Seizures exacerbate excitatory: inhibitory imbalance in Alzheimer's disease with attenuation after rapamycin treatment in 5XFAD mice.

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Abstract

Increasing evidence indicates a bidirectional relationship between epilepsy and Alzheimer's disease (AD) with 22% of AD patients additionally suffering from seizures, which may be a targetable component of disease progression. Since epileptogenesis is associated with changes in excitatory: inhibitory (E:I) balance, we examined postmortem AD brain tissue from patients with and without seizure history and five times familial AD (5XFAD) mice for changes in several markers of E:I balance, including the inhibitory $GABA_A$ receptor, the chloride cotransporters, sodium potassium chloride cotransporter 1 (NKCC1) and potassium chloride cotransporter 2 (KCC2), and the excitatory NMDA and AMPA type glutamate receptors. We hypothesized that seizure history in AD patients would be associated with greater E:I imbalances, and that such changes would also be observed in the 5XFAD mice following pentylenetetrazol (PTZ) kindling. We found that seizures in AD patients were associated with alterations in NKCC1 and KCC2 expression, indicative of depolarizing GABA, and exacerbated cognitive deficits. Seizures also significantly contributed to E:I imbalance in the 5XFAD mouse model, as similar changes in NKCC1 and KCC2 expression were found in PTZ treated 5XFAD mice, along with altered AMPA receptor protein expression indicative of calcium permeable-AMPA receptors. In addition, we found that chronic treatment with the mTOR inhibitor rapamycin at doses we have previously shown to attenuate seizure-induced β-amyloid pathology and cognitive deficits in 5XFAD mice, can mitigate the dysregulation of markers of E:I balance in this model. These data suggest that mTOR activation plays a role in modifying the E:I imbalance and network hyperexcitability in AD and that the FDA-approved mTOR inhibitors such as rapamycin may have potential for therapy in AD patients with a seizure history.

Key words: Alzheimer's disease, epilepsy, GABA receptors, glutamate receptors, NKCC1, KCC2, mTOR.

Introduction

Alzheimer's Disease (AD) makes up 60-70% of all dementia cases (World Health Organization 2022) and is characterized by the accumulation of β-amyloid (Aβ) plaques followed by the emergence of neurofibrillary tangles comprised of hyperphosphorylated tau, which closely correlates with cognitive decline (Bejanin et al. 2017; Xia et al. 2017). There is clear evidence of seizures in AD patients, with 10-22% showing clinically identifiable seizures and up to 64% displaying subclinical epileptiform activity (Vossel et al. 2017). Notably, the co-occurrence of seizures and epileptiform activity in AD patients enhances disease progression and worsens cognitive performance (Vossel et al. 2017; Vossel et al. 2016; Horvath et al. 2021; Baker et al. 2019; Voglein et al. 2020). We and others have demonstrated bidirectional interactions with AD and epilepsy sharing similar pathology and convergence upon common underlying cellular mechanisms. Human temporal lobe epilepsy brain tissue shows increased Aβ plaques and increased hyperphosphorylated tau (pTau) (Gourmaud et al. 2020; Thom et al. 2011; Tai et al. 2016) and the accumulation of these neuropathological proteins in AD animal models are associated with elevated neuronal hyperexcitability and seizure susceptibility (Palop and Mucke 2009; Westmark et al. 2008; DeVos et al. 2013; Johnson, Ho, et al. 2020; Chang et al. 2021; Gourmaud et al. 2022). Indeed, development of late-onset epilepsy substantially increases dementia risk and accelerates the rate of cognitive decline (Johnson, Krauss, Kucharska-Newton, et al. 2020; Johnson, Krauss, Walker, et al. 2020; Kawakami et al. 2018; Keret et al. 2020).

While mechanisms of seizure generation in AD are not fully elucidated, there is evidence of a synaptic excitatory:inhibitory (E:I) similar to that observed in chronic epilepsy, including altered expression and function of select neurotransmitter receptors and ion cotransporters, resulting in neuronal hyperexcitability (Rakhade and Jensen 2009; Bakker et al. 2013; Liu et al. 2019). Chronic epilepsy human tissue and animal model studies show reduced inhibitory neurotransmission, including decreases in the ratio of α1/α3 inhibitory gamma-aminobutyric acid type A receptor ($GABA_AR$) subunits resulting in reduced sensitivity to benzodiazepines and diminished GABA currents (Blair et al. 2004; Noebels et al. 2010; Brooks-Kayal et al. 1998). Inhibitory GABAergic transmission is further diminished by paradoxical depolarizing action of GABA due to reversal of the chloride (Cl^-) gradient caused by increases in the ratio of the sodium (Na^+) potassium (K^+) Cl^- co-transporter 1 (NKCC1), which imports Cl^-, to that of the

potassium Cl^- cotransporter 2 (KCC2), which exports Cl^- (Talos, Sun, Kosaras, et al. 2012; Palma et al. 2006; Barmashenko et al. 2011; Kourdougli et al. 2017).

Commensurate with these changes in inhibitory drive in epilepsy, there is an increase in glutamate-receptor mediated excitability, in part due to alterations in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR) subunits (Rakhade and Jensen 2009; Rogawski 2013). Specifically, human cases and animal models of status epilepticus reveal decreased ratios of GluA2/GluA1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunits resulting in GluA2-lacking, Ca^{2+}-permeable AMPAR (Lippman-Bell et al. 2016; Rakhade et al. 2008; Egbenya et al. 2018) and increased ratios of GluN2B/GluN2A N-methyl-D-aspartate receptor (NMDAR) subunits contributing to extended decay times and elevated Ca^{2+} influx (Loddenkemper et al. 2014; Di Maio et al. 2011; Di Maio et al. 2013).

However, E:I alterations do not appear to be unique to epilepsy, as considerable evidence for neuronal network hyperexcitability have been reported in AD patients and mouse models (Hazra et al. 2016; Krantic et al. 2012; Lei et al. 2016; Li et al. 2009; Verret et al. 2012; Toniolo, Sen, and Husain 2020; Johnson, Ho, et al. 2020; Palop and Mucke 2016). Indeed, there are mechanistic interactions between neurodegenerative pathways and those involved in epileptogenesis (Gourmaud et al. 2020; Farrell, Wolff, and Teskey 2017; Chang et al. 2021). In AD patients, region-specific alterations to $GABA_AR$ subunit regulation have been observed (Kwakowsky et al. 2018), including decreased α1/α3 expression resulting in reduced GABA currents amplitude in membranes isolated from the temporal cortex (Limon, Reyes-Ruiz, and Miledi 2012). Amyloid precursor protein (APP) is elevated in AD (Matsui et al. 2007) and its overexpression results in reduced KCC2 levels *in vitro* (Doshina et al. 2017). *In vivo*, KCC2 is reduced in the AD11 mouse model, resulting in paradoxically depolarizing GABA (Lagostena et al. 2010). Furthermore, $A\beta_{1-42}$ has been shown to regulate E:I balance by increasing hippocampal NKCC1 (Lam et al. 2022) and by altering AMPAR trafficking and increasing Ca^{2+}-permeable AMPARs which can result in memory deficits via reduced long term potentiation and synapse loss (Hara et al. 2012; Guntupalli, Widagdo, and Anggono 2016; Sanderson et al. 2021; Sun et al. 2018). At the network level, E:I imbalance related to selective loss of parvalbumin+ (PV)-expressing interneurons (Zallo et al. 2018) may also contribute to memory loss in AD (Palop and Mucke 2016), as these neurons give rise to gamma

oscillation, thought to underlie executive functions including associative memory and attentional processing (Hu, Gan, and Jonas 2014; Kim et al. 2016).

While there are several candidate signaling pathways that might link network hyperexcitability and epilepsy to neurodegeneration and dementia, there is substantial evidence for the activation of the mammalian target of rapamycin complex 1 (mTORC1) as a key common element. Both AD and epilepsy can independently increase mTORC1 activation, which is associated with phosphorylation of tau at residues Ser202/Thr205 (AT8) and Thr212, Ser214 (AT100), as well as pathological APP processing (Caccamo et al. 2013; Pei et al. 2006; Oddo 2012; Cai et al. 2015; Wang et al. 2013). In addition, there is growing evidence implicating mTORC1 in glutamate and GABA receptor function and fine-tuning of excitatory and inhibitory networks relevant to cognition (McCabe et al. 2020; Kim, Lee, et al. 2021). Studies of models of Tuberous Sclerosis Complex (TSC), a genetic disorder characterized by constitutive mTORC1 overactivation commonly resulting in epilepsy (Talos et al. 2008; Wu et al. 2022), have demonstrated rescue of GABAergic neurotransmission, PV-expressing neuronal connectivity and epilepsy after rapamycin treatment (Amegandjin et al. 2021; Bateup et al. 2013; Koene et al. 2021; Goto et al. 2011), suggestive of restoration of E:I balance. Rapamycin treatment has also been reported to rescue electrophysiological and behavioral dysfunction in other mouse models with mTORC1 hyperactivation (Kim, Lee, et al. 2021; Talos, Sun, Zhou, et al. 2012).

