Reversal of Impaired Hippocampal Long-term Potentiation and Contextual Fear Memory Deficits in Angelman Syndrome Model Mice by ErbB Inhibitors

Supplemental Information

Supplemental Methods

Mice

Angelman syndrome (AS) model mice were generated by a null mutation that was achieved via a deletion of 299 bp of exon-2 that deletes 100 of the most N-terminal amino acids and shifts the reading frame, thereby inactivating all putative isoforms of E6-AP protein (1). Mice were genotyped using specific primers as described previously (1). Mice utilized for all experiments were bred from a female that was heterozygous for the deletion and a wild-type male. For all experiments using AS model mice, wild-type littermates were used as controls and the mice were on a C57BL/6 background. The age of the mice used for all experiments (biochemistry, electrophysiology and behavior) was 10-16 weeks. Mice were housed under standard conditions in the Transgenic Mouse Facility of New York University compliant with the NIH Guide for Care and Use of Laboratory Animals. Mice were kept on a 12-hour light/dark cycle, with food pellets and water *ad libitum*.

For co-immunoprecipitation experiments we used E6-AP-EYFP transgenic mice that were generated as described previously, expressing the fused protein E6-AP with enhanced yellow fluorescent protein (EYFP) (2). Mice for experiments were bred from a female that was heterozygous for the insertion and a wild-type male. Only heterozygous mice carrying the inserted transgene were used for these experiments.

Tissue Preparation and Western Blots

AS mice and wild-type littermates were decapitated, the brain was quickly extracted, and hippocampi from both hemispheres were removed in an ice-cold cutting solution (CS) containing (in mM): 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose, and 0.6 ascorbate, and immediately snap-frozen on dry ice. Frozen hippocampi were placed in ice-cold lysis buffer containing (mM): 40 HEPES (pH = 7.5), 150 NaCl, 10 pyrophosphate, 10 glycerophosphate, 1 EDTA and 0.3% CHAPS, Protease Inhibitor II, Phosphatase Inhibitor Cocktail I, II (Sigma, St. Louis, MO), and homogenized by sonication. Protein quantification was carried out with BCA (bicinchoninic acid) protein assay reagent (Thermo Scientific, Rockford, IL). After homogenization and protein quantification, protein samples were added to β-mercaptoethanol-containing sodium dodecyl sulphate (SDS) sample

buffer and heated for 3 minutes in 60°C prior to loading on SDS-polyacrylamide gels. After heating the samples, 5-40 µg of protein (depending on the specific protein linear range of chemiluminescence) were loaded on gels, resolved, transferred to polyvinylidene difluouride membranes, and probed with primary antibodies using standard techniques. The primary antibodies and the dilutions for the Western blots used in these studies are as follows: NRG1 1:200 (Abcam, Cambridge, MA); ErbB4 1:500 (Abcam, Cambridge, MA); phospho-ErbB4 (Y1056) 1:500 (Santa Cruz, Santa Cruz, CA); phospho-ErbB4 (Y1162) 1:500; phospho-ErbB4 (Y1188) 1:500 (Novus Biologicals, Littleton, CO); ERK 1:1000 (Cell Signaling Technology, Danvers, MA); YFP 1:1000 (GeneTex, Irvine, CA); GluR1 1:1000, phospho-GluR1 (S831) 1:1000, phospho-GluR1 (S845) 1:1000, NR1 1:1000, NR2A 1:1000, and NR2B 1:1000 (Millipore, Billerica, MA); pNR2B (Y1472) 1:1000 (Thermo Scientific, Rockford, IL); β-actin 1:5000 (Santa Cruz, Santa Cruz, CA). All blots were developed using enhanced chemiluminescence detection (GE Healthcare, Fairfield, CT). The bands of each Western blot were imaged and quantified using the KODAK 4000MM imaging system. All signals were obtained in the linear range for each antibody, normalized by total protein, and quantified via densitometry. Data represent mean ± standard error of the mean. A Student's t-test was used for Western blot analysis with $p < 0.05$ as significance criteria.

