Levetiracetam Treatment Normalizes Levels of Presynaptic Endocytosis Machinery and Restores Nonamyloidogenic APP Processing in App Knock-in Mice

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Cite This: J. Proteome Res. 2021, 20, 3580−3589

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ABSTRACT: Toxic amyloid-beta (Aβ) peptides, produced by sequential proteolytic cleavage of the amyloid precursor protein (APP), play a key role in the initial stage of Alzheimer's disease (AD). Increasing evidence indicates that Aβ42 induces neuronal circuit hyperexcitability in the early stages of AD pathology. As a result, researchers have investigated treatments that modulate the excitatory/inhibitory imbalance as potential AD therapies. For example, levetiracetam, an atypical antiepileptic drug used to quell hyperexcitability, has garnered recent interest in the AD field, even though its exact mechanism(s) of action remains elusive. Here, we show that in APP knock-in mouse models of amyloid pathology, chronic levetiracetam administration decreases cortical Aβ42 levels and lowers the amyloid plaque burden. In addition, using multiplexed tandem mass tag-quantitative mass spectrometry-based proteomic analysis, we determined that chronic levetiracetam administration selectively normalizes levels of presynaptic endocytic proteins. Finally, we found that levetiracetam treatment selectively lowers beta carboxyl-terminal fragment levels, while the abundance of full-length APP remains unchanged. In summary, this work reports that chronic treatment with levetiracetam serves as a useful therapeutic in AD by normalizing levels of presynaptic endocytic proteins and altering APP cleavage preference, leading to a decrease in both Aβ42 levels and the amyloid plaque burden. These novel findings provide novel evidence for the previously documented therapeutic value of levetiracetam to mitigate AD pathology.

KEYWORDS: Alzheimer's disease, levetiracetam, APP knock-in, quantitative proteomics, axon terminal, amyloid precursor protein

INTRODUCTION

In Alzheimer’s disease (AD), sequential proteolytic cleavage of the amyloid precursor protein (APP) leads to the production of toxic amyloid-beta (Aβ) peptides. The inability to efficiently degrade Aβ42 has been shown to drive downstream pathologies such as synapse deterioration and formation of amyloid plaques and neurofibrillary tangles.1−4 While downstream repercussions have been documented, there is currently no effective treatment to prevent, reverse, or slow the progression of AD. Notably, Aβ-lowering antibody treatments show promise but have likely been administered too late in the progression of AD.5 As a result, there has been a shift in focus to identifying and investigating early AD pathologies that may serve as potential therapeutic targets.

Hyperactivity and neural network disruption have been observed during the initial stages of amyloid pathology and could represent a pioneering aspect of AD pathogenesis.5−7 These findings have motivated recent investigations focused on the role of brain hyperexcitability in AD and subsequently whether modulating the excitatory/inhibitory imbalance could be an efficacious AD therapy. We recently discovered an early impairment in degradation and turnover of synaptic vesicle (SV) machinery in APP knock-in (App KI) mouse models of amyloid pathology.8 Our findings indicate that targeting or correcting early presynaptic proteostasis could represent an effective therapeutic target. Levetiracetam (LEV) is an atypical antiepileptic drug that, unlike those targeting the GABA-ergic system, binds to the presynaptic SV glycoprotein 2A (SV2A).9 However, despite FDA approval and wide use, levetiracetam’s mechanism(s) of actions remain elusive. In APP transgenic mouse models, levetiracetam administration reduces hyperexcitability, suppresses neuronal network dysfunction, and decreases Aβ plaque burden and associated cognitive deficits.10−13 In a clinical study of patients with mild cognitive impairment, abnormal entorhinal cortex hyperactivity was corrected with chronic levetiracetam administration, and
interestingly, these patients simultaneously had an improved working memory performance. Levetiracetam is, to date, the focus of seven phase 1 or 2 clinical trials for AD.

