SUPPLEMENTARY MATERIALS

Thorase variants cause defects in glutamatergic signaling and behavioral deficits: rescue by Perampanel.

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Material and Methods

Viral plasmid constructions and virus Generation

Three different Thorase viral plasmid constructs were generated encoding Thorase-GFP, Thorase IRES mCherry and Thorase-FLAG. Thorase-GFP and Thorase IRES mCherry were generated as previously described (*1*). Thorase mutations (R9H, D221H and E290K) were generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as described in the manual. All variant constructs were verified by sequencing at Johns Hopkins University sequencing facility. Thorase wild-type or variants coding sequence were cloned into lentiviral expression vectors cFUGW vector (UBC-promoter) between BamH I and Age I sites to generate Thorase-GFP and into pLVX-IRES-mCherry (Clontech, USA) between Xho I and BamH I to generate Thorase IRES mCherry. Thorase-FLAG tagged, wild-type or variants coding sequence were cloned into the AAV viral plasmid (pAM/CBA-pl-WPRE-BGH) under the control of the chicken beta-actin (CBA) promoter to produce recombinant AAV2 (this serotype is also known as AAV2/1;

32). All viral constructs were verified by sequencing at Johns Hopkins University sequencing facility. All AAV2 viruses were produced by Vector BioLabs. The expressions of all proteins were verified by transfecting HEK293 cells with plasmid constructs and cells examined under fluorescence microscope after 24-48 hr of infections. Cells were harvested and lysate were resolved on 10% SDS-PAGE and immunoblotted by probing with anti-Thorase and the antibodies to the various tags.

Recombinant fusion Proteins

GST tagged GluA2C (last 50 residues of GluA2 C-terminus) and GRIP1 fragment (PDZ 4,5 and 6 domains of GRIP1) plasmid constructs were generated as previously described(*1*). GST-Thorase was generated by cloning Thorase wild-type or variants coding sequence into pGEX6P1 (GE healthcare) between BamH I and Xho I. Thorase wild-type or variants coding sequence were cloned into pET32a-TRXtag (Addgene, USA) between BamH I and Xho I to generate thioredoxin-His_6-tagged Thorase. The thioredoxin tag was then deleted to generate His_6-Thorase. GST- and His_6- tagged fusion proteins were expressed in *Escherichia coli* strain BL21-CodonPlus (DE3)-RIPL (Stratagene, Agilent Tech. Div., USA) bacteria and purified by using GSTrap and His-Nickel column (GE Healthcare Lifesciences, USA), respectively following the manufacturers instructions. The purity of the recombinant proteins was assessed by SDS-PAGE followed by Coomassie blue staining. Immunoblotting was used to confirm the presence of proteins in the purified samples.

Circular dichroism spectroscopy.

Recombinant His_6-Thorase proteins eluted from His-Nickel column were further purified by size exclusive chromatography using Superdex 200 10/300GL column (GE Healthcare, Life Sciences). Eluted fractions containing His_6-Thorase were resolved on

10% SDS-PAGE, stained with commassie blue to check the purity of the protein and immunoblotted with anti-Thorase and anti-His_6 antibodies. Circular dichroism (CD) spectroscopy was performed on a AVIV 420 CD spectrometer (Biomedical Inc., Lakewood, NJ, USA). Near-UV (240–320 nm) and Far UV (190–260 nm) CD spectra were recorded at room temperature using a quartz cuvette of 0.5 cm and 0.1 cm path length, respectively with protein samples at a concentration of 2 mg/ml. The spectra were obtained from 0.5 nm data pitch, 1 nm/3 sec scan speed and 0.5 s response time were selected for the recordings.

ATPase Activity Assay

The ATPase activities of Thorase variants were assessed by measuring ATP hydrolysis and [α-^{32}P]-ATP binding. ATP hydrolyses measurement was carried out using ADP colorimetric assay kit (BioVision, USA) according to the instructions from the manufacturer with some modifications. Briefly, 1.0 mg of purified His_6-Thorase recombinant protein was incubated with 1.0 mM ATP in 0.5 ml of ADP assay buffer (supplemented with 2 mM $MgCl_2$) at 37° C for 30 min. The samples were placed on ice to stop the reactions. The amount of ADP formed due to ATP hydrolyses was then determined. The assay was repeated at least three times (each time in triplicates). The ATP binding of Thorase variants was evaluated by photo-labeling technique as described by Babst et al (*33*) with a few modifications. Approximately, 1.0 mg of purified His_6-Thorase proteins in 0.5 ml nucleotide binding buffer (50mM Tris.Cl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 5% glycerol) containing 0.1 mM [α- ^{32}P]ATP were incubated at 4° C for 1 hour. The mixtures were illuminated by UV light to cross-link the bound [α-^{32}P]ATP to Thorase. The proteins were then precipitated by adding trichloroacetic acid (TCA), washed with acetone and resuspended in the nucleotide binding buffer with SDS-PAGE

sample buffer. The samples were resolved on SDS-PAGE, stained with Coomassie blue to check protein input, destained and then exposed to a phosphor screen (Perkin Elmer, CT, USA). Quantitation of ^{32}P protein labeling was performed by scanning the radioautograph with a soft laser scanning densitometer.

GST Pull-Down Assays

For the in vitro Thorase-GluA2-GRIP1 interactions assays, purified GST fused recombinant proteins bound to glutathione beads were incubated with purified His_6-tagged proteins. Approximately 1 mg of GST-GluA2C or GST-GRIP1 bound to glutathione beads were incubated with purified 1 mg purified His_6-Thorase proteins in 0.5 ml of nucleotide binding buffer with 2 mM ATPγS at 4° C for 2 hr. The beads were then thoroughly washed three times with the nucleotide binding buffer containing 0.5 mM ATPγS and then resuspended in SDS-PAGE sample buffer. The samples were separated on 10% SDS-PAGE, stained with commassie, immunoblotted with anti-Thorase antibody to evaluate Thorase binding and also with anti- GST antibody to check GluA2C/GRIP1 input.

GST-Thorase fused recombinant proteins bound to glutathione beads were incubated with Glu2 C-terminal fragment (GluA2C) and GRIP1 PDZ 4 /5 domains fragment in 0.5 ml of nucleotide binding buffer with 2 mM ATPγS at 4° C for 2 hr. The beads were extensively washed four times with the nucleotide binding buffer containing 0.5 mM ATPγS and then resuspended in SDS-PAGE sample buffer. The samples were resolved on 10% SDS-PAGE and proteins were detected by immunoblotting using anti-GluA2C, anti-His6 (GRIP1) and anti-GST (GST-Thorase) antibodies.

Coimmunoprecipitation and Surface Protein-Crosslinking Assay

Primary cortical neuron cultures prepared from embryonic day 15 (E15) mouse pups as described (*34*) were infected with Thorase-FLAG viruses (AAV2) 12 days after plating. The neurons were collected four days after infection and sonicated in nucleotide binding buffer containing protease inhibitors and then Triton X-100 added to a final concentration of 1%. The lysate was incubated by rotation for 1 hour at 4° C and centrifuged at 10,000 x g for 10 min. The supernatants plus 2 mM ATPγS were mixed with magnetic FLAG-beads (Sigma) incubated with end-over-end rotation for 2 hours at 4° C and extensively washed four times with the nucleotide binding buffer containing 0.5 mM ATPγS using a magnetic rack. The beads were suspended in SDS-PAGE samples buffer, resolved on 10% SDS-PAGE and proteins were detected by immunoblotting using anti-Thorase, anti-GluA2, anti-GRIP1 and anti-FLAG antibodies.

To determine the distribution of AMPA receptors in Thorase variant neurons a surface protein-crosslinking assay was performed as previously described (*1*) with some modifications. Primary cortical neuron cultures infected with Thorase-FLAG viruses (AAV2) at 12 days after plating were treated with or without NMDA at 4 days after infection. The medium was replaced with ice-cold artificial cerebrospinal fluid (ACSF) containing 2 mM membrane-impermeant crosslinking agents, Bis(sulphosuccinimidyl)-suberate [BS3, (Pierce Biotechnology)] or 1 mg/ml Sulfo-NHS-SS-Biotin (*34*) to selectively crosslink cell surface proteins for 30 minutes. The reaction mixture was then replaced with ACSF containing 0.1 M glycine to quench the crosslinking (with 10 minute incubation) followed by washing cells 3 times with ACSF containing 0.1 M glycine. The neurons were suspended in lysis buffer (nucleotide binding buffer with 1% Triton-X100, and protease inhibitors), sonicated and centrifuged at 10,000 g for 10 minutes. The total protein concentrations in the supernatants were determined. For the surface biotinylation crosslinking part of the supernatant was incubated with Streptavidin beads

(Pierce) overnight at 4°C with end over end mixing. The beads were washed four times with the lysis buffer and then resuspended in 2X SDS-PAGE loading buffer. The samples were resolved on 4-12% gradient SDS-PAGE and immunoblotting were performed to analyze the surface and intracellular pools of AMPA receptors using anti-GluA2, anti-FLAG and anti-Thorase antibodies.

Thorase oligomer and GluA2-GRIP1 disassembling assays

To evaluate the oligomer formation of the Thorase variants, 1.0 mg purified proteins were treated with ADP and ATP in 1.0 ml nucleotide binding buffer at 4° C. The samples were examined by chemical cross-linking using glutaraldehyde as previously described (*33*). The cross-linked products were TCA-precipitated, washed with acetone and analyzed by SDS–PAGE and Immunoblot with anti-Thorase antibody. To determine the Kinetics of the disassembly of the Thorase oligomer complex, purified proteins with ATP were incubated at 4° C for 1 hour and then transferred to 37° C for ATP hydrolysis. Approximately 50 µl for chemical crosslinking reaction were collected every 10 minutes during the incubation period and the reaction quenched with glycine and SDS-PAGE sample buffer. The samples were resolved on 4-20% gradient SDS-PAGE (Invitrogen) and Immunoblotted with anti-Thorase antibody to evaluate the presence of Thorase oligomer complex.

