IPSCs) were recorded at a holding potential of +10 mV in the presence of bath 10 μM DNQX and 50 μM APV.

Paired-pulse facilitation experiments were carried out to estimate the release probability. The peak amplitude of postsynaptic currents evoked by two identical stimuli separated by 50 ms was measured. The facilitation ratio (the second peak amplitude/the first peak amplitude) was calculated.

Miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs) were respectively recorded at –

60 mV or –80 mV (Fig. S6) and +10 mV using cesium-based internal fluid (above) and a low divalent ion ACSF (in mM): 125 NaCl, 3.5 KCl, 1.25 NaH2PO4, 0.5 MgCl2, 26 NaHCO3, 25 Dextrose, and 1 CaCl2. AMPA receptor-mediated mEPSCs (AMPA-mEPSCs) were recorded in the presence of 20 μM APV, 100 μM picrotoxin, and 1 μM TTX. NMDA receptor-mediated mEPSCs (NMDA-mEPSCs) were recorded at –70 mV in the presence of 10 μM DNQX, 100 μM picrotoxin, 20 μM glycine, 0 Mg^{2+}, and 1 μM TTX. Continuous data were recorded in 10 sec sweeps, filtered at 1 kHz and sampled at 20 kHz, 300 s of synaptic events were randomly chosen and the total number of events was analyzed. Individual events were counted and analyzed with MiniAnalysis software (Synaptosoft) and custom scripts written in MatLab using amplitude as the main identification parameter and a 5 pA cut-off to account for noise. 50 events were randomly chosen from each cell and combined into the total pool of events for each genotype, and the amplitude and the interevent interval histograms were binned at 1 pA and 0.01 s, respectively. Differences between cumulative histograms were evaluated by the Kolmogorov-Smirnov test. The decay times of AMPA-mEPSCs and NMDA-mEPSCs were fitted using one exponential equations.

Spontaneous EPSCs (sEPSCs) were recorded at a holding potential of –50 mV using the same cesium-based internal fluid and regular extracellular ACSF containing 100 μM picrotoxin. Spontaneous IPSCs (sIPSCs) were recorded at a holding potential of +10 mV using cesium-based internal fluid and regular ACSF containing 10 μM DNQX and 50 μM APV. Analysis was similar to mEPSCs and mIPSCs.

Glutamate iontophoresis.: The proximal portion of the apical dendrites of layer 2/3 pyramidal neurons in the barrel cortex was exposed by blowing ACSF onto the surface of the slice *via* ACSF-filled glass pipettes. The pyramidal neurons were then voltage-clamped at -70 mV in the presence of 1 μm TTX and 100 μm picrotoxin. Iontophoretically applied glutamate (10 mM sodium glutamate in 10 mM HEPES, pH 7.4) was delivered through glass pipettes (4–6 MΩ when filled with normal internal solution) placed 1–2 μm away from the main apical shaft (~ 15–20 μm from cell body). The iontophoresis pipette was connected to the second channel of a Heka EPC 10 amplifier and glutamate was expelled using 100 ms –, 100 nA current pulses at 0.1 Hz. 1 nA retention currents were applied between stimuli to prevent glutamate leakage in the baseline conditions.

Minimal stimulation and estimation of vesicle glutamate content: The vesicle glutamate content was estimated by the relative inhibition of mean single fiber EPSC amplitude by the fast off-rate, non-NMDA receptor blocker γ-DGG (300 μM). The higher the percentage inhibition by γ-DGG , the lower the concentration of synaptic glutamate (see *32*). To selectively stimulate a single fiber in layer 2/3 of the barrel cortex, minimal stimuli were delivered through ACSF-filled bipolar glass electrodes pulled from 2.0 mm OD dual barrel theta capillary glass (Warner Instruments). The tip of the stimulating electrodes is about 2 μm. To establish a minimal stimulation, we first sought for the highest stimulus that gave all failures. Then we slightly increased the stimulation intensity to lower the failure rate. To acquire a reasonable number of EPSCs from 40–100 trials in both baseline and γ-DGG conditions at 0.3 Hz, we adjusted the stimulation intensity to give about 10% failures (WT: 9.7 ± 2.4%, $n = 8$; 1x: 9.7 ± 3.4%, $n = 8$; 2x: 11.6 ± 4.6%, $n = 6$; $F(2, 21) = 0.106$, $P = 0.90$).

Under this failure rate, the calculated quantal content was about 2. In addition, EPSC latency should remain stable throughout the experiments (< 20% fluctuations). The other recording conditions were similar to evoked EPSC recordings.

Estimation of readily releasable pool size and release probability. We used 20 Hz train stimulations (40 stimuli) to estimate the size of readily releasable pool. We averaged 10–20 train stimulations (train frequency: 0.033–0.067 Hz). To effectively discharge the readily releasable pool, a slightly higher stimulation intensity than the afore-mentioned minimal stimulation was used (~ 5% more than the minimal stimulation). This stimulation intensity gave < 5% failures. The readily releasable pool size was estimated by linear interpolating the linear portion (normally from 21st to 40th stimuli) of the cumulative EPSC amplitude plot to virtual stimulus 0. The ratio of this readily releasable pool size and the quantal size gave the number of readily releasable sites. To estimate the release probability, the mean amplitude of the 1st EPSC during the train stimulation was divided by the readily releasable pool size. The other recording conditions were similar to evoked EPSC recordings.

Statistical Testing

Behavioral data were analyzed using Prizm (Graphpad). Comparisons between two groups used Student's T-Test, comparisons among multiple groups used one-way ANOVA with Dunnett's post-hoc test comparing each genotype to wild-type; non-significant comparisons are not stated in the manuscript. Comparisons involving multiple independent variables used two-way ANOVAs. Non-normal data (social vocalizations) were tested using the Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc tests comparing each genotype to wild-type.

For electrophysiological data, one-way ANOVA with Dunnett's post-hoc test was used to compare multiple group means. Kolmogorov-Smirnov (KS) test was used to compare distributions. Unbalanced two-way ANOVA with bonferroni's post-hoc test was used to compare multiple group variance. n = number of cells analyzed.

All data is presented as mean ± SEM unless otherwise noted. $P < 0.05$ was considered statistically significant.