In this study, we set out to identify the pathways and mechanisms primarily affected by levetiracetam in diseased brains of amyloid pathology to determine how levetiracetam affects the proteome. In order to avert the possible confounding effects of APP overexpression, we used App Ki mouse models that have the humanized Aβ42 sequence expressed under the endogenous APP promoter and harbor familial mutations. App Ki mice harboring the Swedish mutation (App
\[\text{F/NL} - \text{F}\]) serve as controls for mice with an additional Iberian mutation (App
\[\text{NL/F-NL/F}\]), which increases the ratio of Aβ42 to Aβ40, representing a moderate amyloid pathology. Addition of the male and female mice were used for all experiments. Animals were then hemisected with one-half for immunostaining and the other for biochemical analysis. Methanol-chloroform precipitation was used to separate proteins from lipids and impurities. The extracted protein was then resuspended in 6M guanidine in 100 mM N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES). The proteins were further processed via the reduction of disulfide bonds with dithiothreitol and alkylation of cysteine residues with iodoacetamide. Proteins were then digested for 3 h at room temperature (RT) with 1 μg of LysC (Promega) and then digested overnight at 37 °C with 2 μg of Trypsin. The digest was then acidified with formic acid and desalted using C18 HyperSep columns (ThermoFisher Scientific). The eluted peptide solution was dried before resuspension in 100 mM HEPES. Micro-BCA assay was subsequently performed to determine the concentration of peptides. 100 μg of peptide from each sample was then used for isobaric labeling. TMT-16-plex labeling was performed on peptide samples according to the manufacturer’s instructions (ThermoFisher Scientific). After incubating for 75 min at room temperature, the reaction was quenched with 0.3% (v/v) hydroxylamine. Isobaric labeled samples were then combined 1:1:1:1:1:1:1:1:1:1 and subsequently desalted with C18 HyperSep columns. The combined isobaric labeled peptide samples were fractionated into eight fractions using high pH reversed-phase columns (Pierce). Peptide solutions were dried, stored at −80 °C, and reconstituted in liquid chromatography–mass spectrometry (LC–MS) buffer A (5% acetonitrile, 0.125% formic acid) for LC–MS/MS analysis.

**TMT-MS Analysis**

TMT-MS analysis was performed as previously described. In short, samples were resuspended in 20 μL of buffer A (5% acetonitrile, 0.125% formic acid), and micro-BCA was performed. 3 μg of each fraction was loaded for LC–MS analysis via an auto-sampler with a Thermo EASY nLC 100 UPLC pump onto a vented Pepmap100, 75 μm × 2 cm, nanoViper trap column coupled to a nanoViper analytical column (Thermo Scientific) with a stainless steel emitter tip assembled on the nanospray flex ion source with a spray voltage of 2000 V. Orbitrap Fusion was used to generate MS data. The chromatographic run was performed with a 4 h gradient beginning with 100% buffer A and 0% B and increased to 7% B over 5 min, then to 25% B over 160 min, 36% B over 40 min, 45% B over 10 min, 95% B over 10 min, and held at 95% B for 15 min before terminating the scan. Buffer A contained 5% acetonitrile (ACN) and 0.125% formic acid in H₂O, and buffer B contained 99.875 ACN with 0.125% formic acid. Multinotch MS3 method was programmed with the following parameters: ion transfer tube temp = 300 °C, easy-IC internal mass calibration, default charge state = 2, and cycle time = 3 s. MS1 detector was set to orbitrap with 60 K resolution, wide quad isolation, mass range = normal, scan range = 300–1800 m/z, max injection time = 50 ms, AGC target = 6 × 10⁶, microscans = 1, RF lens = 60%, without source fragmentation, and datatype = positive and centroid. Monoisotopic precursor selection was set to include charge states 2−7 and reject unassigned. Dynamic exclusion was allowed; n = 1 exclusion for 60 s with 10 ppm tolerance for high and low. The intensity threshold was set to 5 × 10⁵. Precursor selection decision = most intense, top speed, 3 s. MS2 settings include isolation window = 0.7, scan range = auto normal, collision energy = 35% CID, scan rate = turbo, max injection time = 50 ms, AGC target = 6 × 10⁵, and Q = 0.25. In MS3, the top 10 precursor peptides selected for analysis were then fragmented using 65% higher-energy collisional dissociation before orbitrap detection. A precursor selection range of 400–
1200 m/z was chosen with mass range tolerance. An exclusion mass width was set to 18 ppm on the low and 5 ppm on the high. Isobaric tag loss exclusion was set to TMT reagent. Additional MS3 settings include an isolation window = 2, orbitrap resolution = 60 K, scan range = 120–500 m/z, AGC target = 6 × 10³, max injection time = 120 ms, microscans = 1, and datatype = profile.