Immunoprecipitation

Transgenic E6AP-EYFP mice were decapitated, and hippocampi were extracted and homogenized in lysis buffer as described previously for tissue preparation for western blots. Cleared homogenate was incubated with either anti-YFP 1:100 (GeneTex, Irvine, CA), anti-ErbB4 1:50 (Abcam, Cambridge, MA), or anti-NRG1 1:25 (LifeSpan BioSciences, Seattle, WA) and gently rotated overnight at 4ºC. 10 µL/ 100 µL supernatant/antibody was added to a slurry of IgG bound to agarose-beads (Pierce, Rockford, IL). The bead/sample slurry was incubated while rocking at 25ºC for two h. Immunoprecipitates (IPs) were washed three times in lysis buffer, and once in wash buffer in mM (50 HEPES pH 7.5, 40 NaCl, 2 EDTA). SDS-PAGE buffer was added to the washed IPs, which were then resolved on SDS-PAGE gels. The specificity of the IP was tested using antibodies against ERK2, β-tubulin and β-actin (data not shown for the last two).

Extracellular Electrophysiology

Brains from AS model mice and their wild-type littermates (10-16 weeks of age) were quickly removed and transverse hippocampal slices (400 µm) were isolated with a Leica

VT1200 Vibratome (Leica, Bannockburn, IL), and placed in ice-cold CS (in mM): 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose, and 0.6 ascorbate. A cut was generally made between the CA1 and CA3 region to prevent recurrent excitation. Slices were allowed to recover for 30 min at room temperature in 50:50 CS: artificial cerebrospinal fluid (ACSF) containing in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose, 2 $CaCl_2$, and 1 $MgCl_2$ ACSF, followed by additional recovery for 30 minutes in room-temperature ACSF.

After initial recovery, slices were placed in an interface chamber (Scientific Systems Design, Mississauga, Ontario, Canada) and maintained at 32°C in ACSF (2 mL/min). Slices were allowed to recover for an additional 120 min on the electrophysiology rig prior to experimentation. All solutions were constantly caboxygenated with 95% O_2 + 5% CO_2. Bipolar stimulating electrodes (92:8 Pt:Y) were placed at the border of area CA3 and area CA1 along the Schaffer-Collateral pathway. ACSF-filled glass recording electrodes (1–3 MΩ) were placed in stratum radiatum of area CA1. Basal synaptic transmission was assessed for each slice by applying gradually increasing stimuli (0.5–15 V), using a stimulus isolator (A-M Systems, Carlsborg, WA) and determining the input:output relationship. All subsequent stimuli applied to slices were equivalent to the level necessary to evoke a field excitatory postsynaptic potential (fEPSP) that was 30-40% of the maximal initial slope that could be evoked. Synaptic efficacy was continuously monitored (0.05 Hz). Sweeps were averaged together every 2 min. fEPSPs were amplified (A-M Systems Model 1800) and digitized (Digidata 1440, Molecular Devices, Sunnyvale, CA) prior to analysis (pClamp, Molecular Devices, Sunnyvale, CA). Stable baseline synaptic transmission was established for at least 30 min. Slices were given high-frequency stimulation (HFS) to induce long-term potentiation (LTP) using two trains of 100 Hz for 1 second, with an interval of 20 seconds between each train. Stimulus intensity of the HFS was matched to the intensity used in the baseline recordings. The initial slopes of the fEPSPs from averaged traces were normalized to those recorded during baseline. PD158780 (10 µM), PD168393 (10 µM), or bicuculline (20 µM) were applied to slices in the perfused ACSF for 2 minutes before and 8 minutes after HFS.

PD158780 and PD168393 were dissolved as stock solutions in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and further dissolved in ACSF at a dilution of 1:1000 to a final concentration of 10 µM. Both compounds were delivered in the perfused ACSF after a stable baseline was established as described. The control groups for these experiments were vehicle solutions of 0.1% DMSO. PD158780 inhibits only ErbB receptors and does not inhibit other tyrosine kinase receptors (3). PD158780 is a reversible inhibitor and does not act as a

partial agonist (4, 5). The specificity of PD158780 for ErbB4 compared to other ErbB subtypes has been studied in non-neural tissues (4, 5); currently there are no other ErbB4 specific inhibitors available. PD158780, PD168393 and other ErbB inhibitors are still used for ErbB4 inhibition (6-8), mainly because in the adult mouse hippocampus ErbB4 is the most prevalent isoform, especially in interneurons (9-12). PD168393, which has a completely different structure than PD158780, is an irreversible inhibitor that has similar specificity and affinity to PD158780 (5, 13, 14).