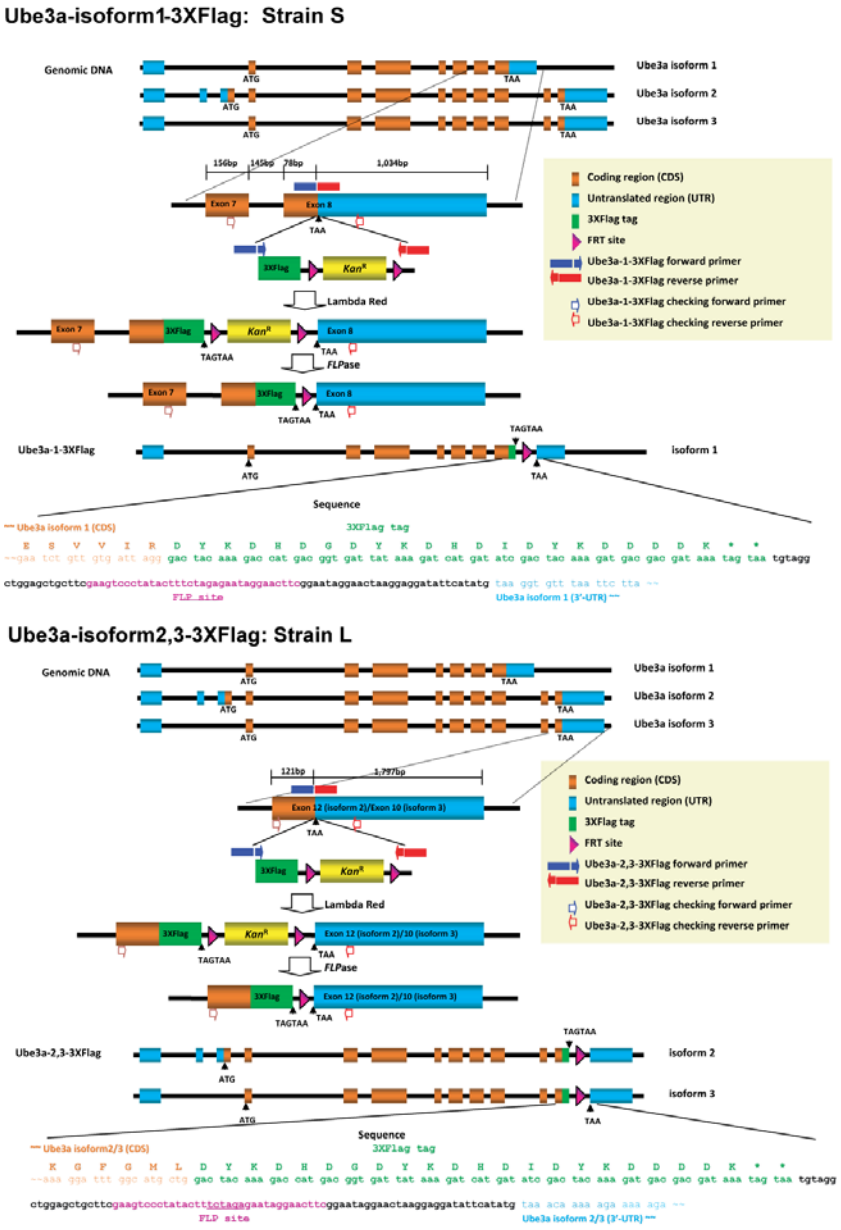

Fig. S1. Recombineering a C-terminal FLAG tag into the three isoforms of the wild-type Ube3a gene (162 kb, BAC vector). **Upper panel**: Ube3a isoform 1 was FLAG-tagged by the lambda red recombinase system in *E. coli* to incorporate DNA sequences encoding the FLAG epitope tag and a selection marker in frame at the 3' coding/untranslated boundary of exon 8. The FRT sequence flanking the kanamycin selection cassette was removed by transfecting a plasmid encoding FLP recombinase into the *E. coli*. DNA sequence of the final construct shows the FLAG tag in frame with the Ube3a isoform1 C-terminus followed by two translational stop codons. A single remaining FLP site is also present. **Lower panel**: an identical method was used to construct the FLAG-tagged mRNA isoforms 2 and 3, except the tag was inserted at the 3' coding/untranslated boundary of exon 12.

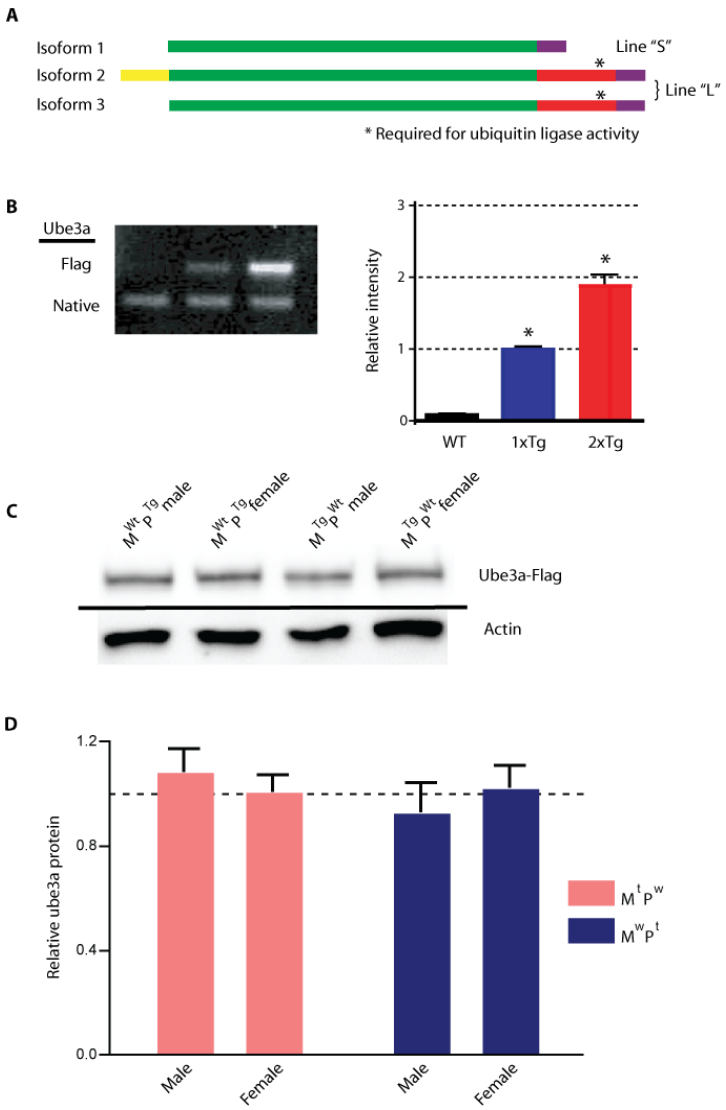

Fig. S2. Design and expression of *Ube3a* BAC transgene. (**A**) Three isoforms of Ube3a. Color scheme: red = C-terminal fragment required for ubiquitin ligase activity is missing in Ube3a isoform 1; yellow = N-terminal fragment present only in isoform 2 (function unknown); and purple= 3xFLAG tag. Line L = mice carrying BAC transgene with FLAG in exon 12. Line S = mice carrying BAC transgene with FLAG in exon 8. (**B**), Gel and graph showing Ube3a transgene copy number by semi-quantitative PCR in single- and double-transgenic mice (1xTg and 2xTg, Ube3a-L form). n = 3-5, * $P < 0.001$ by ANOVA with Dunnett's post-hoc. (**C**) Ube3a transgene is expressed independent of sex or parent-of-origin. Anti-FLAG western blot reveals the transgenic protein is expressed in both males and females, whether inherited from the father ($M^{Wt}P^{Tg}$) or the mother ($M^{Tg}P^{Wt}$). (**D**) The level of anti-FLAG immunoreactivity on western blots was quantified from 4 animals per group. Two-way ANOVA reveals no difference in band intensity based on sex ($F_{(1,12)}$=0.01186, P =.9151) or transgene parent-of-origin ($F_{(1,12)}$=0.6786, P = 0.4261), and no interaction ($F_{(1,12)}$=1.007, P = 0.3354). Mean + S.E.M. are plotted.