**TMT-MS Data Analysis and Quantification**

TMT-MS data analysis was performed as previously described in ref 17. In short, protein identification, TMT quantification, and analysis were performed with The Integrated Proteomics Pipeline-IP2 (Integrated Proteomics Applications, Inc., http://www.integratedproteomics.com/). Proteomic results were analyzed with ProLuCID, DTASelect2, Census, and QuantCompare. MS1, MS2, and MS3 spectrum raw files were extracted using RawExtract 1.9.9 software (http://fields.scripps.edu/downloads.php). Pooled spectral files from all eight fractions for each sample were then searched against the Uniprot mouse protein database and matched to sequences using the ProLuCID/SEQUEST algorithm (ProLuCID ver. 3.1) with 50 ppm peptide mass tolerance for precursor ions and 600 ppm for fragment ions. Fully and half-tryptic peptide candidates were included in the search space, all that fell within the mass tolerance window with no misscleavage constraint, assembled, and filtered with DTASelect2 (ver. 2.1.3) through the Integrated Proteomics Pipeline (IP2 v.5.0.1, Integrated Proteomics Applications, Inc., CA, USA). Static modifications at S7.02146 C and 304.2071 K and N-term were included. The target-decoy strategy was used to verify peptide probabilities and false discovery ratios. A minimum peptide length of five was set for the process of each protein identification, and each dataset included a 1% FDR rate at the protein level based on the target-decoy strategy. Isobaric labeling analysis was established with Census 2 as previously described. TMT channels were normalized by dividing it over the sum of all channels. No intensity threshold was applied. The fold change was then calculated as the mean of the experimental group standardized values, and p-values were then calculated by Student’s t-test with Benjamini-Hochberg adjustment.

**Online Databases for PANTHER and STRING (http://string-db.org)**

Protein ontologies were determined with protein analysis through evolutionary relationship (PANTHER) system (http://www.pantherdb.org) in complete cellular component categories. The statistical overrepresentation test was calculated by using the significant proteins identified from comparing VEH versus levetiracetam experimental groups for each App KI genotype as the query and the aggregated total proteins identified in all three comparisons as the reference. Protein ontologies with Fisher statistical tests with false discovery rate correction less than 0.05 were considered significant.

The search tool for the retrieval of interacting genes (STRING) database was used to determine protein–protein interactions from significant quantified proteins identified by the gene ontology cell component (GO:CC) term. The STRING resource is available at http://string-db.org. The corresponding protein–protein interaction networks were constructed with the highest confidence of interaction score at 0.9.

**Thioflavin Staining**

After transcendental perfusion with cold PBS, hemisected brains were fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose for 2 days, embedded in a cryomold with OCT, flash frozen on dry ice, and stored at −80 °C until cryosectioning. 30 μm sagittal cryosections were prepared and mounted onto gelatin-coated slides (SouthernBiotech). Sections were then prepared for thioflavin S staining following standard procedures. In short, the sections were washed with 70% ethanol for 1 min followed by 80% ethanol for 1 min before being incubated in filtered thioflavin S solution (1% in 80% ethanol) for 15 min in the dark. Slides were then washed sequentially with 80% ethanol, then 70% ethanol, and then distilled water for 1 min each. Coverslips were mounted using Fluoromount-G (SouthernBiotech). Sections were imaged at the Northwestern University Center for Advanced Microscopy with a TissueGnostics system using a 10× objective. Analysis was conducted using Fiji with the analyze puncta tool following thresholding. Cortical area analyzed was kept consistent throughout each section.