Two-way repeated measures analyses of variance (RM-ANOVAs) were used for electrophysiological data analysis with $p < 0.05$ as significance criteria.

Intracellular Recordings

Hippocampal slices were prepared from AS model mice and their wild-type littermates (10-16 weeks of age) in a similar manner to that described for the extracellular electrophysiological recordings, except that after slicing in ice-cold CS the slices were transferred for recovery in ACSF at 37°C for 45 min. Hippocampal CA1 pyramidal cells were illuminated and visualized using a x60 water-immersion objective mounted on a fixed-stage microscope (BX61-WI, Olympus, Center Valley, PA), and the image was displayed on a video monitor using a charge-coupled device camera (Hamamatsu, Bridgewater, NJ). Recordings were amplified by multiclamp 700B and digitized by Digidata 1440 (Molecular Devices, Sunnyvale, CA). Stimulus isolator was AM2200 (A-M Systems, Carlsborg, WA). The stimulating electrode was a bipolar Pt/Ir matrix electrode (FHC Inc., Bowdoin, ME) and was placed in stratum radiatum toward area CA3, ~300 μm from the recording electrode. The recording electrode was pulled from a borosilicate glass pipette (3–5 MΩ) using an electrode puller (P-97; Sutter Instruments, Navato, CA), was filled with an internal solution according to the specific experimental requirement, and was patched onto the soma. Rs was compensated 80% and was readjusted before each experiment. A measured liquid junction potential was corrected by adjusting the pipette offset. All voltage-clamp recordings were low-pass filtered at 10 kHz and sampled at 50 kHz.

For AMPA/NMDA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid / *N*-methyl-D-aspartate) ratio experiments the internal solution contained (in mM): 120 mM cesium methane-sulfonate, 10 mM HEPES, 10 mM EGTA, 4 mM $MgCl_2$, 0.4 mM NaGTP, 4 mM MgATP, 10 mM phosphocreatine, and 5 QX-314 (pH adjusted to 7.3 with CsOH, 290 mOsm). 50 μM bicuculline (Tocris, Ellisville, MI) was added to the bath to block gamma-aminobutyric $acid_A$ receptors. Only series resistance <20 MΩ was included in the data set. Recordings were performed in voltage

clamp, and the AMPA/NMDA current ratio was calculated from the ratio of the EPSC peak amplitude at -70 mV to the current at +40 mV 100 ms after the peak. EPSCs were evoked with stimulation pulses (100 μs) at 0.125 Hz and every 8 traces were averaged to a single trace.

For measuring chloride currents, the internal solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 2 $MgCl_2$, 2.0 Mg-ATP, 4 Na_2-ATP, 0.4 Na_2-GTP, and 5 QX-314 (pH adjusted to 7.3 with CsOH, 290 mOsm), thus yielding a chloride reverse potential of around 2 mV for chloride currents. 6,7-dinitroquinoxaline-2,3-dione (DNQX) 40 μM and 2-amino-5-phosphonopentanoate (D-AP-5) 50 μM (Tocris, Ellisville, MI) were added to the ACSF. For recording miniature inhibitory postsynaptic currents (mIPSCs) tetrodotoxin (TTX) 1 μM was also added to the external ACSF solution. Recording was performed in voltage clamp at -70 mV. IPSCs were evoked with stimulation pulses (100 μs) at 0.125 Hz and every 8 traces were averaged to a single trace. Series resistance, input resistance, and membrane capacitance were monitored during the entire experiment. Changes of these parameters, from beginning to end of experiment, bigger than 10% were criteria for exclusion of data. Peak amplitude of IPSC was measured and normalized to that of baseline.