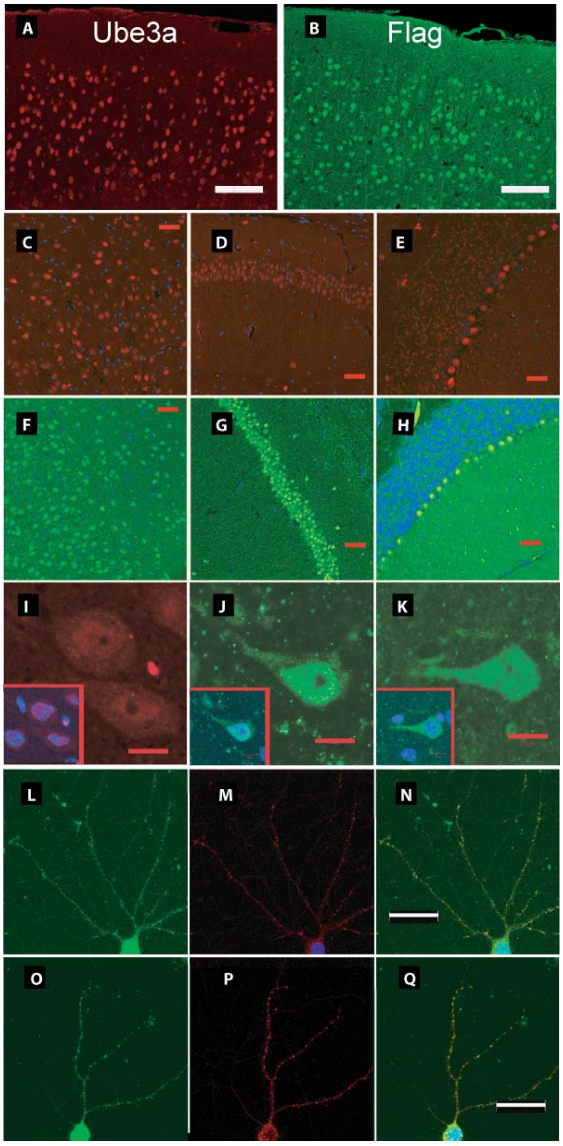

Fig. S3. Distribution of transgenic and native Ube3a protein expression in brain. Brain slice patch-clamp recordings focused on pyramidal in layer 2/3 barrel cortex which strongly express native Ube3a in wildtype animals (**A**) and FLAG-tagged Ube3a in transgenic animals (**B**). Identical patterns of Ube3a staining are found in wild-type (anti-Ube3a, red) and transgenic (anti-FLAG, green) mouse thalamus (**C** and **F**), CA1 hippocampus (**D** and **G**) and cerebellar Purkinje cells (**E** and **H**). Note the absence of transgene and Ube3a in the cerebellar granule cell soma in both. Ube3a is moderately enriched in the nucleus (**I** and **J**, layer V pyramidal neurons) but interestingly, the inactive Ube3a isoform 1 transgene (line S) lacks nuclear enrichment (**K**). In 7DIV cortical neuron cultures from wildtype (**L**-**N**) or transgenic (**O**-**Q**) mice, Ube3a localizes to PSD95-positive dendritic puncta (Green, anti Ube3a (**L**) or anti-FLAG (**O**), Red, anti PSD95 (**M** and **P**)). Scale bars 100 μm (**A**-**H**), 10 μm (**I**-**K**) 30 μm (**L**-**Q**). The results closely match those of Gustin et al. (*S7*).