**Aβ42 ELISA Assay**

Aβ42 levels were measured using a human Aβ42 ELISA kit (Thermo Scientific) following manufacturer instructions. In short, 5% guanidine HCl was added to cortical homogenates (1–2 mg) and kept shaking for 1 h at RT. Samples were then diluted 1:10 for AppNL/NL and AppNL-F/NL-F and 1:1000 for AppNL-G/NL/NL-G in standard diluent buffer. 50 μL of sample was loaded into wells coated with the provided Aβ42 antibody and incubated for 3 h at RT. After three washes, horseradish peroxidase-conjugated antibody was added for 30 min. After another wash step, the samples were incubated with stabilized chromogen for 30 min, and the reaction was stopped with an acid-based stop solution. Finally, OD was measured at 450 nm using a Synergy HTX multimode microplate reader (Biotek) and compared to a standard curve to determine the final concentration.

**Western Blotting**

Cortical brain extracts were homogenized in 500 μL of homogenization buffer (4 mM HEPES, 0.32 M sucrose, 0.1 mM MgCl₂) supplemented with a protease inhibitor cocktail (aprotinin, leupeptin, AEBSF, benzamidine, PMSF, and pepstatin A). The tissue was then homogenized using a bead-based Precellys homogenizer. Protein concentration was then determined by BCA assay (Thermo Scientific) as per manufacturer’s instructions and compared with the respective standard curve. 50 μg of each sample was then prepared for western blots (WB) by adding 6× sodium dodecyl sulfate sample buffer. The mixtures were sonicated and boiled at 96 °C for 5 min each and then loaded in 16% Tris-glycine gel. Gels were run at 80 V for 4 h and then wet transferred to a 0.2 μm nitrocellulose membrane. Membranes were then blocked with Odyssey Blocking Buffer (LI-COR) in PBS for 1 h and then incubated overnight with anti-amyloid beta precursor protein (Y188) rabbit monoclonal antibody at 1:1,000 (Abcam Cat# ab23136) and anti-VCP mouse monoclonal antibody at 1:2000 (Abcam Cat# ab11433). Next day, the membranes were washed and incubated in secondary antibody IRDye 800CW Donkey anti-Rabbit IgG antibody (LI-COR Biosciences Cat# 926-32213) and IRDye 680RD Donkey anti-Mouse IgG antibody (LI-COR Biosciences Cat# 925-68072) for 1 h at RT. Blots were imaged on an Odyssey CLx (LI-COR).
Quantification and Statistical Analysis

Statistical analyses were performed using GraphPad Prism. All values in figures with error bars are presented as mean ± standard error of the mean (SEM). Comparison of VEH versus LEV groups was performed using unpaired Student’s t-tests. Comparisons across all three genotypes were performed by one-way analysis of variance (ANOVA) and post hoc Fisher’s test. P-values < 0.05 were considered statistically significant. Multiple test correction was performed with the Benjamini–Hochberg correction. For Bayesian analysis of variance, we implemented BAMarray 2.0, a Java software package that implements the Bayesian ANOVA for microarray (BAM) algorithm.22 The BAM approach uses a special type of inferential regularization known as spike-and-slab shrinkage, which provides an optimal balance between total false detections (the total number of genes falsely identified as being differentially expressed) and total false nondetections (the total number of genes falsely identified as being nondifferentially expressed).22

 RESULTS

SV-Associated Proteins Have Altered Abundance in App\textsuperscript{NL/F-NL-F} and App\textsuperscript{NL-G/F-NL-G-F} Cortical Extracts