To evaluate the disassembly of the GluA2-GRIP1 complex by Thorase schizophrenia variants, primary cortical neuron cultures 10 days after plating were infected with Thorase-FLAG viruses (AAV2). Four days after infection neurons were suspended in nucleotide binding buffer containing protease inhibitors with 0.5% Triton X-100 and then sonicated. The lysates were divided into three equal aliquots and nucleotides; ADP, ATP or ATPγS were added to a final concentration of 2 mM. The

lysate was incubated by rotation for 1 hour at 4° C and centrifuged at 10,000 x g for 10 minutes. The supernatants were mixed with magnetic G-beads (Sigma) that are prebound to ant-GluA2 antibody and the mixtures incubated by rotation for 1 hour at 4° C. The mixtures transferred to room temperature and incubated by rotation for another 1 hour to allow ATP hydrolysis and protein complex disassembling. The beads were extensively washed using magnetic rack four times with the nucleotide binding buffer. The beads were suspended in SDS-PAGE samples buffer, resolved on 10% SDS-PAGE and proteins were detected by immunoblotting using anti-Thorase, anti-GluA2 and anti-GRIP1 antibodies. To determine the kinetics of Thorase and GRIP1 disassembling from GluA2, similar experiments was set up in the presence of only ATP and samples collected at room temperature every 15 min to determine the rate of disassembling of Thorase and GRIP1 from GluA2.

AMPAR receptor, GluA2 surface expression recycling assay

Wild-type and Thorase heterozygote hippocampal neuron cultures were transfected with GluA2 pHluorin and mCherry (Thorase wild-type or variants) plasmids on day in vitro (DIV) 15. Using a Zeiss LSM 5 Duo confocal microscope the live neurons were imaged with 488 nm (GluA2 pHluorin) and 560 nm (mCherry) excitation with continuously perfused at a rate of 1 ml/min in recording buffer (25 mM HEPES, pH = 7.4, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 30 mM glucose, and 1 mM TTX) to monitor fluorescence changes (*1, 13*). The neurons were imaged for 5 min (1 image/min) to establish a baseline (F_0) before perfusing with NMDA buffer (similar to recording buffer but contained 300 mM $MgCl_2$, 10 mM glycine, and 20 mM NMDA) for 5 min. After perfusion with the NMDA buffer, neurons were continuously perfused with the recording buffer for another 35 min. All images were analyzed using NIH ImageJ

software (Rasband, W.S., NIH, http://rsb.info.nih.gov/ ij/, 1997–2007).

NMDA Induced Endocytosis of GluA2

To estimate the levels of surface GluA2 after NMDA induced endocytosis, live neurons were incubated at 37° C for 30 minutes in neuronal growth media containing 2 mg of mouse-monoclonal anti-N-terminal GluA2 antibodies (*1*). The antibodies were removed by washing neurons with fresh growth medium. The growth media were then replaced with Tyrodes' buffer (119 mM NaCl, 5 mM KCl, 25 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM TTX + 100 mM LY34195) with 20 mM CNQX and incubated for 5 minutess. The neurons were treated with 100 mM NMDA for 5 minutes at 37° C in Tyrodes' buffer containing 300 mM $MgCl_2$, and 10 mM glycine. The neurons were immediately fixed with 4% paraformaldehyde and 4% sucrose in PBS for 15 minutes at room temperature (RT) follow by two washes with PBS followed by treatment with 10% normal goat serum (NGS), 0.3% Triton X-100 in PBS for 1 hour at 4° C and 2 hr incubation with rabbit-monoclonal anti-C-terminal GluA2 antibodies in in PBS with 5% NGS. After three washes with PBS the neurons were incubated with mouse-Alexa 555-conjugated and rabbit-Alexa 350-conjugated secondary antibodies (Invitrogen). The neurons were washed with PBS before imaging. Images were acquired by using a Zeiss LSM 710 laser-scanning confocal microscope using a 40 X oil-immersion Plan Achromat objective and analyzed by using identical acquisition parameters. The fluorescence intensities were measured using NIH ImageJ software (Rasband, W.S., NIH, http://rsb.info.nih.gov/ij/, 1997–2007). Total AMPA receptor endocytosis was calculated using the total internalized fluorescence measurements for each cell normalized to untreated control cells. The internalization index refers to intracellular fluorescence divided by total fluorescence normalized to untreated WT neurons.

Measurement of oxygen consumption rate

Mitochondrial oxygen consumption rate (OCR) was assessed using Thorase heterozygous mouse embryonic fibroblasts (MEFs) expressing GFP or Thorase-GFP in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience), as described previously (*14, 35*). The MEF cells (~0.5 X 10^6 per well) culture media was replaced with XF24 Dulbecco's Modified Eagle Medium (DMEM) containing 10 mM glucose, 2 mM L-glutamine (Life Technologies) and 2 mM sodium pyruvate (Life Technologies). OCR was measured at 37° C with 1 minute mix, 1 minute wait, and 5 minute measurement. After 30 minutes of incubation in a CO_2 free incubator the OCR analyzed in the presence of oligomycin, carbonilcyanide m-cholorophenylhydrazone (CCCP), and rotenone to assess coupling of respiratory chain and mitochondrial respiratory capacity. The OCRs were normalized relative to cell number and basal respiratory in each well and is presented as % change.

Intracerebroventricular (ICV) injection

Approximately 3 μl AAV2 GFP or Thorase-FLAG wild-type, R9H, D221H or E290K (1X10^{13} GC/ml, Vector BioLabs) were intracerebroventricularly injected bilaterally into newborn (postnatal day 2) Thorase heterozygous mice (*15*). The expression of Thorase-FLAG were checked by immunohistochemistry and immunoblotting after 6 months.

Immunostaining of brain sections

Mice were anesthetized with sodium pentobarbital, perfused with 4% paraformaldehyde (PFA) in PBS (phosphate buffer, pH 7.4) and immediately decapitated

and the brains were carefully removed from the skull. Post fixation was performed in 4% PFA in PBS overnight and then cryoprotected by immersion in PBS containing 4% sucrose and 0.01% sodium azide for 48 hours. The fixed brains were frozen in Optimal Cutting Temperature compound (OCT) medium on dry ice and then sectioned on a microtome at 30 μm. The sections were washed three times in PBS to remove the anti-freeze solution and then separated into two sets for staining. One set of the sections were incubated with 0.3% Triton X-100 and the other set without Triton X-100 in PBS containing 0.01% sodium azide and 5% goat serum (Sigma) for 1 hour at room temperature. After a 5 three minute washes with PBS sections were permeabilized with Triton X-100 and were incubated with a mixture of rabbit anti-FLAG (1:1000) and mouse anti-Thorase (1:500) in PBS containing 0.1% Triton X-100, 0.01% sodium azide and 2.5% goat serum overnight at 4° C. Sections that were not permeabilized were incubated with rabbit-monoclonal anti-N-terminal GluA2 antibody for 4 hours followed by three washes with PBS and then incubated with mouse anti-Thorase in PBS containing 0.3% Triton X-100, 0.01% sodium azide and 2.5% goat serum overnight at 4° C. Following washout with PBS, sections were incubated with a mixture of Alexa Fluor® 488 conjugated donkey anti-rabbit IgG and Alexa Fluor® 594 conjugated donkey anti-mouse IgG (1:1000, Molecular Probes) for 3 hours at room temperature (*36*). The sections were washed three times with PBS and cell nuclei counterstained with DAPI (1:10,000, Invitrogen, Molecular Probes). The sections were mounted on slides and Zeiss LSM 710 laser-scanning confocal microscope was used to acquire images and were analyzed by using identical acquisition parameters.

Electrophysiology

Mice were anesthetized with Euthasol and transcardially perfused with ice-cold artificial cerebrospinal fluid (ACSF). Coronal sections (250 μm) containing the prelimbic portion of the medial *prefrontal* cortex (mPFC) were prepared in ice-cold ACSF using a vibrating blade microtome (Leica VT1200). Right after cutting, slices were recovered for 10 minutes at 32˚ C and then transferred to holding ACSF at room temperature. Perfusion, cutting and recovery were performed with ACSF containing the sodium substitute NMDG, 20 HEPES (pH 7.35), 25 glucose, 30 sodium bicarbonate, 1.2 sodium phosphate, 2.5 potassium chloride, 5 sodium ascorbate, 3 sodium pyruvate, 2 thiourea, 10 magnesium sulfate, 0.5 calcium chloride. ACSF used for holding slices prior to recording was identical, but contained 92 mM sodium chloride instead of NMDG and contained 1 mM magnesium chloride and 2 mM calcium chloride. ACSF used to perfuse slices during recording contained, in mM, 125 sodium chloride, 2.5 potassium chloride, 1.25 sodium phosphate, 1 magnesium chloride, 2.4 calcium chloride, 26 sodium bicarbonate, and 11 glucose. All ACSF solutions were saturated with 95% O2 and 5% CO2.