Considering these data and that rapamycin and other rapalogs are approved by the United States Food and Drug Administration for other indications, mTORC1 is a promising target for therapeutic intervention for patients with AD and co-morbid epilepsy. In a recent study, we found that AD patients with a history of seizure display elevated tau and Aβ pathology, associated with increased mTORC1 activity (Gourmaud et al. 2022). In addition, we observed that the induction of seizures in the five times familial AD (5XFAD) mice exacerbates Aβ pathology and cognitive deficits, and that these changes are reversible with chronic low-dose rapamycin treatment. We hypothesized that markers of E:I balance, including $GABA_A$Rs, Cl^- cotransporters, NMDARs, and AMPARs may be dysregulated in AD and that these alterations and cognitive deficits may be exacerbated by seizures. Here, using tissue from the same human subjects (Gourmaud et al. 2022), we measured markers of E:I imbalance in AD temporal neocortex from patients with and without known seizure history and examined their cognitive and

functional scores. In parallel studies, we examined the 5XFAD mouse model for the same markers of neuronal excitability at prodromal and intermediate stages, and in response to pentylenetetrazol (PTZ)-induced seizures. Finally, given that we found meaningful protective effects on pathology and cognition with rapamycin (Gourmaud et al. 2022), we also utilized the tissue from this cohort of animals to further examine whether these therapeutic benefits extend to markers of E:I balance. Overall, we found that seizures significantly contribute to E:I imbalance in human AD and the 5XFAD mouse model, with markers of E:I imbalance correlated with AD severity and pathology and memory deficits in 5XFAD mice. In addition, we show that chronic rapamycin treatment in 5XFAD mice at doses we have previously shown to attenuate cognitive deficits (Gourmaud et al. 2022) can significantly attenuate this E:I imbalance. The data presented here identify novel mechanisms involved in seizure exacerbation of AD neuropathology and cognitive deficits.

Methods

Human subjects

All protocols and procedures were approved under the ethical standards of the Institutional Review Board of the University of Pennsylvania (Philadelphia, PA). The AD cognitive study population consisted of 105 patients, selected by querying the integrated neurodegenerative disease database (Xie et al. 2011) of the Center for Neurodegenerative Disease and Research (CNDR) at the University of Pennsylvania (Philadelphia, PA) for available global clinical dementia rating (CDR) score and CDR Sum of Boxes (CDR-SOB) closest to death. All patients were enrolled in observational research at Penn, with standardized assessments and medical history that includes assessment of seizure history (Hyman et al. 2012). Of the AD cases, 17 (6 males and 12 females; mean age at death= 77.38 years) had a reported clinical seizure history (AD+Sz). The remaining 87 AD patients (43 males and 44 females; mean age at death= 79.7 years) had no known seizure history (AD-Sz). AD clinical diagnosis was established during life based on the clinical history, neurological and neuropsychological assessment, and confirmed by post-mortem histopathological staging of AD neuropathological markers ($A\beta_{42}$ and tau pathology),

according to the National Institute on Aging-Alzheimer's Association (NIA-AA) guidelines (Hyman et al. 2012).

For a subset of the population detailed above, post-mortem brain specimens were acquired from CNDR and consisted of superior/mid-temporal cortex frozen samples (n= 34) and formalin-fixed paraffin-embedded sections (n=15). Detailed clinical characteristics of this AD cohort, obtained from the CNDR INDD database, were previously published (Gourmaud et al. 2022) and included age at onset of cognitive decline (disease duration), cause of death, brain weight and ordinal rating of gross ventricular enlargement at the time of death, Braak and Thal stages, seizure history and medications. Of the AD cases, 14 (6 males and 8 females; mean age at death= 78.4 years; mean PMI= 11h) had a reported clinical seizure history (AD+Sz). The other 20 AD patients (10 males and 10 females; mean age at death= 73.9 years; mean PMI= 12h) had no known seizure history (AD-Sz). Region-matched control tissue (n=15; 11 males and 4 females; mean age at death= 62.4 years; mean PMI=15.8 hours), received from the CNDR and NIH NeurobioBank, had no known neurologic or psychiatric history. AD patients were significantly older than controls at time of death ($p<0.001$). PMI did not differ between control and AD groups. Clinical characteristics for all patients included in the cognitive and biochemical studies can be found in Table S1.

Neurocognitive assessment of AD patients

The global CDR was calculated based on testing of six cognitive domains on a 0-3 scale with a score of 0 indicating cognitively normal and 3 indicating severe dementia (Khan 2016). The CDR-SOB represents the sum score of the six domains (memory, orientation, judgement and problem solving, community affairs, home and hobbies, and personal care), with scores ranging from 0 to 18, which offers a more detailed measure of cognitive and functional impairment (O'Bryant et al. 2008). In addition, the maximum score on the Dementia Severity Rating scale (DSRS) (Clark and Ewbank 1996) was retrieved for each subject in our banked tissue set. The DSRS is a questionnaire completed by the patient's caretaker to rate the patient's memory (0-6), recognition of family members (0-5), orientation to place (0-4), social and community activity (0-5), personal care (0-3), speech and language (0-6), orientation to time (0-4), ability to make decisions (0-4), home activities and responsibilities (0-4), eating (0-3), and

mobility (0-6) with higher scores indicating more severe deficits. Scores from each domain are totaled to determine overall functional performance. No significant differences between AD-Sz and AD+Sz were found in years since onset to final CDR assessment (mean AD-Sz= 8.3 years; mean AD+Sz=9.9 years).

Mice

All animal procedures were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) Office of Animal Welfare of the University of Pennsylvania (Philadelphia, PA). Male and female 5XFAD and WT mice on a mixed B6SJL background second generation (F2) generated from mice originally purchased from Jackson Laboratory (MMRC stock #3840, Bar Harbor ME) were used in these studies. 5XFAD mice harbor the human APP and PSEN1 genes under the Thy1 promoter containing five mutations for aggressive amyloid development (APP: Swedish K670N/M671L, Florida I7516V, and London V717I; PSEN1: M146L and L286V). Genotyping was conducted as specified by Jackson Laboratory. Mice were randomly allocated to treatment groups, which were counterbalanced for sex and litter.

PTZ kindling

PTZ kindling was performed as previously described (Gourmaud et al. 2022). Briefly, mice were administered eight sub-convulsive doses of the $GABA_AR$ antagonist PTZ (i.p., 35 mg/kg of PTZ, Sigma-Aldrich, St Louis, MO) or vehicle (NaCl 0.9%, Sigma-Aldrich) every 48 h for 15 days. Mice were monitored and video-recorded for one hour after PTZ administration and scored for seizure severity to confirm kindling (Gourmaud et al. 2022). One month post-kindling, mice were administered the same dose of PTZ (35 mg/kg) or vehicle to confirm the development of kindling. In our therapeutic cohort, we used a slightly modified kindling procedure to minimize animal loss. Mice were removed from kindling when they reached tonic-clonic seizures on three consecutive days of treatment and were not administered PTZ one month post kindling.

Y-maze spontaneous alternation test

The Y maze is used to measure short term memory given the willingness of mice to explore the novel arm of the maze. Mice underwent behavioral assessment at 6.5 months of age (Gourmaud et al. 2022). Mice were allowed to explore the Y-shaped maze with three arms of equal length (38.1 cm x 7.6 cm x 12.7 cm) and were recorded for 8 minutes. Spontaneous alternation was calculated as the number of arm entries/(total number of arms entered-2) x 100.

Rapamycin treatment

Mice were administered encapsulated rapamycin feed dosed at 14mg/kg (5LG6 w/151ppm Encapsulated rapamycin Irr, Emtora Holdings, San Antonio, TX) or control chow (5LG6 w/151ppm Eudragit Irr, Emtora Holdings)(Lin et al. 2013; Serrano-Pozo et al. 2011; Lin et al. 2017; Gourmaud et al. 2022) beginning at completion of PTZ kindling (3.5 months of age) until euthanasia at seven months of age. Mice were treated with rapamycin 2.24mg/kg/day by ad libitum consumption of 167 mg/one gram body weight/day.

Euthanasia and tissue preparation

Mice were anesthetized with 50 mg/kg pentobarbital (Sagent Pharmaceuticals, Schaumburg, IL) and perfused intracardially with cold PBS. Hippocampi and cortices were dissected and immediately frozen for homogenization.