We designed our experiments to investigate the effect of chronic levetiracetam in App\textsuperscript{Kl} brains with varying degrees of Aβ\textsubscript{42} pathology. The App\textsuperscript{NL/NL} model serves as a relative control that does not develop Aβ\textsubscript{42} pathology, while App\textsuperscript{NL-F/NL-F} mice have a relatively slow progressing Aβ\textsubscript{42} pathology. App\textsuperscript{NL-G/F-NL-G-F} present with aggressive Aβ\textsubscript{42} pathology that is abundantly present by 6 months of age (Figure 1a). Levetiracetam at 75 mg/
kg or VEH saline solution was administered intraperitoneally daily for 30 days beginning at 6 months of age for each App KI model (Figure 1a). To investigate levetiracetam’s mode of action, we performed a quantitative bottom-up proteomic screening using guanidine HCl soluble cortical extracts with 16-plex TMT-MS. We compared protein abundance between cohorts given VEH (N = 6) or levetiracetam (N = 6) of the same App KI genotype along with multiple float channels that allow for comparisons between the multiple TMT-MS experiments (i.e., genotypes).

The overall TMT channel peak intensities were similar in all three experiments, indicating efficient labeling (Figure 1b). To assess the reliability of the TMT-MS data, we plotted the number of total quantified proteins, reporter ion intensities, and fold change distribution and confirmed similar data quality (Figure S1a–d). We compared the protein abundance in VEH AppNL/NL to AppNL/NLG and identified 1704 significantly altered proteins (Figure 1c–e; Table S1). In the parallel VEH AppNL-NL/F-NL-G dataset, we identified 1578 significantly altered proteins compared to AppNL/NL (Figure 1c–e; Table S1). To mine the significantly regulated proteins in AppNL-F-NL-F/NL-FL and AppNL-G-F-NL-G/F-NL-FL, we performed GO:CC enrichment analysis with PANTHER. In both datasets, the regulated proteins are significantly enriched for the GO:CC terms: SV, synapse, presynapse, postsynapse, and others (Figure 1fg; Table S2). This is consistent with our previous report that AppNL-F-NL-F and AppNL-G-F-NL-G-F brains both have synaptic proteome alterations by 6 months of age.5 These results confirm the reliability of our TMT-MS analyses and extend our previous findings that the axon terminal proteome represents an early site of amyloid pathology.

**Chronic Leviteracetam Administration Normalizes Levels of Presynaptic Endocytic Proteins in AppNL-F-NL-G-F Cortex**

To investigate the effect of chronic levetiracetam on Aβ levels, we extracted the relative peptide abundance mapping either inside or outside the Aβ amino acid sequence within APP. We quantified differences relative to the AppNL/NL bridge channel that allows for comparison in the levels of peptide mapping to APP or Aβ across multiple TMT-MS experiments. This allows for quantification of peptide abundance relative to APP levels. APP peptide mapping outside of Aβ showed significant differences when comparing all three App KI genotypes ([F(5,29) = 2.711, p-value = 0.0396]; however, post hoc analysis showed no significant differences in abundance between any of the groups of all three App KI genotypes (Figure 2a). Peptide mapping to the Aβ amino acid sequence was quantified across the App KI genotypes and showed, as expected, that cortical extracts from AppNL/NL cohorts had significantly lower Aβ levels compared to AppNL-NL/F and AppNL-NL-G-F cohorts (p-value = <0.0001). Notably, in cortical extracts from levetiracetam-treated AppNL-F-NL-F and AppNL-G-F-NL-G-F mice, peptide mapping to the Aβ amino acid sequence was significantly reduced compared to their respective VEH-treated controls (p-value = 0.0095, p-value = 0.0211) (Figure 2a). A single tryptic Aβ peptide was quantified in the AppNL-F-NL-F TMT-MS analysis, and one Aβ peptide containing the artif mutation was quantified in three instances during the AppNL-F-NL-G-F TMT-MS analysis. Overall, we found that chronic levetiracetam had no significant effect on global protein abundance [F(5,29) = 1.719, p-value = 0.1617] (Figure 2b). These findings indicate that levetiracetam treatment can lower steady-state Aβ levels without altering the overall APP levels in AppNL-F-NL-F and AppNL-G-F-NL-G-F mice. Importantly, levetiracetam treatment has the ability to lower Aβ levels in AppNL-F-NL-G-F mice, which at 6 months of age already harbor robust Aβ pathology.