For recording, a single slice was transferred to a heated chamber (32˚ C) and perfused with normal aCSF (2.5 ml min−1) using a peristaltic pump (WPI). Visualization of prelimbic neurons was performed with an upright microscope equipped for differential interference contrast (DIC) microscopy (BX51WI, Olympus). Whole-cell patch-clamp recordings were made using a MultiClamp 700B amplifier (1 kHz low-pass Bessel filter and 10 kHz digitization) with pClamp 10.3 software (Molecular Devices). Cells were patched using glass pipets with resistance 2-3.0 MOhms, filled with internal solution containing (in mM) 120 potassium methanesulphonate (KMeSO4), 13 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, and 0.25 NaGTP, pH 7.2–7.3 (270–285 mOsm). Series resistance (10–40 MΩ) was continually monitored on-line with a −20 pA, 300 ms current injection given after every current injection step; if the series resistance changed

by more than 20%, data were not included in the analysis. Membrane potentials were not corrected for junction potentials (estimated to be 10 mV). To measure the amount of current required to reach action potential threshold, a series of current steps (2 ms duration at 2.5 Hz, 0 to 2500 pA range with +20 pA step increments) were injected into the cell until an action potential was generated. Input resistance was collected by a series of hyperpolarizing current injections (1 s duration, 0 to −100 pA in −25 pA step increments) into the cell. Input resistance was taken at the linear part of the trace. Sustained firing was determined from a series of 11 current injections (1 s duration, 50 pA steps).

All values were obtained after the cells had reached a stable response and then were averages of three cycles for each cell. The aforementioned experiments were performed having Picrotoxin (100 μM) and CNQX (10 μM) present throughout the recordings to block inhibitory and excitatory synaptic transmission, respectively. sEPSCs and mEPSCs were collected clamping the cells at −70 mV and sampled at 1 KHz having Picrotoxin (100 μM) alone or Picrotoxin (100 μM) plus lidocaine (500 mM) present throughout the experiment, respectively. Cells were patched using glass pipets with resistance 2-3.0 MOhms, filled with internal solution containing (in mM): 117 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 Mg-ATP, and 0.25 Na-GTP, pH 7.2–7.3 and 280–285 mOsm. Analysis of sEPSCs and mEPSCs were performed off-line using the MiniAnalysis program (v 6.0, Synaptosoft).

Coronal cortical slices (350 μm) were prepared from mice of postnatal 25–35 days old and incubated in artificial cerebrospinal fluid (ACSF) saturated with 95% O_2/5% CO_2. The ACSF contained in mM: NaCl 125, KCl 2.5, $MgSO_4$ 1.0, NaH_2PO_4 1.2, $NaHCO_3$ 26, $CaCl_2$ 2.0, and D-glucose 10. Slices were kept at 34 °C for at least 1 hr before recording. A single slice was transferred into a submerged recording chamber and perfused continuously with oxygenated ACSF at a rate of 2 ml/min. All experiments

were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals, and all procedures were approved by Harvard Medical Area Standing Committee on Animals.

Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were performed using HEKA EPC10 amplifier (HEKA Elektronik, Lambrech, Germany). Presynaptic stimuli (0.1 Hz, 100 μs) were delivered at layer II/III with a concentric electrode (FHC), and fEPSPs were obtained using a glass microelectrode filled with 1 M NaCl placed at Layer V. After 20 min of stable baseline recording, LTP was induced using a theta-burst stimulation (TBS) protocol consisting of five trains of burst with four pulses at 100 Hz, at 200-ms interval, repeated four times at intervals of 10 s. For LTD studies, 1 Hz stimulation was applied for 15 min. The magnitude of LTP or LTD was calculated 35–40 min after stimulation as a percentage of baseline responses. Paired-pulse facilitation (PPF) was measured using interpulse intervals of 20, 50, 100 and 200 ms. Picrotoxin (100 μM) was routinely added to the perfusing medium to block $GABA_A$ receptor-mediated inhibitory synaptic responses. Data were acquired by PatchMaster software (HEKA Elektronik, Lambrech, Germany), sampled at 10 kHz, filtered at 2.9 kHz, and analyzed using Clampfit 10.5 software (Molecular devices, Palo Alto, CA, USA).

Behavioral Experiments

Behavioral experiments were performed using 8-10 month-old Thorase heterozygous mice (n = 12-14) expressing WT (Het-WT), R9H (Het-R9H), D221H (Het-D221H), or E290K (Het-E290K) Thorase-Flag via AAV2 injection of P0-P1 pups. Thorase heterozygous mice injected with AAV2 GFP (Het-GFP) and wild-type mice without injections serve as controls. All mouse behavioral testing occurred in the Johns Hopkins University School of Medicine Behavior Core. All protocols used were approved by the Johns Hopkins University Animal Use and Care Committee and are standard

protocols found in the behavior core manual. The tests were performed in the following order: open field, Y-maze, social interaction, PPI, trace fear conditioning, drug challenge tests.

Open Field

Novelty induced activity and general locomotor activity was measured in the open field test as previously described (*37*). Horizontal and vertical movements where tracked by infrared beams using the PAS Open Field System (San Diego Instruments, San Diego, CA). Beam breaks counted 4 cm x 4 cm from each wall was considered as activity in the periphery while breaks in the rest of the chamber were counted as central activity.

Effects of psychostimulant, dizocilpine (MK-801, 0.1 mg/kg), a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist or amphetamine (1.0 mg/kg) or vehicle on locomotor activity were tested as previously described (*38*). All drug challenge tests were done 1 hour after intraperitoneal injection with drugs or vehicle. Horizontal and vertical beam breaks during the test were counted by the PAS Open Field System and were converted to distance traveled using the PAS Reporter software.

Y-Maze

Spatial and recognition working memory were assessed in the y-maze test as previously described (*37*). Spontaneous alternation sequences were expressed as a percentage of number of correct triads (animal visiting each arm in succession) to maximum possible number of correct triads. Recognition memory was reported as percent of time animal spent in the previously blocked arm during the training trial. Animals were recorded from a video camera mounted from above and tracked using Any-Maze (Stoelting Co., Wood Dale, IL) tracking software.

Social Interaction

Using the Crawley three chambered apparatus mice were tested for sociability and social preference (*37*). After habituation to the apparatus, mice were given one 10 minute trial to assess sociability where a stranger mouse was placed under a cup in one of the end chambers while the cup in the other end chamber remained empty. Video was recorded using an overhead camera and animal tracking was done with Any-Maze tracking software. The amount of time spent interacting with both cups was analyzed from the video. A second trial was done 10 to 15 minutes later for preference of social novelty in which the same mouse was placed in the apparatus with the stranger mouse from trial one as well as a second stranger mouse now under the other previously unoccupied cup. Time spent interacting with both cups for a 10 minute period was analyzed using Any-Maze software from video recorded using an overhead camera.

Pre-pulse inhibition (PPI)

Pre-pulse inhibition and acoustic startle response were measured as previously described (*38*). Briefly, the mice were placed in a Plexiglas cylinder (2 cm in diameter) within a chamber (San Diego Instruments Inc., San Diego, CA, USA) with a loudspeaker mounted above the cylinder. Presentations of the acoustic stimuli were controlled by the SR-LAB software and interface system, which also rectified, digitized and recorded responses from the accelerometer. The maximum voltages within 100-ms reading windows, starting at stimulus onset, were used as the measures of startle amplitudes. Sound levels were measured inside the startle cabinets by means of the digital sound level meter (Realistic, Tandy, Fort Worth, TX, USA). The accelerometer sensitivities within each startle chamber were calibrated regularly and were found to remain constant over the test period.

The experimental session consisted of a 5 minute acclimatization period to a 70 dB background noise (continuous throughout the session), followed by the presentation of ten 40 ms 120vdB white noise stimuli at a 20 second inter-stimulus interval (the habituation session). Upon the completion of the habituation session, each mouse was left in the enclosure for 5 minutes without presentations of any startle stimuli. Immediately after, the pre-pulse inhibition (PPI) session was begun. During each PPI session, a mouse was exposed to the following types of trials: pulse-alone trial (a 120 dB, 100 millisecond, broadband burst); the omission of stimuli (no-stimulus trial); and four pre-pulse–pulse combinations (pre-pulse–pulse trials) consisting of a 2 millisecond broadband burst used as a pre-pulse and presented 80 millisecond before the pulse using one of the four pre-pulse intensities: 74; 78; 82; 86 and 90 dB. Each session consisted of six presentations of each type of the trial presented in a pseudorandom order. PPI was assessed as the percentage scores of PPI (%PPI): 100 × (mean startle amplitude on pulse-alone trials−mean startle amplitude on prepulse–pulse trials/mean startle amplitude on pulse-alone trials) for each animal separately. The percentage of PPI for each animal was used as the dependent variable in statistical analysis.

Trace Fear Conditioning

Control and experimental mice were split into the paired and un-paired group prior to beginning of testing. Testing was conducted over a three days period. Pre-exposure testing on day one consisted of the paired group of mice being placed in the chamber for 12 minutes. Mice in the unpaired group where placed in the chamber (Harvard Apparatus, Bostom, MA) for 120 seconds before receiving a 20 second tone (80 dB, 2000 Hz) followed by an 80 second interval. This was repeated five times so that each mouse received six tones. Training took place on day two. Mice in the paired group were acclimated to the chamber for four minutes before receiving the following

sequence: 20 second interval, 20 second tone, 18 second interval, 2 second foot shock (0.5 mA), 40 second interval. This sequence was repeated six times. Mice in the un-paired group were given a four minute acclimation to the chamber before receiving the following: 58 second interval, 2 second foot shock (0.5 mA), 40 second interval. This was repeated 6 times. All mice were returned to their home cages 40 second after the completion of their trial. On day three, the animal's ability to associate the training context to the shock and the tone to the shock was tested. Both groups of mice received the same trials. For the contextual trial, mice were placed in the training chamber for three minutes. For the tone-based trial, testing took place in a novel context. Following a three minute acclimation, mice received the following: 20 second interval, 20 second tone, 40 second interval. This sequence was repeated four times. Mice were recorded during all trials from a camera mounted to the top of the enclosure and freezing was automatically detected using FreezeScan Software (Clever Sys Inc, Reston, VA).