Western Blot

Human and mouse brain samples were homogenized in a lysis buffer containing 1.1% sucrose, 50 mM Tris HCl pH 7.5, 500 μM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 1X protease inhibitor, 1mM phenylmethanesulfonyl fluoride (PMSF), and 1X HALT™ (Thermo Fisher Scientific, Carlsbad, CA) on ice and centrifuged at 4,100 g for 10 min at 4° C. Membrane fractions were then generated by further centriguation at 3,000 g for 10 min at 4° C. The resultant supernatant was centrifuged at 13,000 g for 10 min at 4° C. The pellet was reconstituted in the lysis buffer above. Protein concentrations were determined using the Bradford Protein Assay kit (Bio-Rad, Hercules, CA). Proteins were separated by electrophoresis on 4-20% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene difluoride

membranes (EMD Millipore, Burlington, MA) following previously used standard protocols (Gourmaud et al. 2020; Gourmaud et al. 2022). Primary and secondary antibodies are listed in Table S2. Detected proteins were imaged with Odyssey Imaging System (LI-COR Biosciences) and quantified with Image Lab (Bio-Rad). Each protein was normalized to β-actin expression level.

Immunohistochemistry

Human tissue was formalin fixed, paraffin embedded, and sectioned at 6 µm. Immunohistochemistry was performed using previously established protocols (Gourmaud et al. 2020; Gourmaud et al. 2022) with anti-PV antibodies (Table S2). Briefly, slides were incubated in primary antibody for 15 min and IgG and HRP-linker conjugates for 8 min. Slides were then exposed to hydrogen peroxide for 5 min before being incubated with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) for 10 min. Finally, slides were counterstained for nuclei with hematoxylin before being mounted with permanent medium. Slides were then imaged with a Nikon Eclipse 80i microscope with a digital Nikon DS-Fi2 camera (Micro Video Instruments, Avon, MA) at 20x objective in a zigzag sequence to sample up to 14 non-overlapping images of the grey matter.

Statistics

For cognitive and functional performances, comparisons between AD+Sz and AD-Sz for ranked ordinal data were made by Mann-Whitney test. For the human tissue studies, all AD to control comparisons were made by unpaired t-tests or Mann-Whitney tests for non-normal data. Control, AD-Sz, and AD+Sz comparisons were made by one-way analysis of variance (ANOVA) or Kruskal-Wallis for non-normal data. For the prodromal mouse study, 5XFAD to WT comparisons were made by unpaired t-test. For the nontherapeutic PTZ study, comparisons were made by two-way ANOVA with genotype and kindling as independent variables. For the therapeutic PTZ study, 5XFAD and WT mice were analyzed separately by two-way ANOVAs with PTZ and rapamycin treatment as independent variables. All ANOVA analyses were followed by Tukey's post hoc test to examine group differences when main effects or interactions were found. Additional multiple linear regressions were performed for all data to determine potential sex effects. Where sex effects were present, data points are displayed as squares (female) or

triangles (male) to visualize these differences. All correlations were calculated by simple linear regression. We considered results to be significant at $p<0.05$. All statistical analyses were performed in GraphPad Prism 9 software (San Diego, CA).

Results

AD patients with a seizure history exhibit more severe dysregulation of proteins that mediate excitation: inhibition balance

We hypothesized that the network hyperexcitability in AD would be associated with alterations in neurotransmitter receptors and/or ion transporter expression, with heightened alterations in AD+Sz as compared to AD-Sz patients. Specifically, we examined inhibitory deficits established in models of epilepsy, including reduced ratio of α1/ α3 subunits of the inhibitory $GABA_A$ receptor ($GABA_AR$) (Noebels et al. 2010) and increased Cl^- cotransporters NKCC1/KCC2 ratio (Talos, Sun, Kosaras, et al. 2012; Palma et al. 2006; Galanopoulou 2008). We also examined AMPA and NMDA glutamate receptors, namely increased ratios of GluA1/GluA2 and GluN2B/GluN2A subunits, to determine if neuronal excitation is heightened (Lippman-Bell et al. 2016; Loddenkemper et al. 2014; Rakhade et al. 2008).

Western blot analysis of AD temporal cortex (Figure 1A-E) showed that the $GABA_ARα1/α3$ ratio was significantly decreased in all AD patients when compared to controls ($p<0.01$, Figure 1B) primarily due to significant reduction in $GABA_AR$ α1 ($p<0.05$, Figure 1B), supporting previous reports (Limon, Reyes-Ruiz, and Miledi 2012). The alterations in $GABA_ARα1$ in the overall AD group were largely driven by AD patients with seizures, as there was a significant decrease in $GABA_ARα1$ expression in AD+Sz compared to controls ($F=3.52$, Tukey's post hoc: $p<0.01$, Figure 1B), which was not found in AD-Sz. The ratio of NKCC1/KCC2 was also significantly increased in the AD+Sz group when compared to the AD-Sz group ($p<0.05$, Figure 1C), likely inducing further GABAergic dysfunction due to reduced hyperpolarization and possibly depolarization at $GABA_AR$ synapses in AD+Sz patients. This difference was largely attributable to downregulation of the extracellular directed KCC2 transporter in the AD+Sz group, compared to the AD-Sz group ($F=3.3$, Tukey's post hoc: $p<0.05$, Figure 1C). In addition, sex was a

significant predictor of KCC2 dysregulation, with decreased levels in males ($p<0.05$, [0.0314, 0.230], Figure 1C).

To further explore inhibitory dysfunction, we examined whether there was a decrease in the predominant PV-expressing interneurons (Rudy et al. 2011) in AD, given that there is a well-established association between inhibitory GABAergic PV cell loss, cognition (Murray et al. 2015) and epilepsy (Drexel et al. 2011; Zamecnik et al. 2006). Quantitative immunohistochemical analysis revealed a decrease in the number of PV+ cells in AD temporal cortex compared to controls ($p<0.01$, Figure S1 A-B), and this was largely due to the decrease measured in the AD+Sz group ($p<0.01$, Figure S1B).

To determine whether alterations at excitatory glutamatergic receptors play a role in E:I imbalance, we examined AMPAR and NMDAR subunit levels and ratios by western blot. The expression levels of the AMPA (GluA1 and GluA2) and NMDA (GluN2A and GluN2B) glutamate receptor subunits were also differentially altered in human AD tissue. While AMPAR GluA1 subunit levels were not different between control and AD tissue, we found increased expression of GluA2 subunits in AD temporal cortex compared to controls ($p<0.05$, Figure 1D) and the corresponding GluA1/GluA2 ratio was decreased in the AD cases compared to controls ($p<0.05$, Figure 1D). However, no differences were found between AD+Sz or AD-Sz in AMPAR subunits or subunit ratios (Figure 1D). In examining NMDA receptor proteins, GluN2A and GluN2B subunits were both expressed at significantly higher levels in AD temporal cortex when compared to controls ($p<0.05$ and $p<0.001$, respectively, Figure 1E), but there was no difference between the AD+Sz and AD-Sz group in terms of the ratio of GluN2B/GluN2A between the cohorts (Figure 1E). Taken together, these results provide strong evidence of decreased inhibition and increased excitation in AD patients overall, regardless of seizure history, and an exacerbation of selective regulators to increase neuronal excitability in the AD+Sz group.

AD patients with seizure history have worsened cognitive and functional performance

To determine potential associations between seizures, altered E:I balance, and cognitive and functional deficits in AD, we examined scores from clinical (global CDR and CDR-SOB) and caretaker questionnaire (DSRS) for subjects with known seizure history. The AD+Sz group showed elevated final global CDR ($p<0.001$, n=18-87, Figure 2A) and CDR SOB ($p<0.0001$, n=18-87, Figure 2B) compared to

the AD-Sz group. AD+Sz patients also showed significantly worsened overall functional rating (DSRS) compared to those in the AD-Sz group ($p<0.01$, n=14-19, Figure 2C), including significantly worse ratings in memory, recognition of family members, mobility, orientation to place, social and community activities, and incontinence ($p<0.05$, n=14-19, Figure S2). Overall, these data indicate seizures are associated with worsened cognitive performance and overall function, corroborating previous studies examining epileptiform activity in AD (Voglein et al. 2020; Baker et al. 2019; Horvath et al. 2021; Vossel et al. 2016).

In addition, we performed linear regressions between clinical outcomes of AD and markers of E:I balance. We found that CDR-SOB was inversely correlated with GluN2A levels across all AD subjects ($r=-0.39$, $p<0.05$, Figure 2D) while gross brain atrophy ratings at autopsy was associated with decreased levels of $GABA_AR\alpha1$ in AD+Sz ($r= 0.90$, $p<0.01$, Figure 2E), indicating an interaction between these markers of E:I balance and cognitive decline and AD severity, respectively.