For mIPSC analysis, mIPSCs were recorded for 30 sec at baseline and again for 30 sec after 4 minutes of PD158780 (10 μM) application. Series resistance, input resistance, and membrane capacitance were monitored during the entire experiment. Changes of these parameters, from beginning to end of experiment, bigger than 10% were criteria for exclusion of data. Data analysis was done with Clampfit (Molecular Devices, Sunnyvale, CA). Two-way RM-ANOVAs were used for electrophysiological data analysis with $p < 0.05$ as significance criteria.

Hippocampal Cannulation and Drug infusion

AS mice and their wild-type littermates were provided with water and rodent chow *ad libitum* and maintained on a 12-h light/dark cycle. Mice were anesthetized with isoflurane, placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and implanted with 22-gauge guide cannulae (Plastics One, Roanoke, VA). Stereotaxic coordinates for intra-hippocampal infusions were: -1.7 mm posterior to bregma, ±1.5 mm (both sides) from midline, and -1.5 mm from the skull surface. Animals were allowed to recover for 1 week before fear conditioning. PD158780 was dissolved in 100% DMSO to 5 mg/mL then diluted with sterile 0.9% saline to a final concentration of 1 μg/mL. Infusions were administered bilaterally in the hippocampus using injection cannulae connected to Hamilton microsyringes via polyethylene tubing. A microinfusion pump (Harvard Apparatus, Holliston, MA) was used to deliver 1 μL drug or vehicle per side (0.5 mL/min for 2 min). One minute was allowed for diffusion of the injectate before the injectors

were removed and obdurators replaced. Infusions were given immediately post-training to limit drug effects to the consolidation phase of memory formation. After injection mice were returned to their home cages and transported back to the colony.

Contextual Fear Conditioning

Mice (ages 10-16 weeks) were singly housed and handled for 1-2 min on three consecutive days in the same room that fear conditioning was conducted. Training and testing occurred during the light phase.

On the conditioning day mice were placed into standard sound attenuated fear-conditioning chambers (Med Associates, St. Albans, VT) equipped with ventilation fans to provide white noise. After 2.5 min mice received a 2 s 0.5 mA footshock. After an additional 2.5 min mice received a second 2 s 0.5 mA footshock. Mice remained in the conditioning chamber for a final 2.5 min before vehicle or drug injections were performed.

For intra-hippocampal infusion experiments mice were tested for long-term (7 days) and remote (36 days) contextual fear memory. We performed the contextual memory test by placing the mice back into the same conditioning chambers where they were trained. Freezing behavior was measured for 5 min during which no further stimuli were presented. Freezing behavior, expressed as percent time freezing, was defined as a lack of motion except that involved in respiration, and was measured by video analysis of movement by an automatic motion detection software system (Clever Systems, Reston, VA). Activity levels were assessed during each 2.5 min interval during conditioning (i.e., before and after the first foot shock and after the second foot shock) to determine if any differences in locomotive behavior or reactivity to shock were present between wild-type and AS groups and to assess acquisition.

To compare the four groups in the long-term (1 week) contextual fear memory tests, we compared the average freezing of the last three minutes, after mice habituated and were able to recognize the context by using 2-way-ANOVA for interaction of genotype and treatment. We also applied an additional comparison of the four groups over the entire period of exposure to the context in the long-term contextual fear memory by using RM-ANOVA and a posthoc Bonferroni analysis.

Primary Neuronal Cell Cultures and NRG1 Stimulation

All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) and all chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Recombinant human NRG1 was from R&D Systems (Minneapolis, MN). The following primary

antibodies were used: anti-ErbB4, anti-ubiquitin (P4D1) and control IgG (Santa Cruz, Santa Cruz, CA), anti-tubulin (B-512; Sigma).