Perampanel rescue activity

The effects of a FDA approved selective GluA2 antagonist, perampanel (0.5 mg/kg), on schizophrenia-like behavioral deficits observed in the Thorase variant mice were examined. The locomotor activities in the open field test, spatial working memory in Y-maze test and PPI were evaluated in the Thorase variant mice one hour after perampanel (0.5 mg/kg) or vehicle treatments. To examine the effects of perampanel on MK-801 induced behavior deficits perampanel (1.0 mg/kg) or vehicle was administered to the test mice 1 hour before MK-801 (0.2mg/kg) was administered immediately before open field and PPI tests were performed.

SUPPLEMENTARY FIGUREs

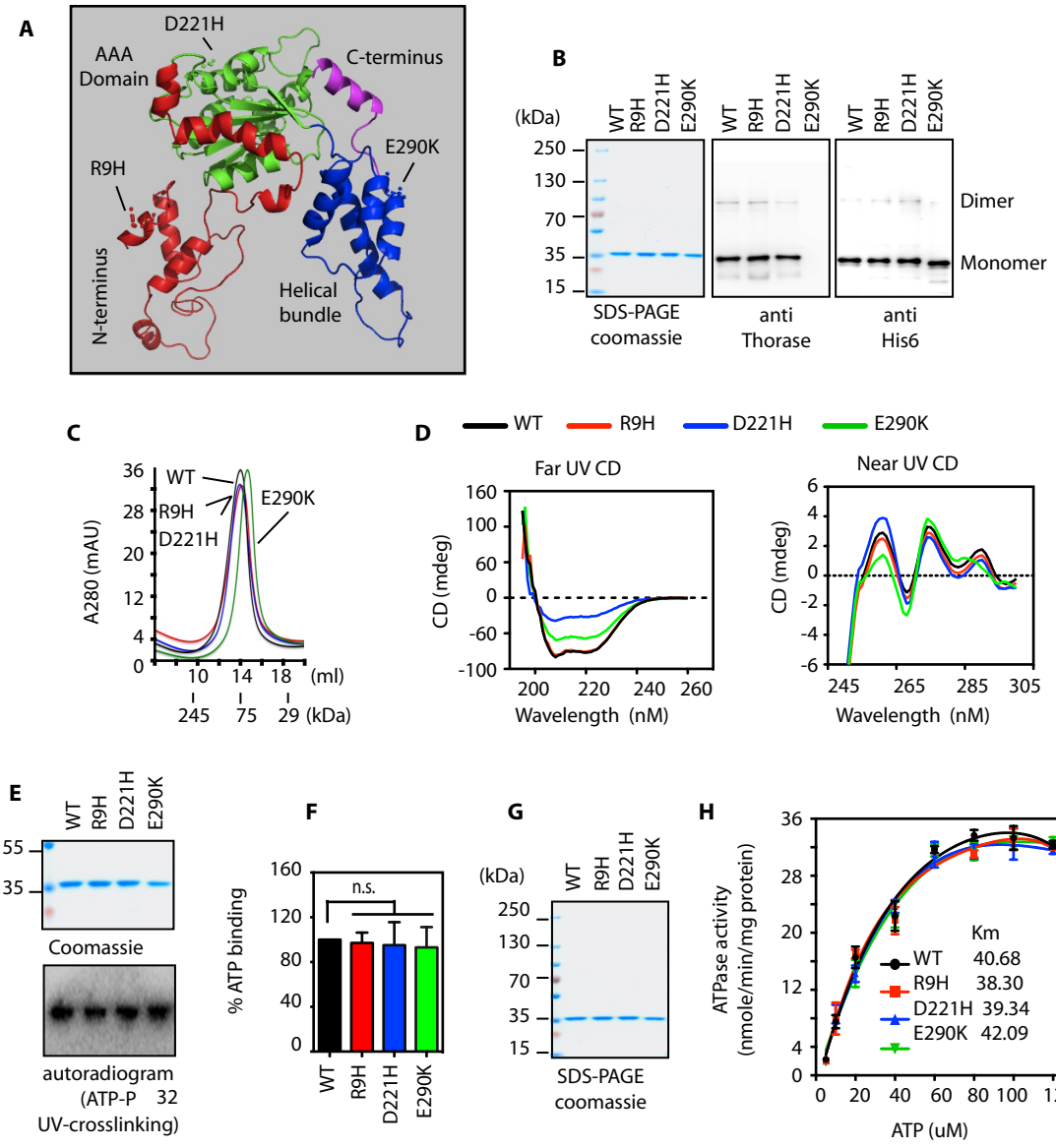

Figure S1. Thorase variants have no defects in ATPase activity but changes in structure. (A) A diagram of predicted 3D structure of Thorase showing the positions of the Thorase residues, R9H, D221H, and E290K variants. (B) Recombinant His_6 tagged Thorase wild-type (WT) and variants resolved on 10% SDS-PAGE stained with coomassie stain and immunoblotted with anti-Thorase antibody and anti His_6-HRP. (C) FPLC profile of recombinant His_6 tagged Thorase. All purified proteins appeared as dimers (~70 kDa) with E290K showing a slight shift from WT. (D) Far and near UV-CD spectra of recombinant His_6 tagged Thorase WT and variants. The D221H and E290K

variants have structural changes. (E) UV light-induced cross-linking of radiolabeled [α-P^{32}]ATP to recombinant His_6 tagged Thorase. The cross-linked proteins were separated on 10% SDS–PAGE. Upper panel is Coomassie stain of the protein input and lower panel is a representative of autoradiogram of [α-P^{32}]ATP bound to proteins. (F) Percent ATP bound to the Thorase in (E). (G-H) Analysis of ATP hydrolysis (ATPase) activities of recombinant His_6 tagged Thorase. The Km (mM) values are shown on the graph. (mean ± standard error of the mean [SEM] of experiments performed in triplicate. n = 3, n.s $p > 0.10$, ANOVA with Holm-Sidak post-hoc test when compared with WT, Power: 1-β err prob = 0.1).

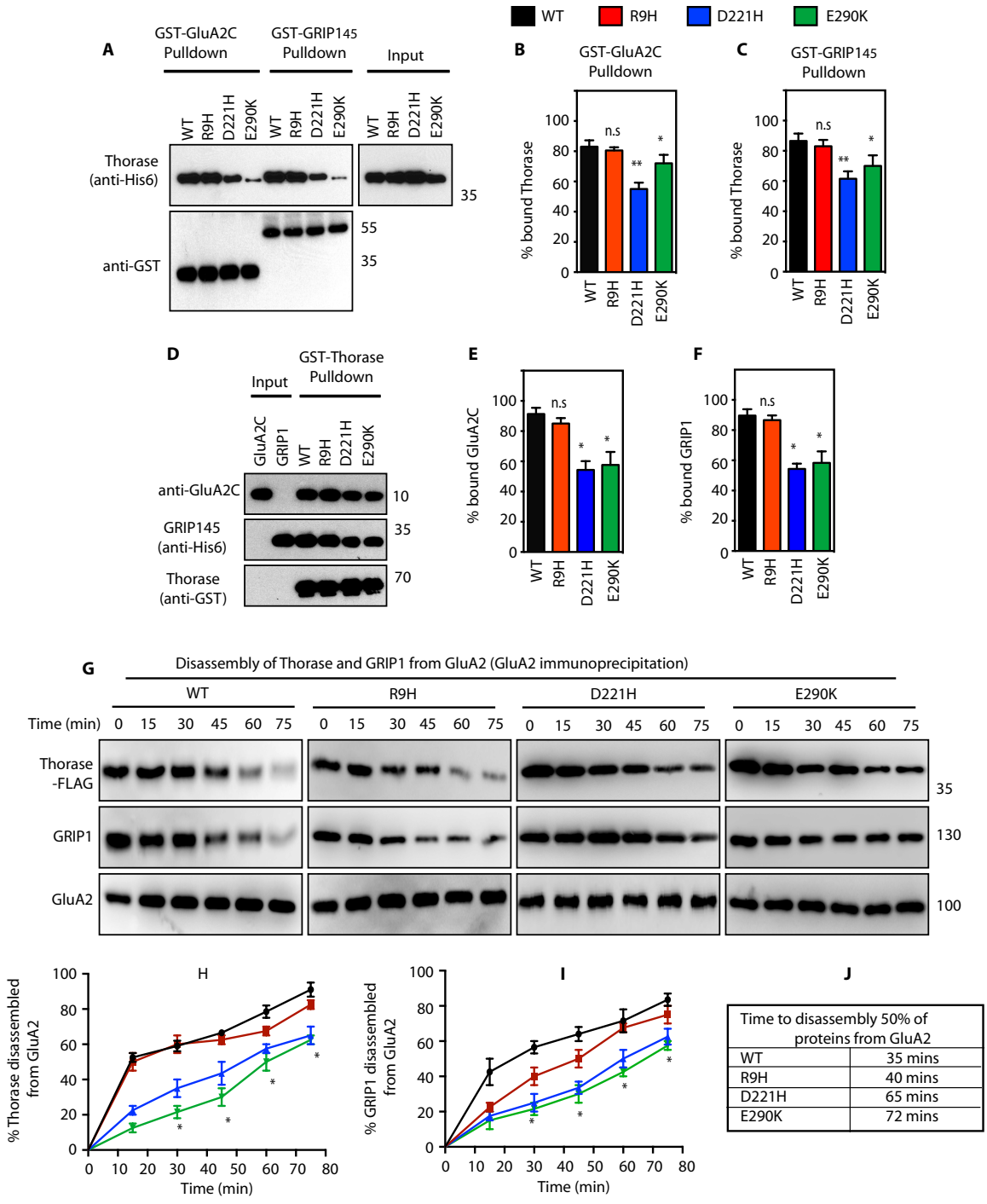

Figure S2. Mutation in Thorase impairs GluA2 and GRIP1 interactions. (A) Immunoblot analyses of GST pull-down of GST tagged GluA2 C-terminal or GRIP1 PDZ 4/5 fragment binding to recombinant His_6 tagged Thorase. The samples were resolved on 10% SDS-PAGE and immunoblotted with anti-His_6-HRP and anti-GST-HRP. (B-C)