Excitation: inhibition imbalance in prodromal stage 5XFAD mice

5XFAD mice first begin to exhibit subclinical epileptiform electroencephalographic activity and increased susceptibility to PTZ at a prodromal stage (around 4 months of age) (Abe et al. 2020; Gourmaud et al. 2022), at which time mild behavioral impairment and AD pathology are present (Kimura and Ohno 2009; Oakley et al. 2006). To further understand the mechanisms and progression of hyperexcitability in 5XFAD mice and to extend the evidence of E:I imbalance reported in other AD models at early (Kim, Kim, et al. 2021; Kazim et al. 2017; Davis, Fox, and Gigg 2014) and more advanced pathological stages (Palop, Mucke, and Roberson 2011; Palop et al. 2007; Verret et al. 2012; Hazra et al. 2016; Krantic et al. 2012) we assessed the hippocampi and cortices from prodromal 5XFAD and WT mice for E:I markers by western blot (Figure S3). We found a significant decrease in $GABA_AR\alpha1/GABA_AR\alpha2$ ratio in the hippocampus ($p<0.05$, n=14-15) and trend towards decreased KCC2 in the cortex ($p=0.09$, n=14-15) of 5XFAD mice without changes seen in AMPAR or NMDAR subunits (Figure S3). These data suggest that impaired inhibition may precede alterations in excitatory neurotransmission.

Kindling further enhances dysregulation of proteins involved in excitation: inhibition balance in 5XFAD mice

In accordance with E:I balance alterations seen in prodromal mice, we have recently demonstrated that PTZ kindling at 3-3.5 months exacerbates cognitive deficits and AD neuropathology in 5XFAD mice (Gourmaud et al. 2022). To determine a causative role of seizure-induced E:I imbalance in these mice, we performed western blot to analyze the expression of excitatory and inhibitory neurotransmitter receptor subunits and Cl^- cotransporters in the hippocampi (Figure 3) and cortices (Figure S4) of the same cohorts of 5XFAD and WT mice. At an intermediate stage of pathological progression (7 months, 3.5 months after the completion of PTZ kindling protocol), the $GABA_AR\alpha1/GABA_AR\alpha3$ ratio was decreased in hippocampal tissue of 5XFAD mice compared to WT mice (genotype effect: $F_{1,61} = 22.76$, $p<0.0001$, Figure 4A), with post hoc analysis showing significant decreases within both vehicle ($p<0.05$) and PTZ-kindled groups ($p<0.01$). These changes to $GABA_AR$ ratio seem to be largely driven by decreases in the $GABA_AR\alpha1$ subunit in 5XFAD mice (genotype effect: $F_{1,61}=40.09$, $p<0.0001$) (Figure 3A), a pattern similar to those observed in the human AD+Sz group. In addition, sex was a significant predictor of $GABA_AR\alpha1/GABA_AR\alpha3$ with greater decreases found in females ($p<0.05$, [0.0156, -0.1591], Figure 3A).

In addition, kindling resulted in an increased NKCC1/KCC2 ratio in the hippocampus of 5XFAD mice (interaction: $F_{1,61}=5.78$, $p<0.05$) compared to WT and vehicle treated 5XFAD mice ($p<0.05$. Figure 3B), due to a significant increase in expression of the Cl^- importer, NKCC1 (interaction: $F_{1,61}=7.93$, $p<0.01$; Tukey's post hoc: $p<0.01$) (Figure 3C). The expression of Cl^- exporter KCC2 was also decreased in the hippocampus of 5XFAD mice regardless of kindling status (genotype effect, $F_{1,61} = 12.35$, $p<0.001$). These data suggest that Cl^- dysregulation contributes to the exacerbation of GABAergic dysfunction in the hippocampus of kindled 5XFAD mice, which has been shown to result in excitatory GABA (Schulte, Wierenga, and Bruining 2018). Furthermore, we used our retrospective quantitative measures of disease severity in this same cohort of mice (Gourmaud et al. 2022) and found that NKCC1/KCC2 was positively correlated with $A\beta_{42}$ ($r=0.57$, $p<0.05$) and pTau AT100 (Thr212, Ser214)/Tau ($r=0.64$, $p<0.05$) in the hippocampus of PTZ kindled 5XFAD mice and with performance on the Y-maze ($r=-0.61$, $p<0.01$) (Figure 3F, G, H) which we previously demonstrated was worsened by PTZ kindling, suggesting a relationship between E:I imbalance, AD pathology, and cognitive decline.

To determine whether excitatory neurotransmission may be dysregulated in kindled 5XFAD mice we examined AMPAR and NMDAR subunit protein levels. In the hippocampus, we found a significant interaction (Genotype x kindling, $F_{1,61}$=4.622, $p<0.05$) to increase GluA1/GluA2 ratios in PTZ kindled 5XFAD mice (Figure 3C, $p<0.05$). Increases to GluA1/GluA2 ratio are a source of E:I imbalance that has been described in both human cases and mouse models of status epilepticus (Loddenkemper et al. 2014). In addition, both subunits were decreased in the hippocampus of 5XFAD mice (GluA1 genotype effect, $F_{1,61}$ = 7.722, $p<0.01$; GluA2 genotype effect, $F_{1,61}$ = 30.28 $p<0.0001$). The NMDAR GluN2A subunit was decreased in 5XFAD mice (genotype effect, $F_{1,61}$=6.31, $p<0.05$) while GluN2B and subunit ratios showed no main effects or interactions between groups in the hippocampus (Figure 3D).

We also examined these E:I aspects in cortical homogenates. Similar changes were seen in $GABA_AR$ subunits with vehicle treated 5XFAD mice showing reduced $GABA_AR$ α1/α3 (interaction: $F_{1,43}$= 6.44, $p<0.05$) (Figure S4A, C). Additionally, NKCC1/KCC2 ratios were increased in the PTZ kindled cortex (Kindling effect: $F_{1,43}$=4.49, $p<0.05$), which was largely driven by a reduction of KCC2 in kindled WT mice (interaction: $F_{1,43}$= 6.75, $p<0.05$). Interestingly, there was a decrease in NKCC1 in vehicle treated 5XFAD mice (genotype effect: $F_{1,43}$= 5.2, $p<0.05$) compared to vehicle treated WT mice (Tukey's post hoc: $p<0.05$), which may indicate a compensatory mechanism, although NKCC1/KCC2 ratios were not significantly different (Figure S4B). 5XFAD mice also showed increased GluA1/GluA2 (genotype effect: $F_{1,43}$= 6.33, $p<0.05$) with post hoc analysis revealing significant increases in PTZ kindled 5XFAD mice compared to WT ($p<0.05$) reflecting changes seen in the hippocampus. GluN2A was decreased in the cortex 5XFAD mice (genotype effect: $F_{1,43}$= 8.98, $p<0.01$) without changes in GluN2B or GluN2A/GluN2B ratio (Figure S4D).

mTORC1 inhibition modifies select seizure-induced excitation:inhibition markers in the hippocampus of 5XFAD mice

Given that chronic low-dose rapamycin treatment (2.24 mg/kg) rescued seizure-induced neuropathology and cognitive deficits in the same cohorts of 5XFAD mice (Gourmaud et al. 2022), we sought to determine whether this treatment also ameliorated the PTZ-exacerbated E:I imbalance. The

5XFAD (Figure 5) and WT (Figure S4) mice used in our previous report were analyzed separately by two-way ANOVA with PTZ kindling and rapamycin treatment as independent variables.

Rapamycin ameliorated the reduction in $GABA_AR$ α1/$GABA_AR$ α3 in PTZ kindled 5XFAD mice (kindling effect: $F_{1,45}$=6.1, p<0.05; rapamycin effect: $F_{1,45}$=4.1, p<0.05) with post hoc analysis showing a strong trend towards an increase in rapamycin treated, non-kindled 5XFAD mice compared to control 5XFAD (p=0.058). These shifts corresponded to a rescue in GABARα1 subunit expression (kindling effect: $F_{1,45}$= 4.2, p<0.05; rapamycin effect: $F_{1,45}$=6.1, p<0.05) (Figure 4A). When assessing Cl^- cotransporter levels, we found an interaction between PTZ and rapamycin in NKCC1/KCC2 ratio (interaction: $F_{1,45}$=9.8, p<0.01) with post hoc analysis revealing elevated NKCC1/KCC2 in PTZ kindled, control treated, 5XFAD mice compared to control 5XFAD mice (p<0.05), similar to the observed change in the nontherapeutic cohort, and a rescue of this effect in PTZ kindled, rapamycin treated 5XFAD mice (p<0.05) (Figure 4B). The changes in Cl^- transporter ratio was largely driven by changes in the Cl^- importer, NKCC1 (Figure 4B). These results likely reflect a rescue of $GABA_AR$ function in kindled 5XFAD mice by rapamycin.