Primary hippocampal and cortical neurons were isolated from E18 mouse embryos of AS heterozygous x AS heterozygous crosses as previously described. Briefly, hippocampi and cortices of individual embryos were dissociated separately by incubation with 0.05% trypsin at 37°C for 8 min followed by trituration. Cells were plated on poly-D-lysine coated dishes and grown in Neurobasal medium containing B27, 0.5 mM glutamine and 10 µM 5-fluorodeoxyuridine. After plating, embryos were genotyped and only cultures derived from knockout or wild-type littermates embryos were used for the experiments. The primary cell cultures contained approximately 20% interneurons. Where indicated, cortical cultures were treated with 50 ng/ml NRG1 for 15 min before lysis.

Tissue processing for immunoprecipitation and Western blots was performed as follows: at DIV8, cells were lysed in buffer (20 mM Tris-HCl pH8.0, 140 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 2 µg/ml aprotinin and 2 µg/ml leupeptin) and lysates were cleared by centrifugation (1 min at 20000 x g). Hippocampal lysates were separated by SDS-PAGE and analyzed by Western blot for total ErbB4 and tubulin levels. Cortical lysates were incubated with 2 µg anti-ErbB4 or rabbit IgG overnight, followed by protein A-Sepharose for 2 h. Beads were washed extensively in lysis buffer and precipitates were analyzed by Western blot for ubiquitin and ErbB4 levels.

Table S1. A comparison of various clinical abnormalities and neuropathologies between schizophrenia and Angelman syndrome.

Parameter	Schizophrenia	Angelman syndrome
Main brain regions affected	Hippocampus, PFC, cerebellum (15-19)	Hippocampus, PFC, cerebellum (2, 20, 21)
Autism	Part of the disorder (22-24)	Part of the disorder (25-28)
Deterioration of language	Deterioration of language skills, verbal fluency and others, sometimes to a state of mutism in severe cases (22-24)	Deterioration of language to a state of loss of language and a vocabulary of 2-3 words (25-28)
Hyperactivity	States of hyperactive motor behavior. Easily excitable. Sometimes the opposite (Cataleptic) (29-31)	States of hyperactive motor behavior. Easily excitable (25-28)
Affect	Flattening of affect, or inappropriate affect (32)	Inappropriate affect. Giggling without any identified reason (25-28)
Attention	Impaired. Short attention span (29, 32, 33)	Impaired. Short attention span (25, 26, 28)
Spine Morphology	Decreased neuropil, and a tendency to decreased spine density. Also decreased brain volume in general (34-39)	Decreased neuropil, and a tendency to decreased spine density. Unlike most cases of autism (2)
Movement disorder	Controversial, but some consider movement disorders as part of the pathology. Usually dyskinesia type (40-43)	Movement disorder that is usually ataxia. Also tongue thrusting mouthing and chewing, resembling dyskinesia (25-28)
Epilepsy	Controversial. There is research evidence in favor and against any connection (41, 44-46)	80-90% of patients suffer epilepsy (25-28)
Ube3A gene involvement	Susceptibility gene for the disorder (47)	The gene deletion is the etiology of the syndrome (1)

PFC, prefrontal cortex.