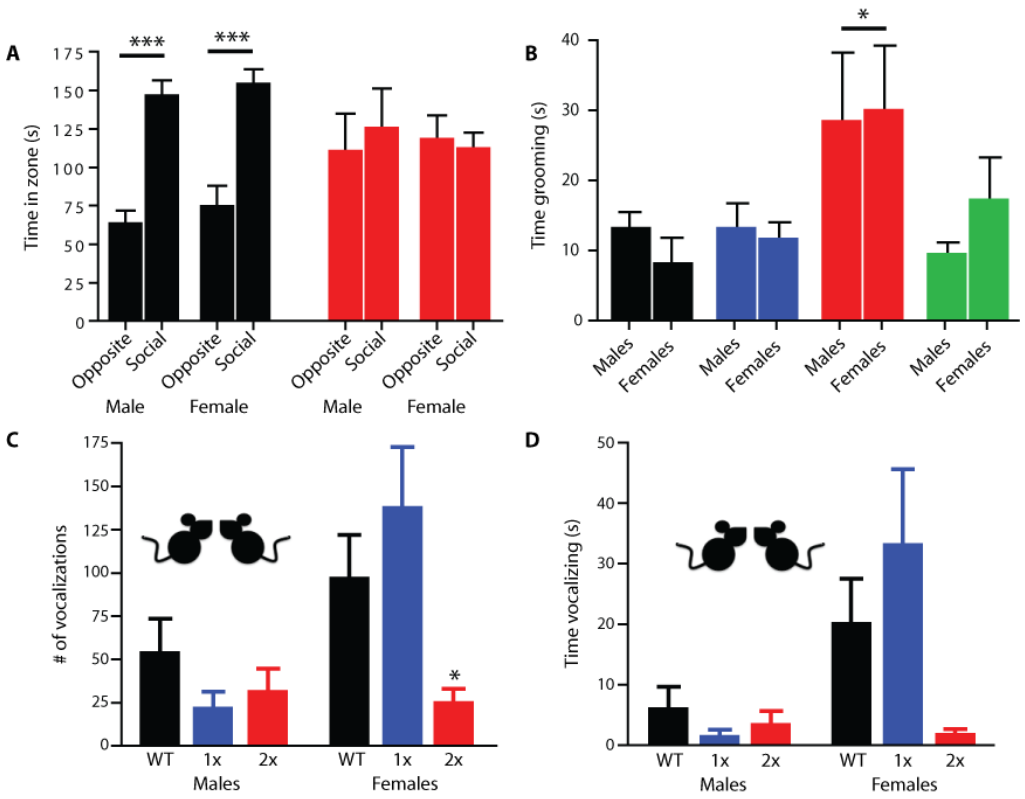

Fig. S4. Gender-specific effects of increased *Ube3a* gene dosage on social, repetitive, and communication behaviors. (**A**) Male and female wild-type mice (black, $N = 5, 6$) show a significant preference for the social zone (within-genotype T-test, *** $p < 0.005$) in the three chamber social interaction paradigm. Neither male nor female double transgenic Ube3a-L mice (red, $N = 7, 8$) show a preference for the social zone. (**B**) Both male and female double transgenic Ube3a-L mice show increased grooming. Two-way ANOVA of time spent grooming with gender and genotype as independent variables shows a significant effect of genotype ($F_{(3,38)} = 3.61$, $^{**}P = 0.0218$), with no effect of gender ($F_{(1,38)} = 0.03$, $P = 0.8716$), and no interaction ($F_{(3,38)} = 0.35$ $P = 0.7881$). (**C**) Ultrasonic vocalization responses of male and female mouse pairs (sex and genotype matched). Number of vocalizations: 2-way ANOVA reveals a significant effect of both gender ($F_{(1,49)} = 7.81$, $P = 0.0074$) and genotype ($F_{(2,49)} = 3.32$, $P = 0.0445$) and a significant gender x genotype interaction ($F_{(2,49)} = 3.80$, $P = 0.0291$). * $P < 0.05$ wt vs. 2x females by Bonferroni post-hoc test; other comparisons (Wt vs. 1xTg and 2xTg males, Wt vs. 1xTg females) were not significant. (**D**) Time spent vocalizing: Two-way ANOVA also shows a significant effect of gender ($F_{(1,49)} = 7.38$ $P = 0.0091$), but no significant effect of genotype ($F_{(2,49)} = 2.61$ $P = 0.0841$) and no genotype x gender interaction ($F_{(2,49)} = 3.17$ $P = 0.0505$). While there appears to be a trend towards lower numbers of vocalizations and less time spent vocalizing in males, male mice vocalized so infrequently that the differences are not statistically significant. Color code: wild-type (WT, black), single-transgenic *Ube3a* (1x, blue), double-transgenic *Ube3a* (2x, red) double transgenic short form *Ube3a* (green). Mean + S.E.M. are plotted.

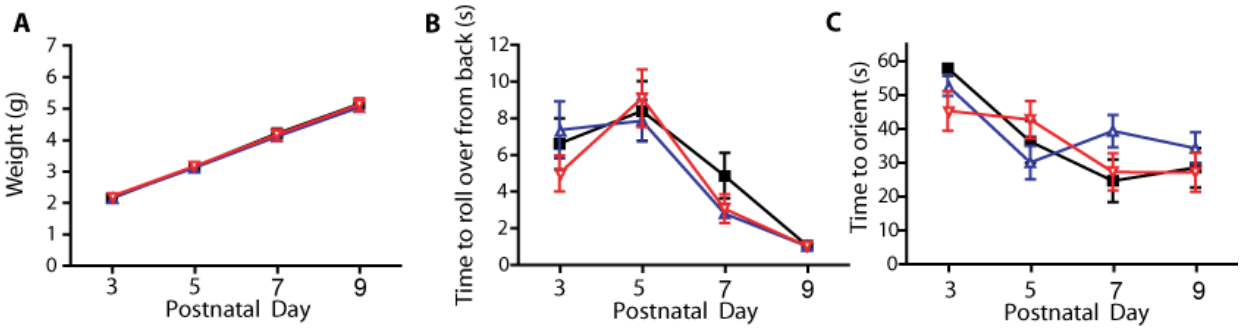

Fig. S5. Effects of increased *Ube3a* gene dosage on developmental milestones. (**A**) Weight and weight gain is normal. (**B**) Time to roll over when placed on back was similar across genotypes. (**C**) Time to orient head up when placed head-down on an inclined plane was similar across genotypes. $N_{(Wt,\ 1xTg,\ 2xTg)}$ =16, 24, 15 for all pup tests. Mean + S.E.M. are plotted.