We next sought to investigate how levetiracetam affects the AppNL-F-NL-G-F cortical proteome as we were primarily interested in investigating how it mitigates amyloid pathology in the brain. First, in order to investigate proteomic alterations resulting from levetiracetam treatment in the AppNL-NL/F-NL-G-F cortex, we performed a Bayesian analysis of variance. This statistical technique is used for identification of differentially expressed genes or proteins using a unique type of signal-to-noise detection strategy that allows for the detection of less

Figure 2. Chronic levetiracetam administration selectivity lowers levels of Aβ1-42 in AppNL-NL/F-NL-G-F cortex. (A) Normalized TMT intensities relative to AppNL/NL of APP peptides mapping outside or within the Aβ1-42 sequence comparing VEH and LEV groups of AppNL/NL, AppNL-NL/F, and AppNL-G-F animals. Aβ amino acid sequences for each App KI genotype: AppNL/NL, LDAEFRHDSGYEVHHQKIL; AppNL-NL/F, KLVFAEDVSNKG; AppNL-G-F, KLVFFAGVSNKG. (B) Normalized global TMT intensities for all proteins in AppNL/NL, AppNL-NL/F, and AppNL-G-F versus VEH and LEV groups. Each circle represents an individual biological replicate. N = 6 for each group. Data represents mean ± SEM analyzed with unpaired Student’s t-test or one-way ANOVA with post hoc Sidak test. *p-value < 0.005, †p-value < 0.05, **p-value < 0.01, and ***p-value < 0.001. LEV, levetiracetam.
Figure 3. Chronic levetiracetam administration normalizes levels of presynaptic endocytic proteins in App<sup>NL-G-F/NL-G-F</sup> cortex. Presynaptic endocytic proteins are significantly upregulated with levetiracetam treatment in App<sup>NL-G-F/NL-G-F</sup>. (A) Shrinkage plot from Bayesian analysis of variance showing proteins that are differentially expressed when directly comparing VEH and levetiracetam treatment in App<sup>NL-G-F/NL-G-F</sup> cohorts. Pink and blue dots indicated significantly elevated and decreased proteins, respectively. Gray dots indicate nonsignificant proteins. (B) GO:CC enrichment analysis of the panel of significantly upregulated proteins plots depict p-value (−log2) for each GO:CC term. Categories of high interest are indicated in pink. (C) Pie chart depicts significantly altered proteins identified by comparing VEH groups App<sup>NL-G-F/NL-G-F</sup> / App<sup>NL-NL</sup>. White indicates the number of proteins that remain significantly altered after LEV. Dark green denotes the proteins that no longer significantly altered after LEV (Table S3). (D) GO:CC enrichment analysis plots depict fold enrichment vs p-value (−log2) analyzed by Fisher’s exact test. GO terms related to presynaptic endocytosis (pink), postsynapse (light purple), synapse (dark purple), and all other terms (light pink) (Table S4). (E) Percent change of presynaptic endocytosis proteins (GO:0098833) between VEH and LEV App<sup>NL-G-F/NL-G-F</sup> and App<sup>NL-NL</sup> VEH and LEV groups. (G) Protein–protein interaction hub of presynaptic endocytosis proteins based on STRING functional enrichment analysis. Data represents mean ± SEM analyzed with unpaired Student’s t-test and BH correction. N = 6 per genotype, N = 6 per treatment group. Each circle represents an individual biological replicate. Data represents mean ± SEM analyzed with unpaired Student’s t-test or one-way ANOVA with post-hoc Sidak test. *p-value < 0.005, **p-value < 0.05, ***p-value < 0.01, and ****p-value < 0.001. LEV, levetiracetam.
In order to further probe how levetiracetam alters the proteome, we identified proteins that were significantly altered in the levetiracetam-treated AppNL-NL animals, which were homed in on the proteins that were significantly altered between the VEH- and LEV-treated cohorts of AppNL-NL and AppNL-NL. Next, we studied the effects of treatment on a subset of proteins identified in these comparisons.