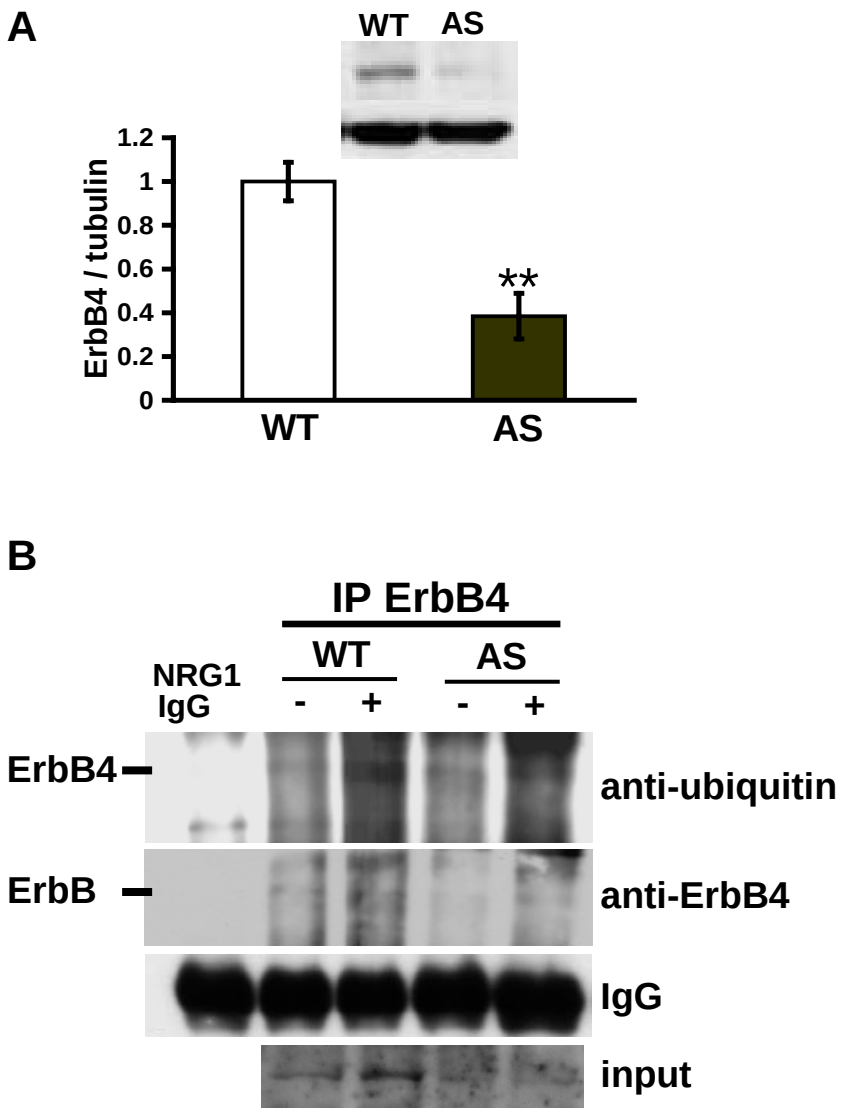

Figure S1. ErbB4 expression is decreased in primary hippocampal neuronal cultures from Angelman syndrome (AS) model mice. (**A**) Western blot of primary hippocampal neuronal culture homogenates probed with antibodies to ErbB4 and tubulin. (n = 4 for AS and wild-type (WT) mice). ** denotes $p < 0.01$ in t-test. (**B**) Western blots of ErbB4 immunoprecipitated (IP) from lysates of primary cortical neuronal cultures treated either with or without NRG1 (50 ng/ml). First lane marked as IgG is a control of IgG and cell lysate without the ErbB4 antibody used for the IP. Blots were probed with antibodies for ubiquitin and ErbB4. Loading control is the IgG band from the same blot. Input of the respective samples is displayed in the blot below.