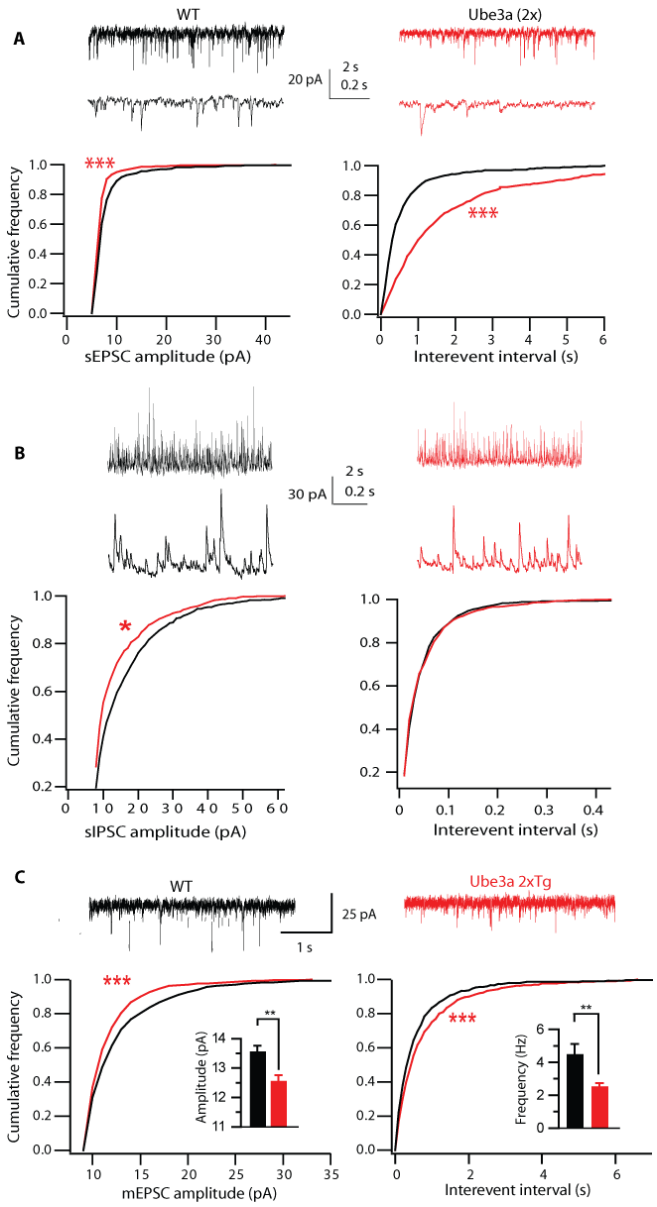

Fig. S6. Effects of increased *Ube3a* gene dosage on spontaneous EPSCs, spontaneous IPSCs, and mEPSCs at -80mV in layer 2/3 pyramidal neurons from the barrel cortex. (**A**) Spontaneous excitatory postsynaptic current (sEPSC) traces (top) and cumulative frequency plots of amplitude (left) and frequency (right) show decreased amplitude and frequency of sEPSCs (*** $P < 0.001$; K-S test, $N = 4$-8). (**B**) Spontaneous inhibitory postsynaptic current (sIPSC) traces (top) and cumulative amplitude (left) and frequency (right) plots (* $P < 0.05$, K-S Test, $N = 4$-8). (**C**) Miniature excitatory postsynaptic current (mEPSC) traces recorded at -80mV to increase detection of low-amplitude events. Cumulative frequency plots of amplitude (left) and frequency (right) show decreased amplitude and frequency of mEPSCs (KS test *** $P < 0.0001$). The mean amplitude and frequency were also significantly reduced (** $P < 0.01$, t test). Mean + S.E.M. are plotted.

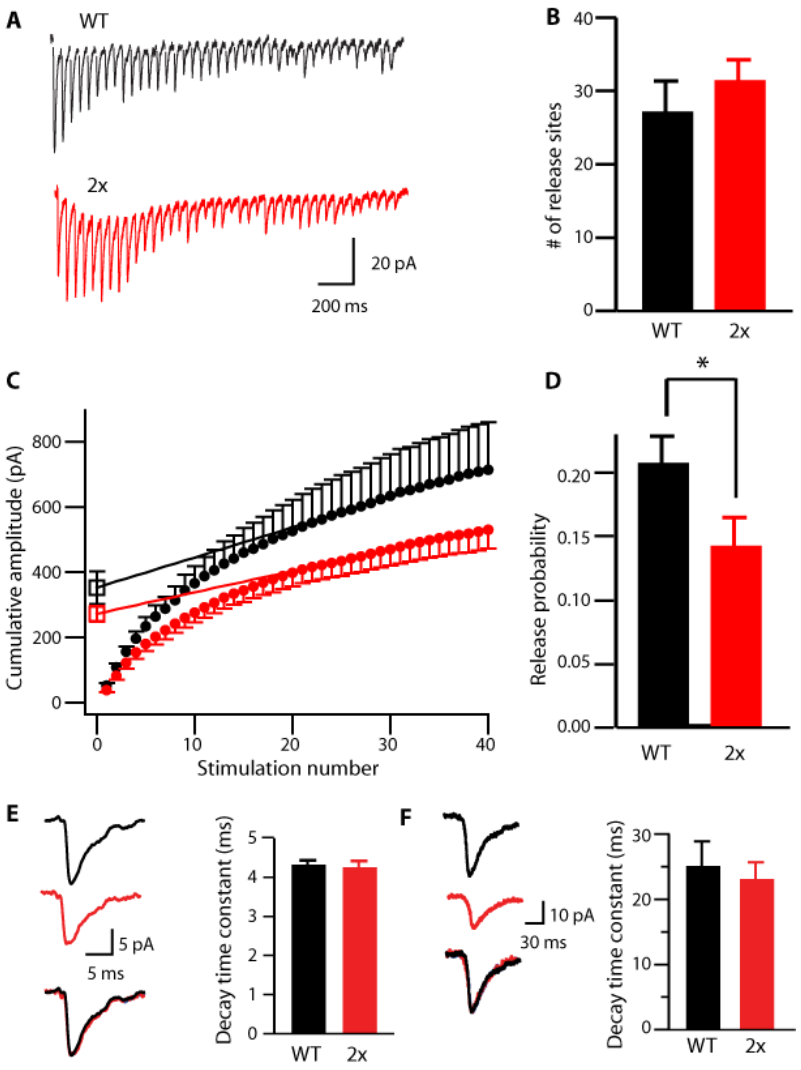

Fig. S7. Effects of increased *Ube3a* gene dosage on release probability, readily releasable pool size, and AMPA and NMDA kinetics. (**A**) Representative traces showing the response to 20Hz stimulation, 200μm from the cell body in Wt and 2x animals. (**B**) The number of vesicles in a single readily releasable pool estimated as readily releasable pool size divided by quantal size, is similar (P = 0.405, N = 10, 9). (**C**) Cumulative amplitude graph showing the magnitude of the cumulative amplitude is increased in 2x animals (ANOVA, P < 0.0001, N = 10, 9), but readily releasable pool size, defined as the y-intersept of the linear portion of the curve, is not different (P = 0.224, t test). (**D**) The release probability, calculated as mean EPSC amplitude (the mean value of the 1st EPSCs) divided by readily releasable pool size, is significantly reduced in 2x transgenic mice (P = 0.0206, t test, N = 10, 8). Averaged AMPA (**E**) and NMDA (**F**) traces from representative cells (~200 events per trace). When scaled so that the amplitude equals wildtype (bottom trace), the kinetics are similar. Decay time constants are also similar (t test: AMPA, P = 0.715, n = 11, 11; NMDA, P = 0.669, N = 8, 5). Mean ± S.E.M. are plotted.