We then focused on the proteins belonging to the GO:CC term presynaptic endocytosis as this term was significantly enriched in both methods of analysis and investigated how their levels changed with VEH and LEV treatment. We found that levetiracetam treatment in AppNL-NL mice that were age-matched wild-type mouse age-matched cortical homogenates from AppNL-NL, AppNL-NL, and AppNL-NL mice in VEH and LEV treated groups as measured by Aβ1-42 sandwich ELISA. N = 6 for each genotype and treatment group. Each circle represents an individual biological replicate. Data represents mean ± SEM analyzed with an unpaired Student’s t-test or one-way ANOVA with post hoc Sidak test. *p-value < 0.05, **p-value < 0.01, and ***p-value < 0.001.

LEV, levetiracetam.

**Figure 4.** Chronic levetiracetam administration alters APP CTF production and decreases Aβ levels in AppNL-NL/F/NL-NL animals. (A) Cortical amyloid pathology in AppNL-NL/F/NL-NL animals comparing VEH and LEV treatment groups. Representative thioflavin S stained sagittal brain sections are shown. White box indicates area of magnified image. (B) Quantification of amyloid plaque puncta normalized to cortical area. (C) Aβ1-42 levels in cortical homogenates from AppNL-NL, AppNL-NL, and AppNL-NL mice in VEH and LEV treated groups as measured by Aβ1-42 sandwich ELISA. N = 6 for each genotype and treatment group. Each circle represents an individual biological replicate. (D) Representative WB analysis of full-length APP and APP cleavage products, β-CTF, and α-CTF from cortical homogenates from AppNL-NL VEH and LEV groups. Age-matched wild-type mouse age-matched cortical homogenates were used as a negative control. VCP was used to control loading and normalization. (E) Representative WB analysis of full-length APP and APP cleavage products, β-CTF, and α-CTF from cortical homogenates from AppNL-NL/F/NL-NL groups. Age-matched wild-type mouse age-matched cortical homogenates were used as a negative control. VCP was used to control loading. (F) Quantification of (D) showing the abundance of APP-FL normalized to VCP for AppNL-NL and AppNL-NL/F/NL-NL VEH and LEV groups. (G) Quantification of (E) showing the abundance of β-CTF/α-CTF ratio normalized to VCP. N = 4 for each genotype and treatment group. Each circle represents an individual biological replicate. Data represents mean ± SEM analyzed with an unpaired Student’s t-test or one-way ANOVA with post hoc Sidak test. *p-value < 0.05, **p-value < 0.01, and ***p-value < 0.001.

We then performed GO:CC enrichment analysis of the 985 proteins with PANTHER and found that the normalized proteins were most significantly enriched for GO:CC terms: presynaptic endocytosis, postsynapse, and presynaptic endocytosis complex, AP-2 adaptor complex, presynaptic endocytic zone) GO:CC terms related to presynaptic endocytosis (e.g., HOPS complex) were significantly enriched (Figure 3b; Table S4). We then performed GO:CC enrichment analysis and investigated how their levels changed with VEH and LEV treatment by comparing VEH to levetiracetam datasets for AppNL-NL/F/NL-NL animals. Notably, nearly all of the presynaptic endocytosis proteins had elevated levels after levetiracetam treatment (Figure 3e). We found that levetiracetam treatment in AppNL-NL/F/NL-NL normalized presynaptic endocytosis protein levels back toward AppNL-NL control levels (Figure 3f). To investigate the possibility that the levetiracetam-modulated
proteins physically interact, we subjected the group of normalized proteins to STRING analysis and uncovered a robust protein–protein interaction hub (Figure 3g). These regulated endocytic factors participate in all three predominant steps (i.e., initiation, assembly, and fission), suggesting that the entire process of endocytosis is modulated by levetiracetam (Figure S2a). At the 6 month time point, AppNL−F/NL-F animals do not have significant Aβ pathology or plaque burden and therefore serve as an additional negative control. We performed parallel analyses for the AppNL−F/NL-F datasets and found no synapse-associated proteomic alterations as a result of levetiracetam treatment (Figure S2b–e and Tables S3 and S4).