Normalized percent bound Thorase to GluA2 and GRIP1 in (A). (D) Immunoblot analyses of GST pull-down of GST-Thorase binding to GluA2 C-terminal or GRIP1 PDZ 4/5 fragment (GRIP1$_{45}$) recombinant His$_6$ tagged. (E-F) Normalized percent bound GluA2C and GRIP1 to GST-Thorase in (D). (G) Immunoblot analyses of GluA2 IP from Thorase-heterozygous cortical neurons expressing Thorase-FLAG WT or variants in the presence of ATP. (H-I) Normalized percent bound Thorase and GRIP1 in the GluA2 IP samples in (G) at different times. (J) The evaluated time required to disassembly 50% of the GluA2/GRIP1 complex. (mean ± standard error of the mean [SEM], n = 3, **$p < 0.05$, *$p < 0.10$, n.s $p > 0.10$, ANOVA with Holm-Sidak post-hoc test when compared with WT, Power: 1-β err prob = 1.0).

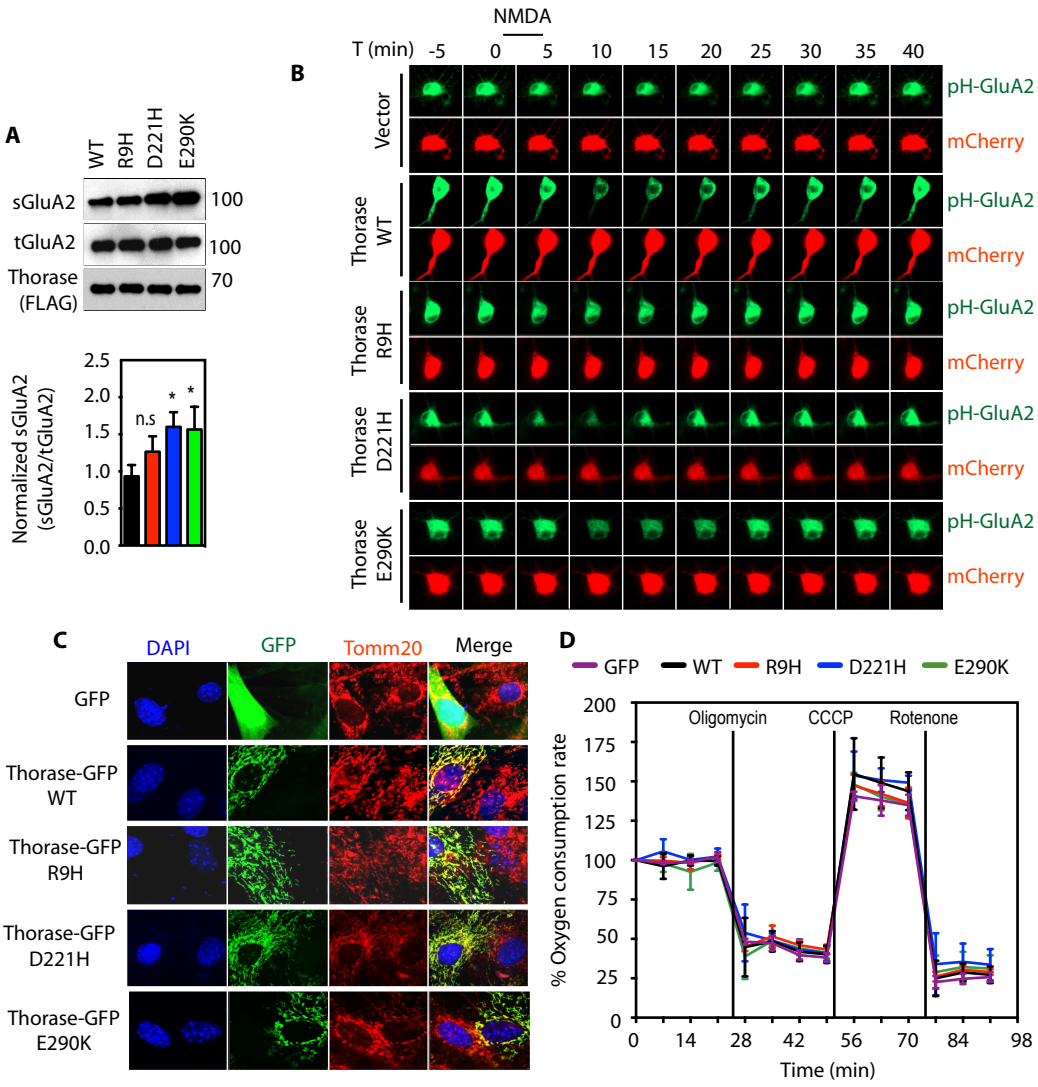

Figure S3. Mutations in Thorase impair GluA2 trafficking. (A) Immunoblot analyses of surface biotinylation assay for GluA2 surface expression in hippocampal neurons expressing Thorase-FLAG WT or variants. The graph represents the quantification of sGluA2 normalized to the total GluA2 (tGluA2). (mean ± standard error of the mean [SEM], n = 3, *p < 0.10, n.s. p > 0.10, Holm-Sidak post-hoc test compared with WT, Power: 1-β err prob = 0.7). (B) Representative images of hippocampal neurons transfected with pHluorin pH-GluA2 and mCherry showing fluorescence changes for pH-GluA2 during NMDA perfusion/washout experiments. (C) Representative images of the mitochondrial morphology of Thorase heterozygous mouse embryonic fibroblasts

(MEFs) expressing GFP or Thorase-GFP. The cells were stained with anti-Tomm20 antibody (red) to label the mitochondria and with DAPI (blue) to label the nuclei. (D) Oxygen consumption rate (OCR) of the MEFs expressing GFP or Thorase-GFP. The data was normalized to the amount of protein in each well and represents % control. (mean ± standard error of the mean [SEM], n = 12, n.s $p > 0.10$, ANOVA with Holm-Sidak post-hoc test when compared with WT, Power: 1-β err prob = 1.0).

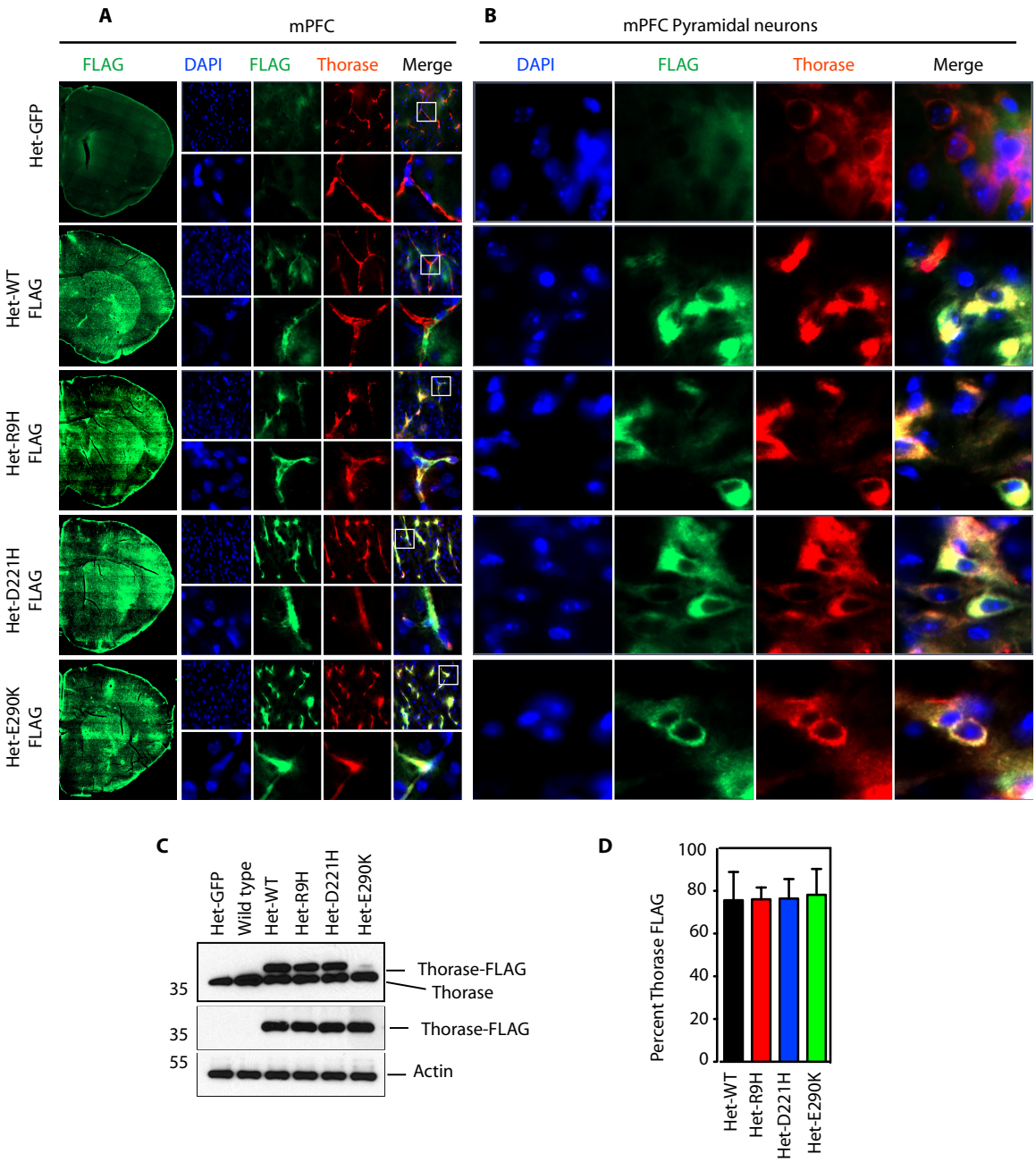

Figure S4. AAV2 Thorase-FLAG expression in mice. (A) Representative images of Thorase-FLAG and Thorase staining of brain sections from Thorase heterozygous (Het) mice expressing AAV2-GFP, AAV2-Thorase-FLAG wild-type (WT) or variants. Expression of Thorase-FLAG is shown in the prelimbic cortex pyramidal (mPFC). FLAG is labeled green and Thorase is labeled red. The nuclei of all cells are stained with DAPI