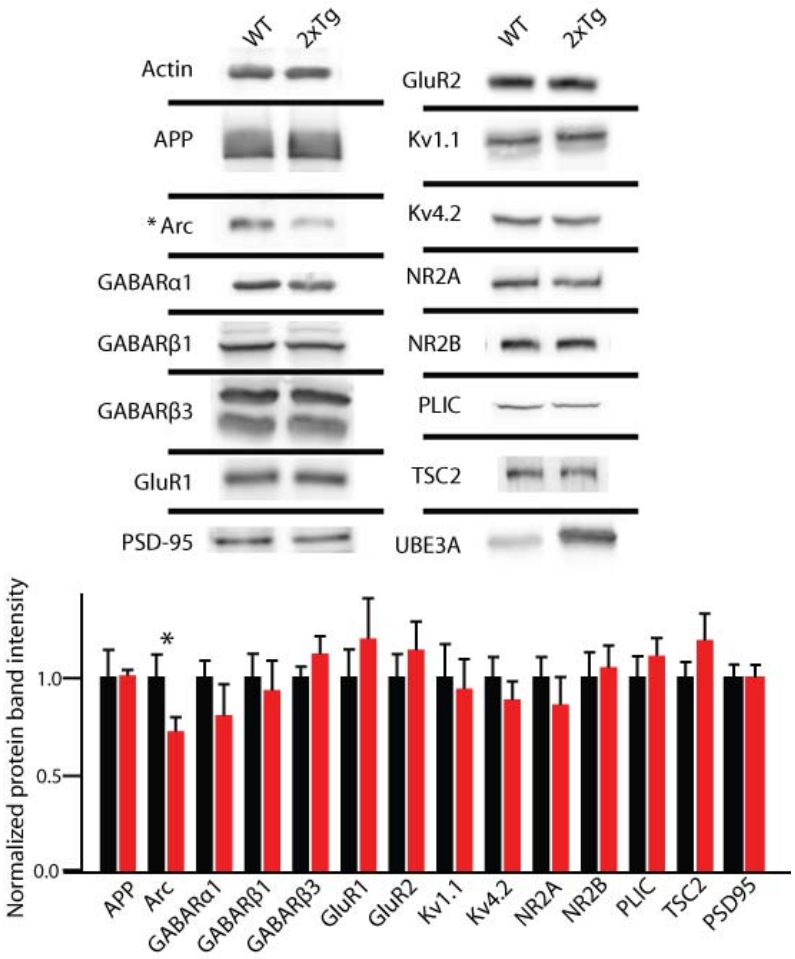

Fig. S8. Effects of increased *Ube3a* gene dosage on synaptic and other neuronal proteins. Single housed male mice were exposed to a novel object for three hours before sacrifice (as in *S3*), and protein expression in the barrel cortex was assayed by western blot. * $P = 0.03$, two-tailed, unpaired T-test, $n = 10\text{-}12$. All unmarked comparisons not significant ($P > 0.05$, $N = 4\text{-}12$). Mean ± S.E.M. are plotted. Color code: wild-type (black) and double- (2x, red) *Ube3a* transgenic.

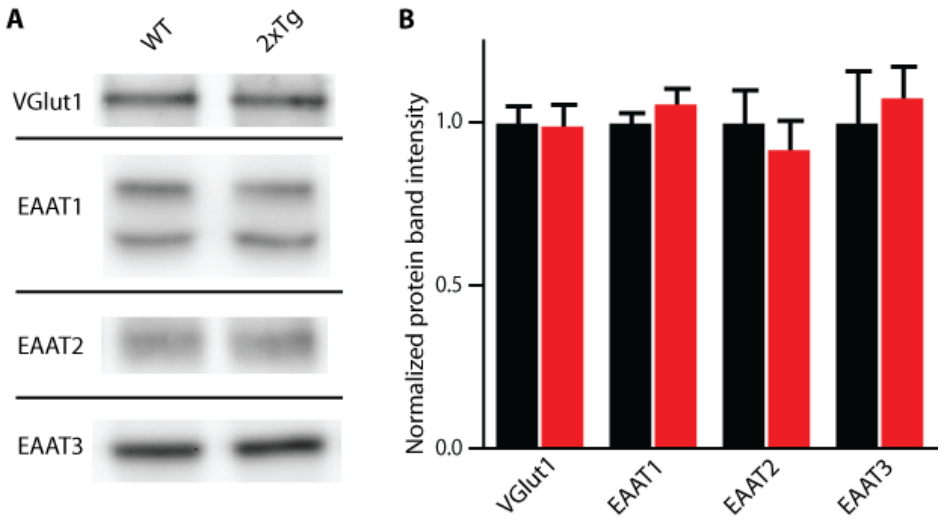

Fig. S9. Effects of increased *Ube3a* gene dosage on quantity of proteins regulating synaptic glutamate concentration. (**A**) Representative western blot bands resulting from glutamate transporter antibodies immunostaining. (**B**) Bar graph of normalized band intensity. Single housed male mice were exposed to a novel object for three hours before sacrifice (as in *S3*), and protein expression in the barrel cortex was assayed by western blot. All unmarked comparisons not significant by t-test ($P > 0.05$, $N = 8$). Mean $\pm$ S.E.M. are plotted.

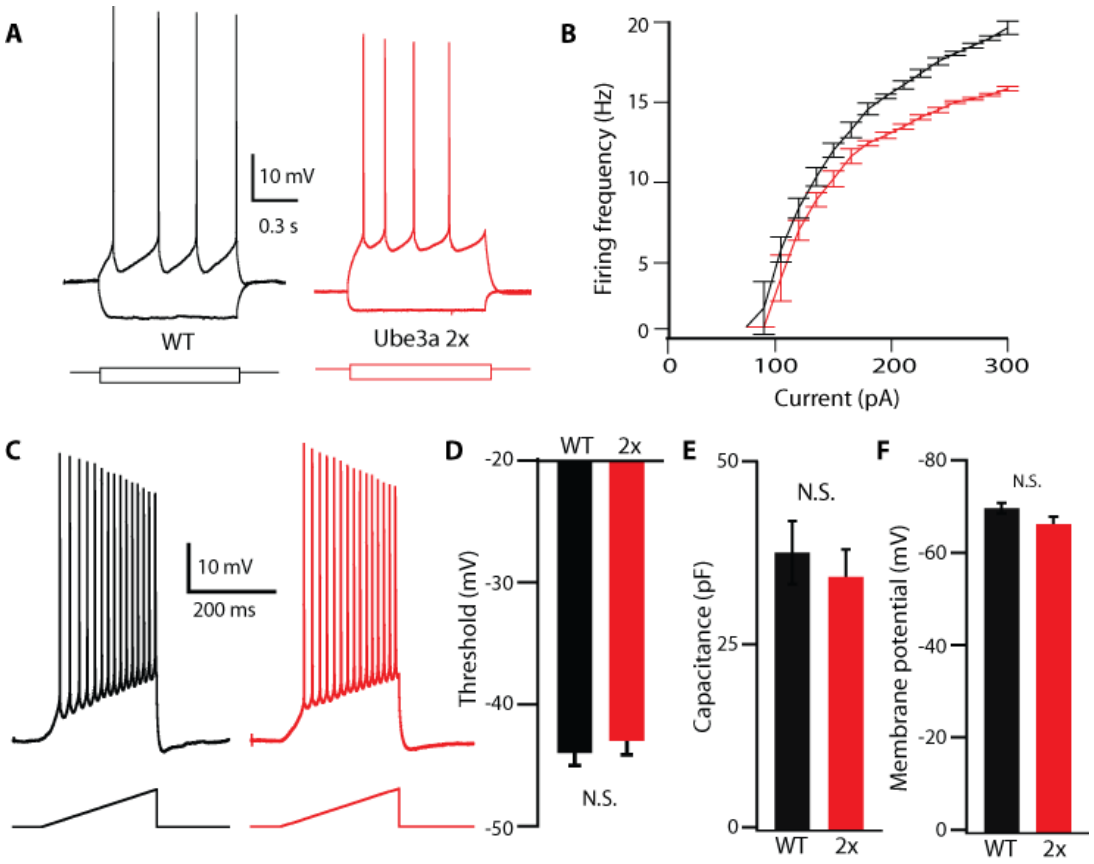

Fig. S10. Effects of increased *Ube3a* gene dosage on other biophysical properties of layer 2/3 barrel cortex pyramidal neurons. Representative voltage traces (**A**) and graph (**B**) showing firing frequency in response to 1 s current pulses of 0-280 pA in 15 pA steps. Repeated-measures ANOVA revealed no differences between genotypes ($P > 0.05$; n = 8-10). Representative traces (**C**) and graph (**D**) showing firing response to a ramped current injection, showing no difference in the threshold to fire ($P > 0.05$, t test, n = 9-17). Capacitance (**E**) and resting membrane potential (**F**) were also similar between groups ($P > 0.05$, t test, n = 9-17). N.S. not significant. Mean ± S.E.M. are plotted. Color code: wild-type (black) and double (2x, red) *Ube3a* transgenic.