Analysis of AMPAR subunits, showed that PTZ increased GluA1/GluA2 ratios in 5XFAD mice, consistent with the nontherapeutic cohort (Figure 3C), and this was reversed by rapamycin treatment (Figure 4C). Additionally, rapamycin increased GluA1/GluA2 ratio in vehicle-treated 5XFAD mice. These changes were largely driven by GluA1 expression, which reflected similar alterations (interaction: $F_{1,45}$: F=4.3, p<0.05). Additionally, rapamycin treatment reduced GluN2A and GluN2B levels regardless of PTZ treatment (GluN2A rapamycin effect: $F_{1,45}$=5.2, p<0.05; GluN2B rapamycin effect: $F_{1,45}$=4.1, p<0.05) while PTZ kindled, rapamycin treated 5XFAD mice had elevated GluN2B/GluN2A ratio compared to control 5XFAD mice (Tukey's post hoc: p<0.05) (Figure 4D).

In WT mice, no significant differences were found due to PTZ kindling or rapamycin treatment for $GABA_ARs$, AMPARs, or NMDARs (Figure S5.), although kindling did increase NKCC1 expression in WT mice across rapamycin and vehicle treatments (Kindling effect: $F_{1,47}$=5.2, p<0.05) (Figure S5).

Discussion

The data presented here suggest novel mechanisms involved in neuronal dysfunction that may underlie seizure-exacerbated cognitive and functional deficits in AD. Markers of both inhibitory and excitatory synaptic dysfunction were found in both AD patients and 5XFAD mice, with further perturbations seen in AD patients with seizure history and PTZ-kindled 5XFAD mice. Together with our recent study demonstrating enhanced AD pathology in AD+Sz patients and PTZ kindled mice (Gourmaud et al. 2022), our data suggest that dysregulation of proteins involved in E:I balance may play a role in seizure exacerbation of neuropathology and cognitive decline in AD through dysregulation of proteins involved in E:I balance. Specific E:I alterations include decreased $GABA_AR$ subunit expression and shifts in the balance between the Cl- cotransporters NKCC1/KCC2 and the AMPA receptor GluA1/GluA2 subunit ratios as factors in this interaction between AD and seizures (Tables 1 and 2). Importantly, we found that markers of E:I balance were correlated with clinical measures of decline and disease severity in rare autopsy-confirmed tissue samples with prospective collection of seizure history data, justifying follow up studies to further examine clinical outcomes associated with seizure history in AD with more comprehensive statistical modeling that was not possible in the dataset here. Furthermore, our data indicate that using mTORC1 inhibitors such as rapamycin to therapeutically target the E:I imbalance associated with mTORC1 upregulation may prove to be a promising therapy to alleviate AD progression due to neuronal hyperexcitability.

GABAergic deficits are well-documented in AD patients and our human data largely corroborate and build upon these findings. Here we found decreased $GABA_AR\alpha1/GABA_AR\alpha3$ ratios in AD patients as compared to controls, supporting previous reports of reduced GABA sensitivity in microtransplanted cell membranes isolated from temporal cortex of AD brains into Xenopus oocytes (Limon, Reyes-Ruiz, and Miledi 2012). In addition, AD patients with seizure history displayed significantly lower expression of both $GABA_AR\alpha1$ and $GABA_AR\alpha3$ subunits with $GABA_AR\alpha1$ levels correlating with brain atrophy in AD patients with seizures. The Cl^- extruder KCC2 was decreased and NKCC1/KCC2 ratios were increased in AD with a history of seizures as compared to patients with AD alone, consistent with collapse of neuronal Cl^- gradient. In addition, we found a loss of PV containing GABAergic interneurons in the temporal cortex of AD patients compared to controls, and a further decrease in the AD+Sz group, consistent with the previously reported reduction of somatostatin and PV cells within the AD parahippocampal gyrus

(Sanchez-Mejias et al. 2020) and the overall significantly lower GABA neurotransmitter levels in the CSF and parietal cortex from AD patients (Bareggi et al. 1982; Bai et al. 2015). Previous studies have found profound alterations in synaptic GABAergic signaling including downregulation of $GABA_AR$ α1-3, α5, β2, β3, and γ2 subunits (Limon, Reyes-Ruiz, and Miledi 2012; Kwakowsky et al. 2018; Govindpani et al. 2020; Rissman et al. 2003), decreased expression of the inhibitory synapse scaffolding protein gephyrin (Agarwal, Tannenberg, and Dodd 2008) and the loss of perisomatic GABAergic terminals contacting cortical neurons adjacent to amyloid plaques (Garcia-Marin et al. 2009), indicative of reduced $GABA_AR$ synapses overall. Fast-spiking PV interneurons are major contributors to pyramidal neurons inhibition and to generation of gamma oscillations, which are disrupted in AD patients (Stam et al. 2002; Klein et al. 2016). Greater loss of this GABAergic population in AD patients with epilepsy may promote network hyperexcitability and further contribute to worsened cognitive and functional performance found in these subjects (Figure 2). Taken in the context of prior studies, these data suggest that a history of seizures in AD is associated with diminished GABAergic transmission via reduced $GABA_AR$ synapses, loss of interneurons, and possibly, depolarizing GABA at those synapses that remain due to elevated intracellular Cl^-.

In addition to the changes in modulators of inhibitory GABAergic neurotransmission balance found in the human samples, we found similar alterations in $GABA_AR$ subunits and Cl^- cotransporters in 5XFAD mice. In prodromal 5XFAD mice (four months of age), we found decreased $GABA_AR\alpha1$/ $GABA_AR\alpha2$ ratios without significant changes in excitatory receptor subunits, suggesting an early preferential vulnerability of inhibitory circuitry that may underlie increased seizure susceptibility of 5XFAD mice at this stage (Gourmaud et al. 2022). Indeed reductions in $GABAR\alpha1$ and $\alpha5$ transcript have been found in patients with mild cognitive impairment suggesting $GABA_AR$ vulnerability at initial disease stages (Rissman, Bennett, and Armstrong 2004). Non-kindled seven month old 5XFAD mice also showed decreased expression of $GABA_AR\alpha1$, which we and others previously showed to be associated with decreased PV immunoreactivity (Gourmaud et al. 2022; Ali et al. 2019; Flanigan et al. 2014; Giesers and Wirths 2020; Caccavano et al. 2020), suggesting a broader deficit of GABA inhibition and reflecting our results in AD temporal cortex without seizures. Indeed, $GABA_AR$ loss has been linked to seizures and cognitive impairment in mouse models of AD (Verret et al. 2012; Li et al. 2021; Ulrich 2015). This

mechanism may be responsible for such changes in the 5XFAD model and may underlie the correlation we found between the loss of $GABA_AR\alpha1$ and increased atrophy in AD patients with seizures. Given that hyperexcitability and epilepsy can lead to neurodegeneration and vice versa, it is a challenge to determine the initiating factor in AD. However, our data demonstrate that in PTZ kindled 5XFAD mice, induced seizures further exaggerate GABAergic dysfunction via upregulation of NKCC1 and increased NKCC1/KCC2 ratios, recapitulating our human data, which were reversed in rapamycin treated mice. Furthermore, our correlations establish a direct link between E:I balance and levels of AD pathology, supporting previous literature positing links between neuronal excitability and AD progression (Targa Dias Anastacio, Matosin, and Ooi 2022).

With respect to glutamatergic neurotransmission, studies of AD human hippocampus have highlighted a decreased expression of GluA2 compared to normal controls (Carter et al. 2004; Ikonomovic et al. 1997; Mohamed et al. 2011), a configuration known to increase AMPAR Ca^{2+} permeability (Lippman-Bell et al. 2016; Loddenkemper et al. 2014; Rakhade et al. 2008; Burnashev et al. 1992; Rajasekaran, Todorovic, and Kapur 2012). Notably, the human AD temporal cortex we examined was late stage (Table S1) and we instead found an increase in GluA2 with a decrease in corresponding GluA1/GluA2 ratio in AD compared to controls (Figure 1E). These changes may represent a compensatory mechanism to neuronal hyperexcitability, but the significant GluA2 elevation may actually result in further impairments, given that Ca^{2+}- permeable AMPAR are critical for the induction of late-phase LTP, essential for the formation of long-term memory (Park et al. 2018; Pang and Lu 2004), as seen in neurodevelopmental disorder models (Banke and Barria 2020). Examination of glutamatergic NMDAR subunit protein revealed an increase in NMDAR subunits in AD patients, which is consistent with studies of the AD parietal cortex demonstrating increased excitatory to inhibitory synaptic ratio (Lauterborn et al. 2021). Elevated GluN2A in AD patients may again represent a compensatory mechanism, as lower GluN2A was associated with worsened CDR-SOB scores across all AD patients in our dataset, consistent with data showing correlations in working memory performance and GluN2A in aged rats (McQuail et al. 2016). Elevated GluN2B is associated with epileptogenesis (Rakhade and Jensen 2009; Loddenkemper et al. 2014) and extrasynaptic excitotoxic signaling in AD (Babaei 2021).