(blue). (B) Representative images showing Thorase-FLAG expression in the mPFC pyramidal neurons. (C) Immunoblot analyses of total brain lysates obtained from wild-type and Thorase heterozygous (Het) mice expressing AAV2-GFP, AAV2-Thorase-FLAG WT or variants. The samples were probed with anti-Thorase and anti-FLAG antibodies and actin-HRP (for loading control). (D) Optical densitometry quantification of percent Thorase-FLAG expression compared to the endogenous Thorase. (mean ± standard error of the mean [SEM], n = 3, **$p < 0.05$, *$p < 0.10$, n.s $p > 0.10$, ANOVA when compared with neurons expressing AAV2-GFP, Power: 1-β err prob = 1.0).

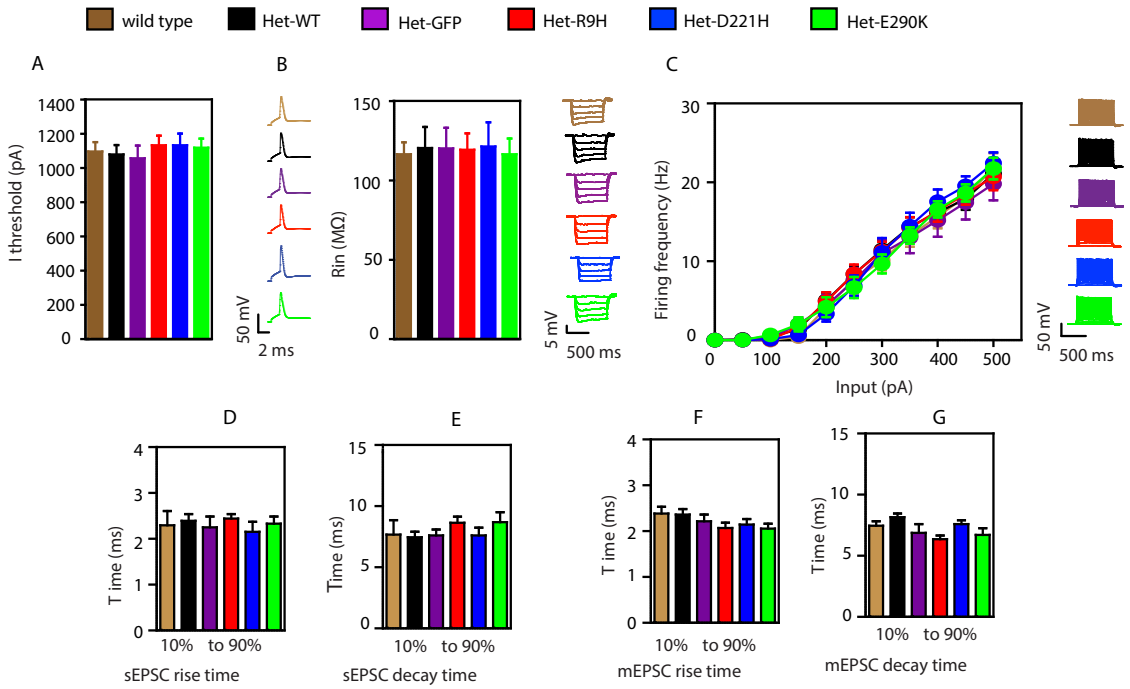

Figure S5. Membrane properties and biophysical features of AMPARs are not affected in prelimbic cortex pyramidal neurons of Thorase variant mice. (A) Representative examples of the first action potential elicited by current injection for prelimbic cortex pyramidal neurons. Each group required the same amount of current to elicit firing with a 2-ms current step in controls and Thorase variant groups. Error bars indicate s.e.m. (B) Neuronal responses elicited by hyperpolarizing steps (1 second duration, 0 to -100 pA in -25 pA steps increments). Input resistance is not affected by Thorase variants. (C) Firing frequency versus current injection (1 second duration, 0 to 500 pA, 50 pA steps). On the right, prelimbic cortex firing in response to current injection. (D-E) Mean sEPSC parameters: rise and decay times. (F-G) Mean mEPSC parameters: rise and decay times. (mean ± standard error of the mean [SEM], n = 4 - 8, n.s $p > 0.05$, ANOVA with Holm-Sidak post-hoc test compared with wild-type, Power: 1-β err prob = 1.0).

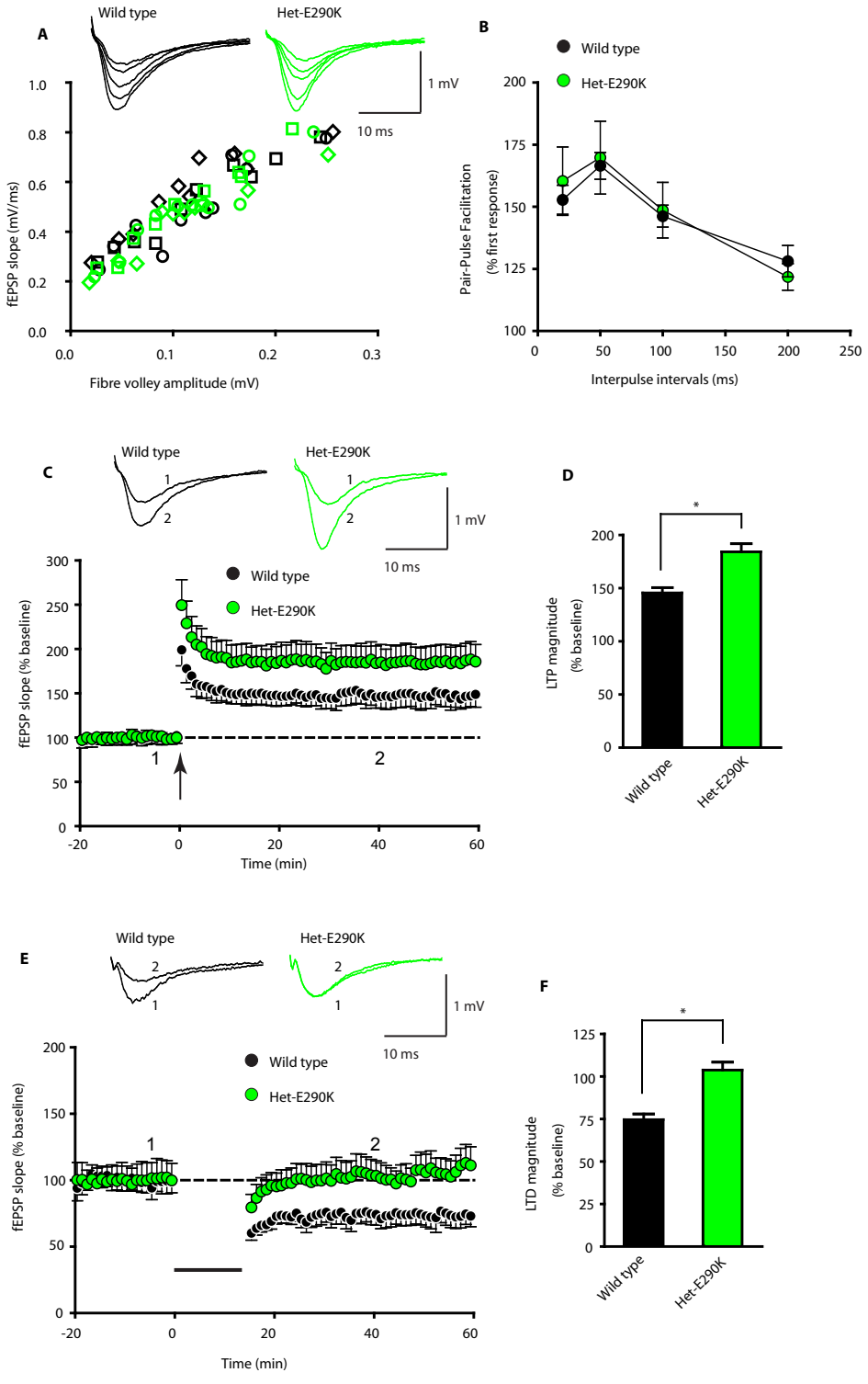

Figure S6. Defects in LTP/LTD in Thorase variant mice. (A) Input-output relationships (I/O) of fEPSPs in wild type (black color) and E290K Thorase variant (green color) slices.