Table S1. Effects of increased *Ube3a* gene dosage on behavior: statistical analyses.

Three-chamber tests

		Mean +/- SEM				withing-genotype ttest comparing time spent in "target" vs "opposite" zones	
		Target	Middle	Opposite	N	t value	P Value
Juvenile Social Interaction (25-32 days)							
Time interacting (s)	Wt	203.3 ± 19.47	-	138.7 ± 14.08	11	t=2.639 df=20	p = 0.0157
	1xTg	176.9 ± 12.38	-	140.2 ± 7.88	15	t=2.499 df=28	p = 0.0186
	2xTg	138.3 ± 10.56	-	167.5 ± 15.90	12	t=1.531 df=22	p = 0.1399
	ANOVA comparing time sniffing social among genotypes				Dunnets post-hoc test comparing Wt vs Each genotype		
			F(2,37)=4.897	p=0.0133	Wt vs 1x	q=1.321, p > 0.05	
					Wt vs 2x	q=3.097, p < 0.01	
Time in Zone (s)	Wt	288.7 ± 19,91	89.2 ± 10.35	222.1 ± 15.77	11	t=2.625 df=20	p = 0.0162
	1xTg	258.2 ± 10.76	102.0 ± 10.19	239.8 ± 10.26	15	t=1.237 df=28	p = 0.2264
	2xTg	235.1 ± 9.86	120.3 ± 9.83	244.7 ± 14.71	12	t=0.5459 df=22	p = 0.5907
	ANOVA comparing time in social zone among genotypes				Dunnets post-hoc test comparing Wt vs Each genotype		
			F(2,37)=3.596	p=0.0380	Wt vs 1x	q=1.600, p > 0.05	
					Wt vs 2x	q=2.677, p < 0.05	
Adult Social Interaction (8-10 wks)							
Time in Zone (s)	Wt	151.2 ± 5.3	74.1 ± 5.4	74.7 ± 7.3	17	t=8.481 df=32	p < 0.0001
	1xTg	142.4 ± 8.7	78.4 ± 14.6	79.20 ± 10.0	10	t=4.773 df=18	p = 0.0002
	2xTg	120.0 ± 11.8	63.7 ± 6.1	116.3 ± 12.5	15	t=0.2167 df=28	p = 0.8300
	2xInactiveTg	175.0 ± 21.0	54.4 ± 6.3	70.6 ± 15.5	6	t=3.998 df=10	p = 0.0025
	ANOVA comparing time in social zone among genotypes				Dunnets post-hoc test comparing Wt vs Each genotype		
			F(3,47)=3.899	p=0.0148	Wt vs 1x	q=0.6115, p > 0.05	
					Wt vs 2x	q=2.440, p < 0.05	
					Wt vs 2xInactive	q=1.392, p > 0.05	
Object Interaction (9-11 wks)							
Time in Zone (s)	Wt	126.1 ± 8.4	76.0 ± 6.0	98.0 ± 8.5	11	t=2.340 df=20	p = 0.0297
	2xTg	141.4 ± 10.1	57.9 ± 5.2	100.7 ± 9.9	13	t=2.878 df=24	p = 0.0083

Other behavior tests

	Mean +/- SEM						
	Wt	1xTg	2xTg	2xInactive	Test	Test statistic	P Value
Grooming (12-14 wks)							
Time spent grooming (s)	10.3 ± 2.1	13.4 ± 2.1	28.7 ± 7.0	13.6 ± 3.6	1-way ANOVA	F(3,42) = 4.073	p = 0.0126
N (males in parenthesis)	12(7)	12(5)	11(7)	11(5)			
					Dunnett's multiple	wt vs 1xTg	P > 0.05
					comparison post-hoc:	wt vs 2xTg	P < 0.01
						wt vs 2xInactive	P > 0.05
Males, time spent grooming (s)	12.8 ± 2.6	13.1 ± 3.8	28.15 ± 10.1	9.3 ± 1.9	2-way ANOVA, Genotype	F(3,38)=3.609	p = 0.0218
Females, time spent grooming (s)	8.0 ± 3.9	11.6 ± 2.5	29.74 ± 9.6	17.1 ± 6.3	2-way ANOVA, Sex	F(1,38)=0.026	p = 0.8716
					2-way ANOVA, Interaction	F(3,38)=0.351	p = 0.7881
Elevated plus (10-12 wks)							
Entries: open arms	15.5 ± 2.5	-	13.2 ± 1.4	-	ttest, wt vs 2xTg	t=0.7420 df=22	p = 0.466
Entries: closed arms	22.5 ± 1.8	-	23.3 ± 1.2	-	ttest, wt vs 2xTg	t=0.4083 df=22	p = 0.6870
Time spent: open arms (s)	66.5 ± 11.4	-	51.8 ± 5.1	-	ttest, wt vs 2xTg	t=1.244 df=22	p = 0.2266
Time spent: closed arms (s)	146.7 ± 12.9	-	162.4 ± 7.1	-	ttest, wt vs 2xTg	t=1.114 df=22	p = 0.2773
Ratio open/total entries	0.385 ± 0.043	-	0.352 ± 0.024	-	ttest, wt vs 2xTg	t=0.7124 df=22	p = 0.4837
Ratio open/total time	0.313 ± 0.056	-	0.244 ± 0.025	-	ttest, wt vs 2xTg	t=1.186 df=22	p = 0.2481
N =	11		13				
Open Field (11-13 wks)							
Distance Traveled (cm)	5612 ± 165.0	-	5369 ± 310.3	-	ttest, wt vs 2xTg	t=0.6514 df=20	p = 0.5222
Entries into center	37.7 ± 2.4	-	31.5 ± 4.0	-	ttest, wt vs 2xTg	t=1.252 df=20	p = 0.2250
Time in center (s)	31.6 ± 2.3	-	29.6 ± 3.3	-	ttest, wt vs 2xTg	t=0.4889 df=20	p = 0.6303
N =	10		12				
Object recognition (12-13 wks)							
Baseline: touches of object A	12.3 ± 1.3	-	8.8 ± 1.4	-	ttest, wt touches of A vs B	t=0.5680 df=20	p = 0.5764
Baseline: touches of object B	11.2 ± 1.4	-	9.9 ± 1.1	-	ttest, 2xTg touches of A vs B	t=0.6173 df=22	p = 0.5434
Baseline: Total touches	23.5 ± 2.4	-	18.8 ± 2.3	-	ttest, wt vs 2xTg total touches	t=1.420 df=21	p = 0.1703
Memory: touches of object A	8.9 ± 0.9	-	7.9 ± 0.7	-	ttest, wt novel vs familiar	t=3.671 df=38	p = 0.0007
Memory: Touches of object C	13.9 ± 1.0	-	10.8 ± 0.9	-	ttest, 2xTg novel vs familiar	t=2.562 df=40	p = 0.0143
Memory: Total touches	22.1 ± 1.6	-	18.7 ± 1.8	-	ttest, wt vs 2xTg total touches	t=1.445 df=32	p = 0.1583
N =	20		21				
Rotorod (3-4 months)							
Latency to fall: day 1	197.9 ± 74.2	171.0 ± 37.9	163.7 ± 39.7	210.7 ± 63.4	2-way ANOVA, Genotype	F(3,204)=0.1154	p = 0.951
Latency to fall: day 2	270.5 ± 90.5	295.9 ± 60.4	281.9 ± 69.6	242.9 ± 62.9	2-way ANOVA, Day	F(2,204)=47.5	p < 0.0001
Latency to fall: day 3	299.7 ± 84.5	312.4 ± 58.6	317.9 ± 70.4	335.1 ± 61.5	2-way ANOVA, Interaction	F(6,204)=1.574	p = 0.1564
N =	31	14	20	7			