Further, elevations in AMPAR and NMDAR subunits are suggestive of elevated glutamatergic synapses overall, which may further tip E:I balance towards excitation.

In prodromal 5XFAD mice, we found no changes in AMPARs or NMDARs, but increased expression of GluA1/GluA2 AMPAR subunit ratios in the hippocampus of seven-month 5XFAD mice, which was exacerbated by PTZ kindling and ameliorated with rapamycin treatment. Overall, these results are indicative of selective vulnerability of inhibitory transmission at early disease stages, and suggest that AMPAR and NMDAR subunit composition is dynamic through AD progression and certain changes could be compensatory or neuroprotective from excitotoxicity, as often seen in acute epilepsy models (Russo et al. 2013; Leo et al. 2018). Recent epidemiologic research has underscored strong associations between epilepsy, late onset seizures, and AD (Vossel et al. 2017; Zhang et al. 2022), and while in the past, seizures were assumed to be an unfortunate biproduct of AD, we and others have provided increasing preclinical and clinical evidence that seizures significantly contribute to neuropathology and cognitive decline, and may also be a treatable component of this complex disease. Indeed, in a recent phase 2a clinical trial, levetiracetam, an antiepileptic drug, was shown to improve memory and executive function in AD patients with epileptiform activity (Vossel et al. 2021). Beyond seizure suppression, our data suggests that targeting specific E:I imbalances also holds therapeutic potential. Notably, a recent study using a computational drug repurposing algorithm has identified bumetanide, a NKCC1 antagonist, as a top candidate to treat AD, which when applied to AD model mice ameliorated transcriptomic, electrophysiological, and cognitive deficits (Taubes et al. 2021). Our data demonstrated significant exacerbation of NKCC1/KCC2 in both AD+Sz and seizure kindled mice. NKCC1/KCC2 ratios were also negatively correlated with performance related to spatial working memory in kindled 5XFAD mice, supporting the notion that restoration of the Cl^- gradient, perhaps with bumetanide, is therapeutically relevant, particularly for AD patients with seizure history.

Since we have recently demonstrated that seizures exacerbate mTORC1 in AD patients and 5XFAD mice, and that chronic low dose rapamycin treatment is sufficient to ameliorate AD pathology and cognitive dysfunction in PTZ kindled 5XFAD mice (Gourmaud et al. 2022), we sought to further examine the therapeutic potential of rapamycin through investigation of its effects on markers of E:I balance in the same mice. Rapamycin is FDA approved and has been proven to benefit both AD and epilepsy models

(Kim and Lee 2019; Kaeberlein and Galvan 2019; Gourmaud et al. 2022). While rapalogs may affect numerous signaling pathways downstream of mTORC1 to attenuate disease course, including autophagy and neuroinflammation (Kodali et al. 2021; Zeng et al. 2008), ours is the first to our knowledge to examine the effects of mTORC1 blockade on these protein ratios involved in E:I balance. In the studies presented here, rapamycin reduced NKCC1/KCC2 and GluA1/GluA2 ratios in PTZ kindled 5XFAD mice and showed a trend to increase $GABA_AR\alpha1/GABA_AR\alpha3$ in non-kindled 5XFAD mice, suggesting reversal of seizure-exacerbated E:I imbalance which may indicate restoration of proper circuitry involved in memory deficits observed in these mice (Gourmaud et al. 2022). However, we found that chronic rapamycin treatment induced small, but significant elevations in GluN2B/GluN2A in PTZ kindled 5XFAD mice and increased GluA1/GluA2 in non-kindled 5XFAD mice, in contrast to prior studies demonstrating that mTORC1 blockade decreases GluN2B and GluA1, but these studies were performed with acute rapamycin treatments (James et al. 2014; James et al. 2016; Calabrese et al. 2020; Zhang et al. 2018). Thus, the relative increases in GluN2B and GluA1 that we found may represent a compensatory mechanism in response to chronic rapamycin administration. Indeed, one prior study demonstrated that chronic rapamycin induced increases in surface GluN2B, and that these changes were associated with amelioration of age-dependent cognitive decline (Majumder et al. 2012). Given the role of these glutamate receptors subunit configurations in epileptogenesis, these interactions should be further examined in the context of AD treatment. Nevertheless, these data indicate that targeting E:I balance with rapamycin, should be explored for clinical efficacy in AD patients with seizure history.

In summary, the data presented here identified novel mechanisms of seizure and Alzheimer's disease interactions to induce neuronal dysfunction that may play a role in worsened cognitive outcomes associated with seizures in AD. These data suggest that targeting E:I imbalance, perhaps with rapamycin as well as other antiepileptic agents, holds therapeutic promise and is particularly relevant for AD patients with comorbid seizures.

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Conflicts of interest

The authors declare no conflicts of interest.

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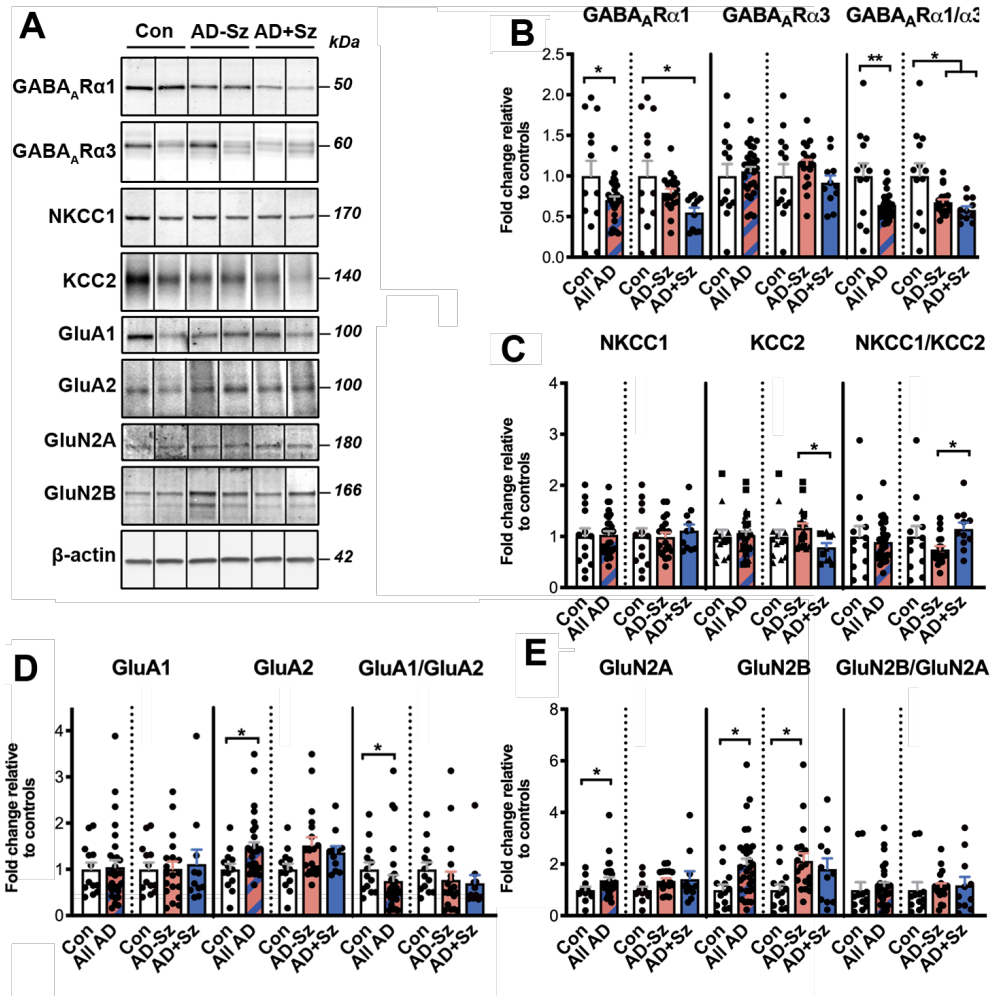

Figure 1. Dysregulation of proteins involved in excitatory: inhibitory balance in AD patients with and without seizure history. (**A**) Representative western blot images for (B-E) showing non-adjacent bands originating from the same blot of the temporal cortex from control cases (Con; n=13) and Alzheimer's disease patients (AD; n=30) split into subgroups, those without (AD-Sz; n=19) or with (AD+Sz; n=11) known seizure history. Semi-quantitative analysis of (**B**) GABA$_A$R subunits GABA$_A$Rα1 and GABA$_A$Rα3 and corresponding ratio GABA$_A$Rα1/GABA$_A$Rα3; (**C**) Cl^-cotransporters NKCC1 and KCC2 and corresponding ratio NKCC1/KCC2; (**D**) AMPAR subunits GluA1 and GluA2 and corresponding ratio GluA1/GluA2; (**E**) NMDAR subunits GluN2A and GluN2B and corresponding ratio GluN2B/GluN2A. *p<0.05, **p<0.01.