Each symbol represents a set of experiments from a single slice. Traces show representative fEPSPs obtained with different stimulus intensities. (B) Paired-pulse facilitation (PPF) of fEPSPs was measured using pairs of presynaptic fiber stimulation pulses separated by 20, 50, 100, and 200 ms. For each group, the means ± SEM are indicated. (C) Enhanced LTP expression in E290K Thorase variant slices. A single train of tetanus (100 Hz for 1 s) was applied to induce LTP. Traces show typical fEPSPs recorded at the indicated time points. Data are expressed as means ± SEM, wild type, n = 8 slices from 8 mice; E290K Thorase variant, n = 8 slices from 8 mice. (D) The magnitude of LTP was calculated 40 min after LTP induction as a percentage of baseline responses (means ± SEM, fEPSP slope at 40 min, $p < 0.01$; two-tailed t test). (E) Lack of LTD in E290K Thorase variant slices. A low frequency train of 1 Hz for 15 min was applied to evoke synaptically-induced LTD. Traces show typical fEPSPs recorded at the indicated time points. Data are expressed as means ± SEM, wild type, n = 6 slices from 6 mice; E290K Thorase variant, n = 6 slices from 6 mice. (F) The magnitude of LTD was quantified 40 min after low frequency stimulation as a percentage of baseline responses. (mean ± standard error of the mean [SEM], n = 6, * $p < 0.1$, ANOVA with Holm-Sidak post-hoc test when compared with Het-WT, Power: 1-β err prob = 0.7 – 0.9).

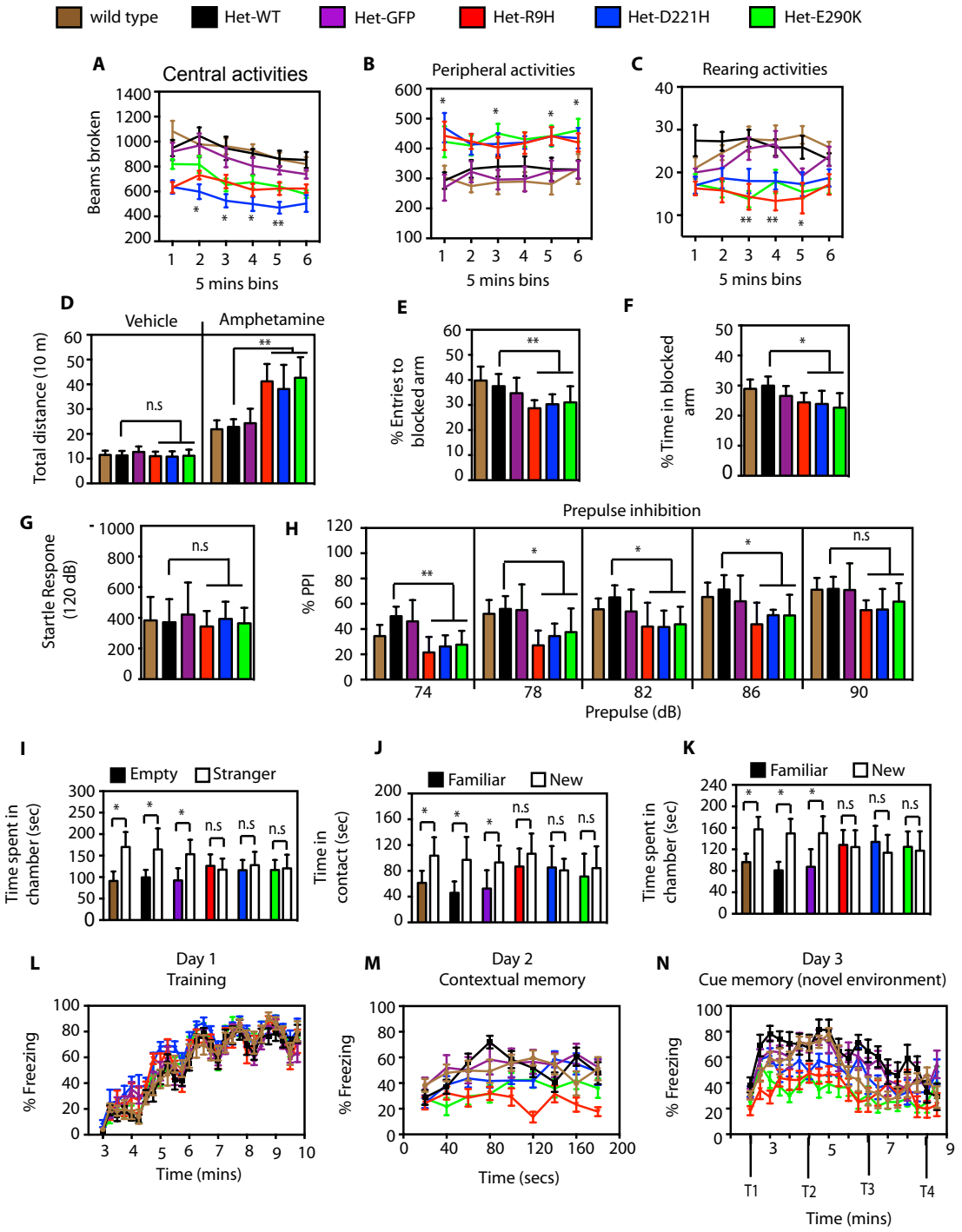

Figure S7. Impaired social behavior and learning deficits in Thorase variant mice.

(A-C) Open field assessments in wild-type (WT) and Thorase heterozygous (Het) mice expressing AAV2-GFP, AAV2-Thorase-FLAG WT or variants. (A) Central activity. (B) Peripheral activity. (C) Rearing activity. (mean ± standard error of the mean [SEM], n =

11, *p < 0.1, ANOVA with Holm-Sidak post-hoc test compared with wild-type, Power: 1-β err prob = 1.0). (D) Graphical representation of the total distance travelled by mice treated with vehicle (control) or amphetamine. (mean ± standard error of the mean [SEM], n = 6, **p < 0.05, n.s p > 0.1, ANOVA with Holm-Sidak post-hoc test compared with wild-type, Power: 1-β err prob = 1.0). (E-F) Y-maze test to assess the spatial working memory component in mice. (E) The percentage of entries into blocked arms. (F) The percentage of time spent in blocked arms. (G) Basic startle response at P120 dB. (H) Prepulse inhibition (PPI) of controls and Thorase variant mice. (I) Social interactions assessment of Thorase variant mice. The graph represents the time mice spent in the chamber with a stranger mouse versus empty chamber. (J-K) Evaluation of social memory of controls and Thorase variant mice. (J) Time in contact with familiar stranger mouse versus new stranger mouse. (K) Time spent in the chamber containing familiar stranger mouse versus chamber containing new stranger mouse. (L-N) Trace fear conditioning to assess contextual memory and associative learning in controls and Thorase variant mice. (L) Mean percent freezing for mice during training of 6 trails. (M) Mean percent freezing of mice during the context test to assess contextual memory. (N) Mean percent freezing of mice during the cue test. Mice were placed in novel environment 24 hours after training with introduction of 4 tones similar to training to assess associative learning in mice. Graphical representation of the average freezing time for the first 2 tones is shown. (Power: 1-β err prob = 1.0). (mean ± standard error of the mean [SEM], n = 10-12, **p < 0.01, *p < 0.05, n.s p > 0.05, two-way ANOVA Holm-Sidak post-hoc test compared with wild-type, Power: 1-β err prob = 0.8 - 1.0).

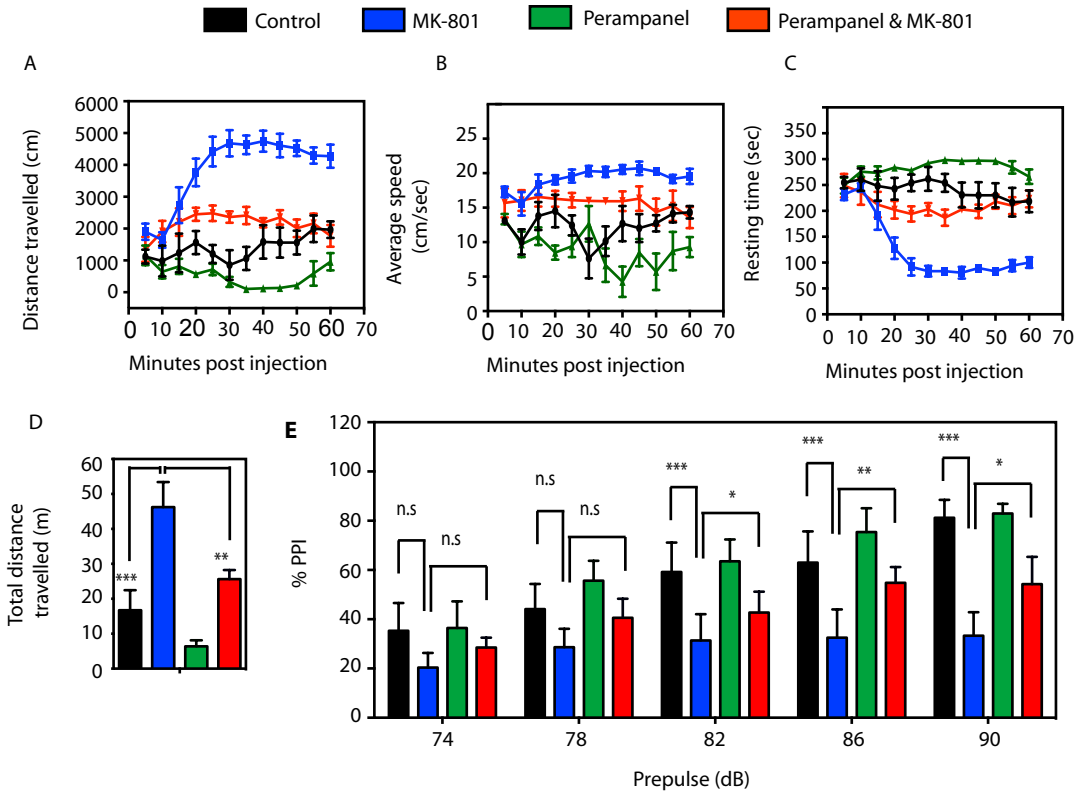

Figure S8. Thorase variant mice fear conditioning assessment and the effects of perampanel on MK-801-induced behavior deficits. (A-D) The effects of perampanel on locomotor activity and MK-801-induced hyperactivity in the open field test. Perampanel (1.0 mg/kg) or vehicle was administered to the test mice 1 hour before MK-801 (0.2mg/kg) was administered immediately before test. (A) The graph represents the distance travelled in the open field. (B) Graphical representation of the average speed of mice treated with vehicle (control) or perampanel and/or MK-801. (C) Graphical representation of the average resting time of mice treated with vehicle (control) or perampanel and/or MK-801. (D) Graphical representation of the total distance travelled of mice for (A). (E) The effects of perampanel on prepulse inhibition (PPI) in the acoustic startle response test. Perampanel (1.0 mg/kg) or vehicle was administered to the test mice 1 hour before MK-801 (0.2mg/kg) was administered 30 min before test. The graph represents the distance travelled in the open field. (mean ± standard error of the mean