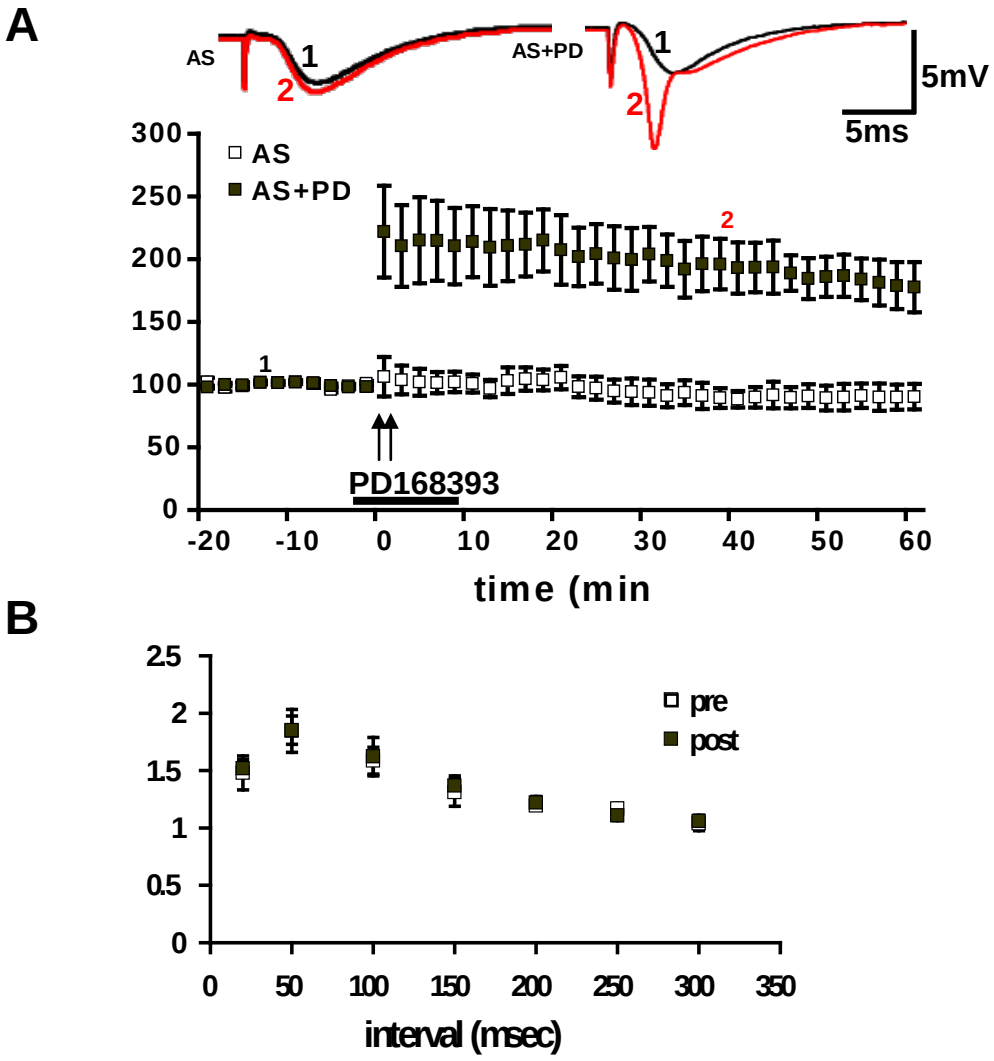

Figure S2. Long-term potentiation in hippocampal area CA1 in Angelman syndrome (AS) model mice is rescued by ErbB receptor inhibition with PD168393 (10 μM), an ErbB inhibitor with a different mechanism than PD158780. (**A**) Hippocampal slices from AS mice were treated with either vehicle (0.1% DMSO) or PD168393 (10 μM) for 10 minutes, beginning 2 minutes prior to two trains of high-frequency stimulation (HFS, indicated by the arrows). For both conditions *n* = 6 (number of mice and slices). (**B**) Paired-pulse facilitation curves for AS slices treated with PD168393 10 μM before and after HFS were similar, suggesting a postsynaptic mechanism for potentiation.

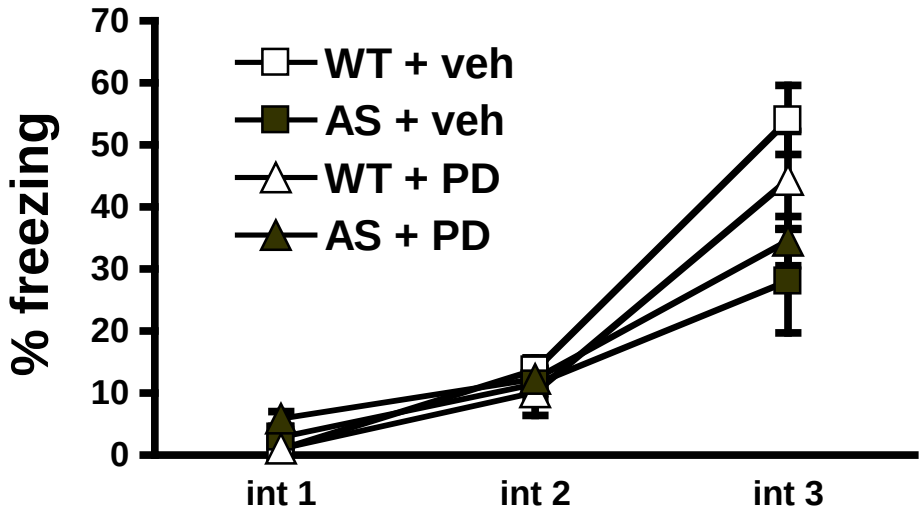

Figure S3. Acquisition curves during the conditioning session just prior to the intra-hippocampal infusion of PD158780 are similar between all four experimental groups of mice. % freezing was divided for 3 intervals before, between and after the two shocks. Each interval (int) was 2.5 minutes long. AS, Angelman syndrome; veh, vehicle; WT, wild-type.