Urine-induced vocalizations (2-3 months, males)							
Number of vocalizations	476.9 ± 117.3	175.5 ± 61.1	186.6 ± 47.9	-	1-way ANOVA	F(2,22) = 4.515	p = 0.0228
					Dunnett's multiple comparison post-hoc:	wt vs 1xTg	p < 0.05
						wt vs 2xTg	p < 0.05
Time spent vocalizing	17.8 ± 5.1	5.8 ± 2.0	5.2 ± 1.3	-	1-way ANOVA	F(2,22) = 5.306	p = 0.0132
N =	7	11	7		Dunnett's multiple comparison post-hoc:	wt vs 1xTg	p < 0.05
						wt vs 2xTg	p < 0.05
Social Vocalizations (2-3 months)							
Number of vocalizations	80.3 ± 16.3	84.9 ± 23.6	29.2 ± 6.9	-	Kruskal wallis test	H = 7.76 df = 2	p = 0.0206
N(pairs of animals)	24	15	16		Dunn's multiple comparison post-hoc	wt vs 1xTg	p > 0.05
						wt vs 2xTg	p < 0.05
Males	55.1 ± 18.6	23.0 ± 8.3	32.4 ± 12.2	-	Kruskal wallis test	H = 3.01, df = 2	p = 0.2225
N =	10	7	8		no post-hoc		
Females	98.3 ± 23.9	139.1 ± 33.6	26.0 ± 7.2	-	Kruskal wallis test	H=10.34 df=2	p = 0.0057
N =	14	8	8		Dunn's multiple comparison post-hoc	wt vs 1xTg	p > 0.05
						wt vs 2xTg	p < 0.05
					2-way ANOVA, Genotype	F(2,49)=3.318	p = 0.0445
					2-way ANOVA, Sex	F(1,49)=7.812	p = 0.0074
					2-way ANOVA, Interaction	F(2,49)=3.805	p = 0.0291
Time spent vocalizing (S)	14.56 ± 4.6	18.74 ± 7.6	2.95 ± 1.0	-	Kruskal wallis test no post-hoc	H = 5.48 df = 2	p = 0.0645
Males	6.34 ± 3.3	1.81 ± 0.8	3.79 ± 1.9	-	Kruskal wallis test no post-hoc	H = 2.89, df = 2	p = 0.2354
Females	20.42 ± 7.1	33.6 ± 12.1	2.1 ± 0.6	-	Kruskal wallis test	H = 9.10 df=2	p = 0.0106
					Dunn's multiple comparison post-hoc	wt vs 1xTg	p > 0.05
						wt vs 2xTg	p < 0.05
					2-way ANOVA, Genotype	F(2,49)=2.606	p = 0.0841
					2-way ANOVA, Sex	F(1,49)=7.376	p = 0.0091
					2-way ANOVA, Interaction	F(2,49)=3.175	p = 0.0505
Pup vocalizations							
P3 (n = 16, 24, 15)	8.13 ± 0.53	9.34 ± 0.32	9.64 ± 0.41	-	2-way ANOVA, Age	F(4,262)=11.55	p = 0.0001
P5 (n = 16, 24, 15)	27.4 ± 1.03	31.55 ± 0.85	21.26 ± 1.31	-	2-way ANOVA, Genotype	F(2,262)=0.8747	p = 0.4182
P7 (n = 16, 24, 15)	39.45 ± 1.5	35.99 ± 1.13	41.02 ± 1.55	-	2-way ANOVA, Interaction	F(8,262)=0.4107	p = 0.914
P9 (n = 16, 24, 15)	28.75 ± 1.15	31.67 ± 0.65	23.53 ± 0.72	-			
P11 (n = 6, 10, 6)	22.18 ± 5.29	21.98 ± 2.31	12.7 ± 3.64	-			
Olfactory Habituation/dishabituation (10-12 wks)							
Time Sniffing q-tip (s)							
Water 1	2.38 ± 0.51	-	3.48 ± 0.37	-			
Water 2	1.65 ± 0.65	-	1.11 ± 0.19	-			
Water 3	0.49 ± 0.19	-	1.02 ± 0.48	-	2-way ANOVA, Genotype	F(1,132)=2.723	p = 0.1248
Cage 1	15.8 ± 3.08	-	20.24 ± 2.62	-	2-way ANOVA, Session	F(11,132)=27.45	p < 0.0001
Cage 2	6.9 ± 2.5	-	8.39 ± 3.33	-	2-way ANOVA, Interaction	F(11,132)=0.5377	p = 0.8747
Cage 3	2.71 ± 1.23	-	5.14 ± 2.24	-	2-way ANOVA, Subjects (matching)	F(12,132)=2.868	p = 0.0015
Banana 1	2.98 ± 0.6	-	6.51 ± 1.22	-			
Banana 2	1.28 ± 0.69	-	1.79 ± 0.49	-			
Banana 3	0.77 ± 0.32	-	1.73 ± 0.46	-			
Almond 1	6.01 ± 1.81	-	8.1 ± 0.92	-			
Almond 2	1.04 ± 0.56	-	1.67 ± 0.55	-			
Almond 3	0.39 ± 0.19	-	1.57 ± 0.56	-			
N	7		7				

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