[SEM] n = 4, ***p < 0.005, **p < 0.01, *p < 0.05, n.s p > 0.05, one-way ANOVA with Tukey-Kramer post-hoc test compared with control, Power: 1-β err prob = 0.9 – 1.0).

Tables:

Table S1: Summary of *ATAD1* variants identified in schizophrenia cases of Ashkenazi Jewish origin

GENOME LOCATION (hg19)	nucleotide change	aminoacid change	aa position	CASES	CONTROLS
chr10:89,574,331	C/T	Arg/His	9	1/696	0/582
chr10:89,536,107	C/G	Asp/His	221	1/691	0/617
chr10:89,516,623	C/T	Glu/Lys	290	2/701	1/598

Table S2: Summary of Thorase schizophrenia variants' activities.

Mutations	Phenotypes				
	ATPase activity	**GluR2-GRIP1 interactions**	**AMPARs trafficking**	**Electro-physiology**	**Mice behavioral tests**
R9H	Normal ATPase activity and oligo-merization	Similar to wild-type	Normal surface expression of AMPARs and defective NMDA induced AMPARs trafficking and recycling	Increased frequency and amplitude of mEPSCs	Abnormal exploratory behavior, MK801 and amphetamine hypersensitivity, decreased social interactions, deficits in spatial and associative learning, memory and PPI
D221H	Normal ATPase activity but defective oligo-merization	Impaired interactions	Increased surface expression of AMPARs and defective NMDA induced AMPARs trafficking and recycling	Increased frequency and amplitude of sEPSCs and mEPSCs	Abnormal exploratory behavior, MK801 and amphetamine hypersensitivity, decreased social interactions, deficits in spatial and associative learning, memory and PPI
E290K	Normal ATPase activity but defective oligo-merization	Impaired interactions	Increased surface expression of AMPARs and defective NMDA induced AMPARs trafficking and recycling	Increased frequency and amplitude of sEPSCs and mEPSCs	Abnormal exploratory behavior, MK801 and amphetamine hypersensitivity, decreased social interactions, deficits in spatial and associative learning, memory and PPI

Table S3: Statistical analyses.

Figure	P Value	N	Power	Statistical Test	Post-hoc Test
Fig 1B	<0.001	3	1.0	2-Way ANOVA	Holm-Sidak
Fig 1D GluR2	=0.016	3	0.8	1-Way ANOVA	Holm-Sidak
Fig 1D GRIP1	=0.013	3	0.8	1-Way ANOVA	Holm-Sidak
Fig 1F Thorase ADP	=0.876	3	0.1	1-Way ANOVA	None
Fig 1F Thorase ATP	=0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 1F Thorase ATPgS	=0.009	3	0.9	1-Way ANOVA	Holm-Sidak
Fig 1F GRIP1 ADP	=0.893	3	0.1	1-Way ANOVA	None
Fig 1F GRIP1 ATP	=0.002	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 1F GRIP1 ATPgS	=0.013	3	0.8	1-Way ANOVA	Holm-Sidak
Fig 1G Thorase	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 1G GRIP1	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 2B Control	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 2B NMDA	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 2C Control	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 2C NMDA	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Fig 2E Control	=0.180	3	0.2	1-Way ANOVA	None
Fig 2E NMDA	=0.066	3	0.8	1-Way ANOVA	None
Fig 2G	<0.001	7	1.0	1-Way ANOVA	Holm-Sidak
Fig 2H	<0.001	7	1.0	1-Way ANOVA	Holm-Sidak
Fig 3B	<0.001	≥ 5	1.0	1-Way ANOVA	Holm-Sidak
Fig 3D	=0.007	≥ 5	0.8	1-Way ANOVA	Holm-Sidak
Fig 3H	=0.002	≥ 4	0.9	1-Way ANOVA	Holm-Sidak
Fig 3J	=0.173	≥ 4	0.2	1-Way ANOVA	Holm-Sidak
Fig 4B Vehicle	=0.129	10	0.3	1-Way ANOVA	None
Fig 4B MK801	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Fig 4C	=0.006	10	1.0	2-Way ANOVA	Holm-Sidak
Fig 4D Vehicle	<0.001	7	1.0	2-Way ANOVA	Holm-Sidak
Fig 4D Perampanel	<0.001	7	1.0	2-Way ANOVA	Holm-Sidak
Fig 4E	=0.013	8	1.0	2-Way ANOVA	Holm-Sidak
Fig 4F Vehicle (74)	=0.002	10	0.9	1-Way ANOVA	Holm-Sidak
Fig 4F Vehicle (78)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Fig 4F Vehicle (82)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Fig 4F Vehicle (86)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Fig 4F Vehicle (90)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Fig 4F Perampanel (74)	=0.587	10	0.1	1-Way ANOVA	None
Fig 4F Perampanel (78)	=0.804	10	0.1	1-Way ANOVA	None
Fig 4F Perampanel (82)	=0.158	10	0.2	1-Way ANOVA	None
Fig 4F Perampanel (86)	=0.494	10	0.1	1-Way ANOVA	None
Fig 4F Perampanel (90)	=0.067	10	0.4	1-Way ANOVA	None
Fig 4G	=0.005	12	1.0	2-Way ANOVA	Holm-Sidak
Fig 4H	<0.001	12	1.0	2-Way ANOVA	Holm-Sidak
Fig 4I	<0.001	12	1.0	1-Way ANOVA	Holm-Sidak
Fig 4J Tone 1	<0.001	12	1.0	1-Way ANOVA	Holm-Sidak
Fig 4J Tone 2	<0.001	12	1.0	1-Way ANOVA	Holm-Sidak

Table S3: Statistical analyses (continue).

Figure	P Value	N	Power	Statistical Test	Post-hoc Test
Sup Fig S1F	=0.990	3	0.1	1-way ANOVA	Holm-Sidak
Sup Fig S1H	=0.049	3	0.1	2-Way ANOVA	Holm-Sidak
Sup Fig S2B	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S2C	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S2E	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S2F	<0.001	3	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S2H	<0.001	3	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S2I	<0.001	3	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S3A	=0.021	3	0.7	1-Way ANOVA	Holm-Sidak
Sup Fig S3D	<0.001	12	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S4D	=0.811	3	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S5A	=0.926	≥ 10	0.1	1-Way ANOVA	None
Sup Fig S5B	=0.881	≥ 10	0.1	1-Way ANOVA	None
Sup Fig S5D	<0.001	≥ 5	0.9	1-Way ANOVA	Holm-Sidak
Sup Fig S5E	=0.007	≥ 5	0.8	1-Way ANOVA	Holm-Sidak
Sup Fig S5F	=0.002	≥ 4	0.9	1-Way ANOVA	Holm-Sidak
Sup Fig S5G	=0.173	≥ 4	0.8	1-Way ANOVA	Holm-Sidak
Sup Fig S6D	=0.017	6	0.7	1-Way ANOVA	Holm-Sidak
Sup Fig S6F	=0.006	6	0.9	1-Way ANOVA	Holm-Sidak
Sup Fig S7A	<0.001	11	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S7B	<0.001	11	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S7C	<0.001	11	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S7D Vehicle	=0.657	6	0.1	1-Way ANOVA	None
Sup Fig S7D Amphe	<0.001	6	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S7E	<0.001	12	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S7F	<0.001	12	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S7G	=0.860	11	0.1	1-Way ANOVA	None
Sup Fig S7H (74)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S7H (78)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S7H (82)	=0.002	10	0.9	1-Way ANOVA	Holm-Sidak
Sup Fig S7H (86)	<0.001	10	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S7H (90)	=0.009	10	0.7	1-Way ANOVA	Holm-Sidak
Sup Fig S7I	<0.001	12	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S7J	=0.007	≥ 11	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S7K	<0.001	≥ 11	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S8A	<0.001	4	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S8B	=0.048	4	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S8C	<0.001	4	1.0	2-Way ANOVA	Holm-Sidak
Sup Fig S8D	<0.001	4	1.0	1-Way ANOVA	Holm-Sidak
Sup Fig S8E (74)	=0.075	4	0.4	1-Way ANOVA	None
Sup Fig S8E (78)	=0.006	4	0.9	1-Way ANOVA	Holm-Sidak
Sup Fig S8E (82)	=0.004	4	0.9	1-Way ANOVA	Holm-Sidak
Sup Fig S8E (86)	<0.001	4	1.0	1-Way ANOVA	Holm-Sidak