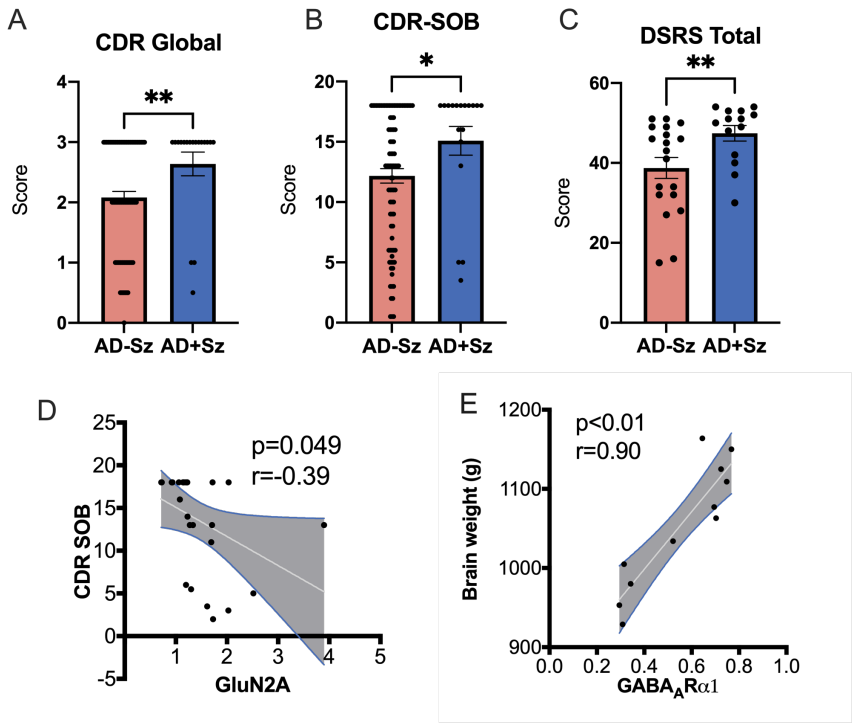

Figure 2. Seizure history is associated worse cognitive and functional performance in AD. Comparisons between AD-Sz and AD+Sz in Final global CDR (**A**), CDR sum of boxes (**B**), and DSRS Total (**C**). CDR: n=18(AD+Sz) – 87 (AD-Sz); DSRS: n=14 (AD+Sz) – 19 (AD-Sz). (**D**) Pearson correlations in all AD showing relationship between GluN2A and CDR SOB score and (**E**) showing the relationship between $GABA_A R\alpha 1$ expression and brain weight at death in grams in AD+Sz patients. Grey areas indicate 95% confidence interval for the two means. *p<0.05, **p<0.01.

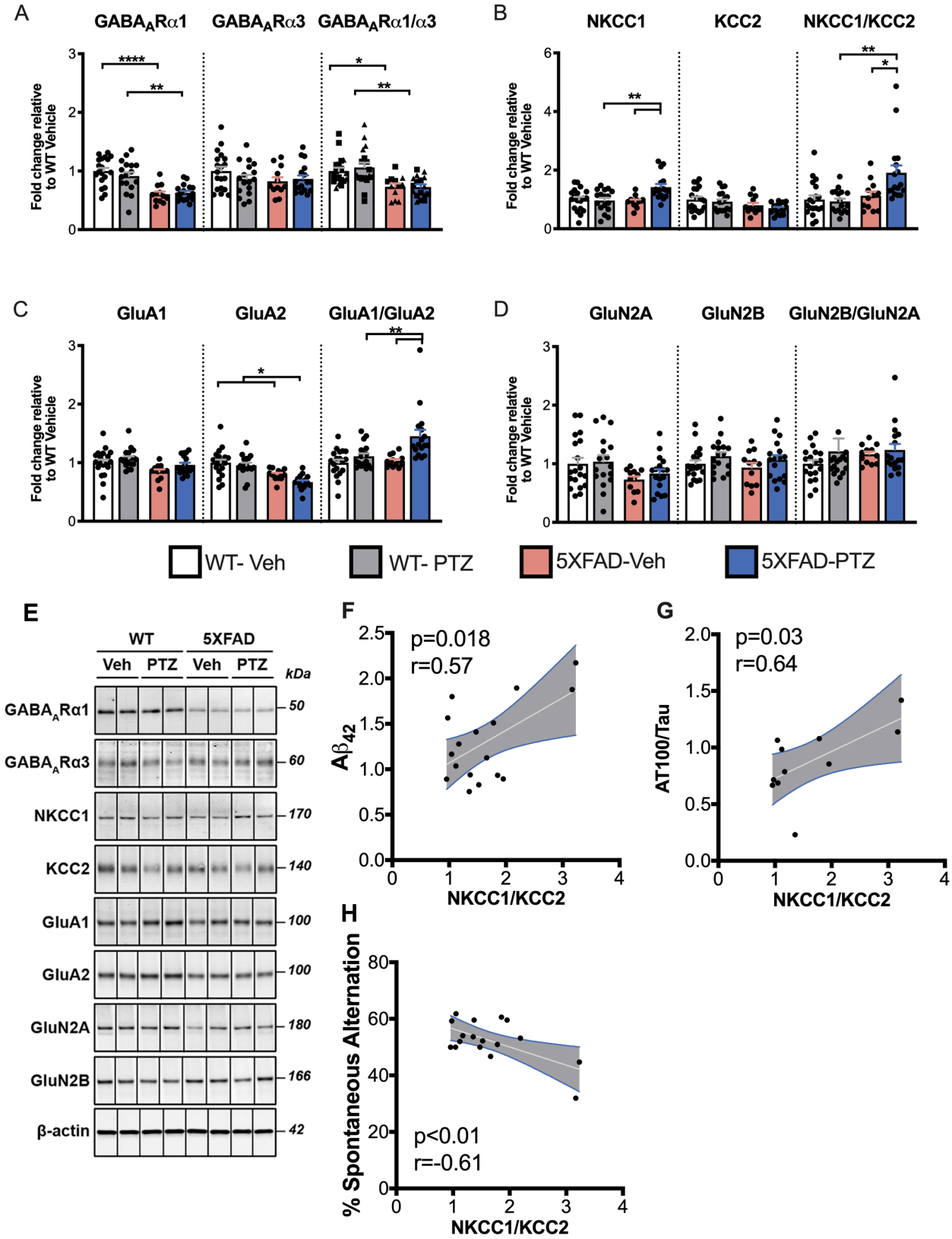
A
GABA$_A$Rα1
GABA$_A$Rα3
GABA$_A$Rα1/α3
Fold change relative to WT Vehicle
B
NKCC1
KCC2
NKCC1/KCC2
C
GluA1
GluA2
GluA1/GluA2
D
GluN2A
GluN2B
GluN2B/GluN2A
WT- Veh
WT- PTZ
5XFAD-Veh
5XFAD-PTZ
E
WT
5XFAD
Veh
PTZ
kDa
GABA$_A$Rα1
GABA$_A$Rα3
NKCC1
KCC2
GluA1
GluA2
GluN2A
GluN2B
β-actin
F
p=0.018
r=0.57
Aβ_{42}
NKCC1/KCC2
G
p=0.03
r=0.64
AT100/Tau
NKCC1/KCC2
H
% Spontaneous Alternation
p<0.01
r=-0.61
NKCC1/KCC2

Figure 3. Excitatory:inhibitory imbalance in the hippocampus of 5XFAD mice following induced seizures. (**A-E**) Quantification of (**A**) $GABA_AR$ subunits $GABA_ARα1$ and $GABA_ARα3$ and corresponding ratio $GABA_ARα1/GABA_ARα3$; (**B**) Cl^-cotransporters NKCC1 and KCC2 and corresponding ratio NKCC1/KCC2; (**C**) AMPAR subunits GluA1 and GluA2 and corresponding ratio GluA2/GluA1; (**D**) NMDAR subunits GluN2A and GluN2B and corresponding ratio GluN2B/GluN2A. (**E**) Representative western blot images for (A-D) showing non-adjacent bands originating from the same blot. (**I**) Pearson correlation of PTZ kindled 5XFAD mice showing the relationship between NKCC1/KCC2 and Aβ normalized to 5XFAD-Veh, (**F**) pTau AT100(Thr212, Ser14):Tau normalized to WT-Veh (**G**), and % spontaneous alternations in the Y-maze. Group comparisons for Aβ ELISA, pTau western blots and the Y-maze were previously published (Gourmaud et al. 2022) (**H**). Grey areas indicate 95% confidence interval for the two means. n = 12-19 WT-vehicle, 12-17 WT-PTZ, 12 5XFAD-vehicle and 17 5XFAD-PTZ. *$p<0.05$, **$p<0.01$. Females and males are designated by square and triangle data points, respectively, where sex effects were found.