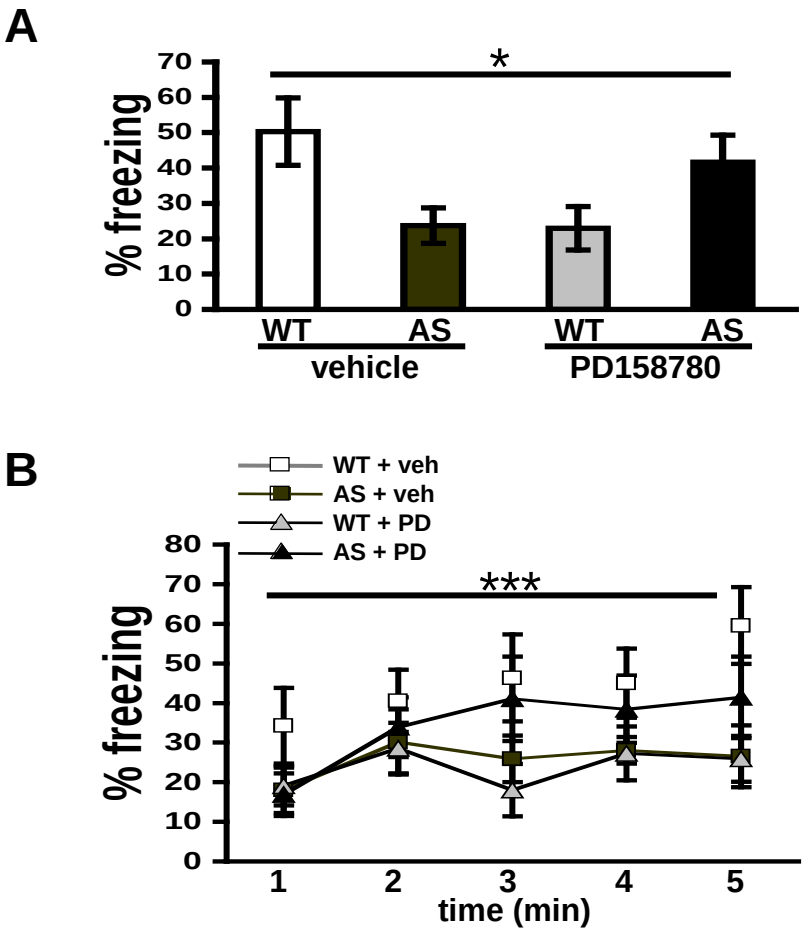

Figure S4. Remote contextual fear memory remains enhanced after 36 days in Angelman syndrome (AS) model mice when the ErbB receptor inhibitor is delivered immediately after acquisition. AS model mice and their wild-type (WT) littermates were retested by exposure to context 36 days after conditioning. All results are displayed as % freezing. $n = 8$ for WT and $n = 7$ for AS. (**A**) Long-term memory 36 days after training as measured by averaged % freezing at the last three minutes of exposure to context. * denotes statistical significance of $p < 0.05$ for interaction of group and treatment in 2-way-ANOVA. (**B**) The freezing response curve during 5 minutes of exposure at 36 days after training. *** denotes statistical significance $p < 0.001$ with repeated measures ANOVA. Posthoc Bonferroni analysis revealed no difference between WT mice treated with vehicle (veh) and AS mice treated with PD158780, and a significant increase between AS mice treated with vehicle and AS mice treated with PD158780 ($p < 0.05$). WT mice treated with vehicle and AS mice treated with vehicle are significantly different as well ($p < 0.01$). ANOVA, analysis of variance.

Supplemental References

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