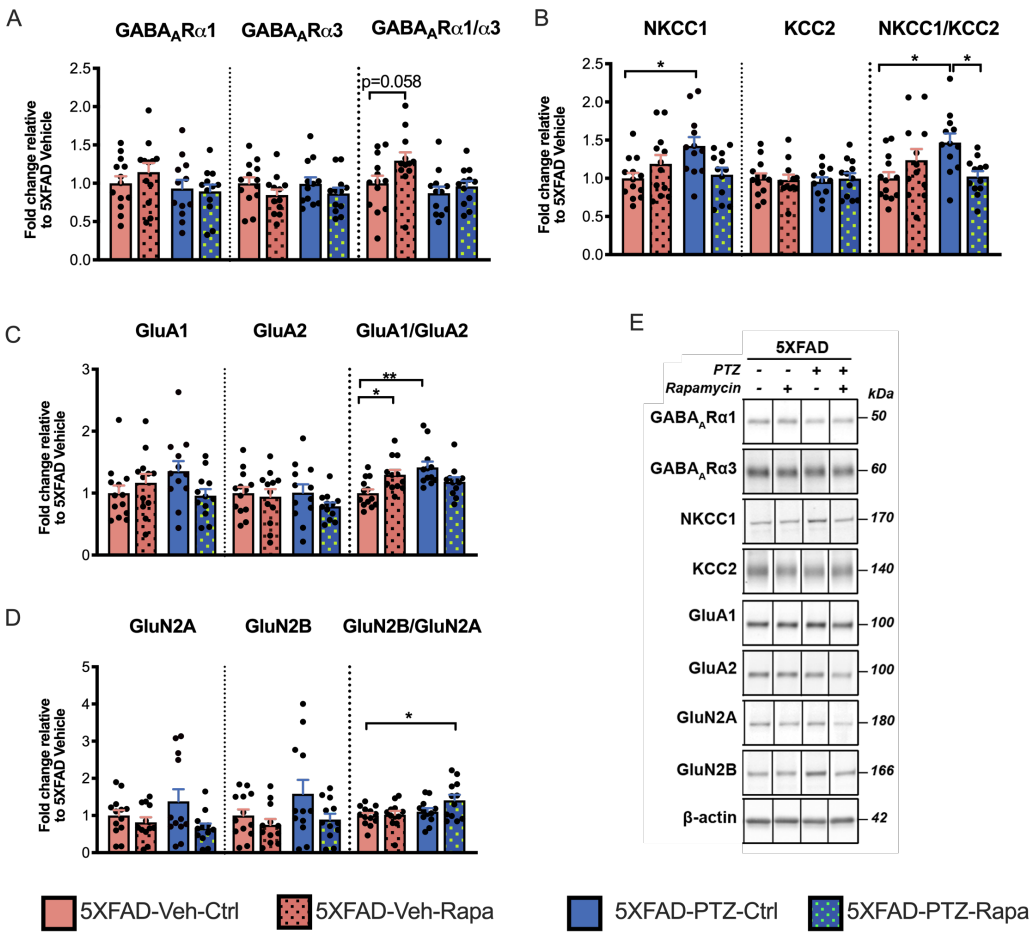

Figure 4. Differential effects of mTORC1 inhibition on seizure-induced excitation:inhibition imbalance in 5XFAD mice. (**A-D**) Quantification of (**A**) GABA$_A$Rα1 and GABA$_A$Rα3 and corresponding GABA$_A$Rα1/GABA$_A$Rα3 ratio, (**B**) Cl^- cotransporters NKCC1 and KCC2 and corresponding NKCC1/KCC2 ratio, (**C**) AMPAR subunits GluA1 and GluA2 and corresponding ratio GluA2/GluA1, and (**D**) NMDAR subunits GluN2A and GluN2B and corresponding GluN2B/GluN2A ratio. (**E**) Representative Western blot images for (**A-D**) showing non-adjacent bands originating from the same blot. n = 12-13 for each group. *p<0.05, **p<0.01.

		Prodromal 5XFAD	Intermediate 5XFAD	Human
$GABA_AR$	Hippocampus	➖$GABA_AR$ α1 ➖$GABA_AR$ α2 ⬇️$GABA_AR$ α1/α2	⬇️$GABA_AR$ α1 ➖$GABA_AR$ α3 ⬇️$GABA_AR$ α1/α3	⬇️$GABA_AR$ α1 ➖$GABA_AR$ α3 ⬇️$GABA_AR$ α1/α3
	Cortex	➖$GABA_AR$ α1 ➖$GABA_AR$ α2 ➖$GABA_AR$ α1/α2	⬇️$GABA_AR$ α1; ⬇️GABAAR α3; ⬇️$GABA_AR$ α1/α3	
Cl^- cotransporters	Hippocampus	➖NKCC1 ➖KCC2 ➖NKCC1/KCC2	➖NKCC1 ➖KCC2 ➖NKCC1/KCC2	➖NKCC1 ➖KCC2 ➖NKCC1/KCC2
	Cortex	➖NKCC1 ➖KCC2 ➖NKCC1/KCC2	⬇️ NKCC1 ➖KCC2 ➖NKCC1/KCC2	
AMPAR	Hippocampus	➖GluA1 ➖ GluA2 ➖ GluA1/GluA2	➖GluA1 ⬇️ GluA2 ➖ GluA1/GluA2	➖GluA1 ⬆️GluA2 ⬇️ GluA1/GluA2
	Cortex	➖GluA1 ➖GluA2 ➖ GluA1/GluA2	➖ GluA1 ⬇️ GluA2 ➖ GluA1/GluA2	
NMDAR	Hippocampus	➖GluN2A ➖ GluN2B ➖ GluN2B/GluN2A	⬇️ GluN2A ➖ GluN2B ➖ GluN2B/GluN2A	⬆️GluN2A ⬆️GluN2B ➖ GluN2B/GluN2A
	Cortex	➖GluN2A ➖ GluN2B ➖ GluN2B/GluN2A	⬇️ GluN2A ➖ GluN2B ➖ GluN2B/GluN2A	

Table 1. Effects of AD and 5XFAD genotype on E:I balance. Prodromal = 4 months old; intermediate = 7 months old.

		Intermediate 5XFAD	Human AD
$GABA_AR$	Hippocampus	➖$GABA_AR$ α1 ➖$GABA_AR$ α 3 ➖$GABA_AR$ α1/α3	⬇️$GABA_AR$ α1 ⬇️$GABA_AR$ α 3 ➖$GABA_AR$ α1/α3
	Cortex	➖$GABA_AR$ α1 ➖$GABA_AR$ α 3 ➖$GABA_AR$ α1/α3	
Cl^- cotransporters	Hippocampus	⬆️NKCC1 ➖KCC2 ⬆️NKCC1/KCC2	➖NKCC1 ⬇️KCC2 ⬆️NKCC1/KCC2
	Cortex	➖NKCC1 ➖KCC2 ➖NKCC1/KCC2	
AMPAR	Hippocampus	➖ GluA1 ➖ GluA2 ⬆️GluA1/GluA2	➖GluA1 ➖GluA2 ➖GluA1/GluA2
	Cortex	➖ GluA1 ➖ GluA2 ⬆️GluA1/GluA2	
NMDAR	Hippocampus	➖GluN2A ➖ GluN2B ➖GluN2B/GluN2A	➖GluN2A ➖ GluN2B ➖GluN2B/GluN2A
	Cortex	➖GluN2A ➖ GluN2B ➖GluN2B/GluN2A	

Table 2. Effects of seizures in AD and 5XFAD mice.