**Levetiracetam Restores Nonamyloidogenic APP Processing in AppNL−G/F/NL−G/F and Decreases Aβ12 Levels**

To further investigate the effect of levetiracetam treatment on amyloid deposition, we performed thioflavin S staining on App KI sagittal sections. Quantification of thioflavin S puncta revealed that the treatment significantly decreased the amyloid plaque load in AppNL−G/F/NL−G/F cortex compared to VEH treatment (p-value = 0.0046) (Figure 4a,b). In line with previous literature, VEH-treated AppNL−F/NL-F mice had significantly more Aβ12 compared to VEH AppNL−NL/NL mice based on sandwich ELISA (p-value = <0.0001; p-value = <0.0001) (Figure 4c). Interestingly, Aβ12 ELISA analysis revealed that levetiracetam-treated AppNL−G/F/NL−G/F cortical extracts have significantly reduced Aβ12 levels (p-value = 0.0010) compared to VEH-treated AppNL−G/F/NL−G/F cortical extracts (Figure 4c). Since Aβ12 levels are reduced without altering the levels of full-length APP protein, we investigated if the levels of APP cleavage products, β-CTF, and α-CTF were altered by levetiracetam. WB analysis of β-CTF and α-CTF bands from cortical homogenates were quantified in both AppNL−NL/NL and AppNL−G/F/NL−G/F VEH and levetiracetam groups (Figures 4d–g and S3). Notably, analysis of the β-CTF/α-CTF ratio from AppNL−G/F/NL−G/F mice indicated that levetiracetam significantly decreases β-CTFs and correspondingly increases α-CTFs compared to VEH controls (p-value = 0.0010) (Figure 4g). There was no significant difference between the β-CTF/α-CTF ratio in the two AppNL−NL/NL groups. Importantly, in both AppNL−NL/NL and AppNL−G/F/NL−G/F, full-length APP showed no change in abundance (p-value = 0.1117; p-value = 0.5334) with levetiracetam treatment (Figure 4f). This finding suggests that chronic levetiracetam administration shifts APP processing toward the nonamyloidogenic pathway, which in turn limits Aβ12 production.

**DISCUSSION**

Taken all together, our work shows that chronic levetiracetam treatment in App KI mouse models normalizes levels of presynaptic endocytosis machinery and alters APP proteolytic processing corresponding with lower levels of Aβ12 and decreased amyloid plaque deposits. Using guanidine HCl-soluble cortical extracts from App KI mouse models, we were able to develop a profound understanding of the proteomic alterations that chronic levetiracetam treatment has on brains with varying stages of amyloid pathology. We note that while guanidine HCl extracts will contain both soluble and insoluble pools, it is possible that the varying abundance of insoluble proteins may affect our TMT-MS experiments. As a growing body of evidence has demonstrated an association between AD and brain hyperexcitability, understanding the relationship between neural network dysfunction and Aβ pathology is crucial.6,14,24,25 Interestingly, in a study of AD patients with epilepsy, a comparison of levetiracetam versus typical epilepsy drugs, lamotrigine and phenobarbital, demonstrated that while all drugs were equally effective in reducing seizures, only levetiracetam treatment led to improved performance on cognitive tasks.26 Furthermore, in AD mouse models of APP overexpression such as APP/PS1 and hAPP J20, only levetiracetam reduced hyperexcitability while also decreasing Aβ plaque burden and cognitive deficits.9–12 These findings suggest that while hyperactivity contributes to increased Aβ pathology, treating hyperactivity alone is not sufficient to alleviate AD pathology. Our lab recently identified an impairment in the turnover of SV-associated proteins at early stages of AD pathology. In this study, we hypothesized that levetiracetam’s unique beneficial effect on AD pathology could result from the atypical nature of this antiepileptic targeting the presynaptic SV2A protein.7 This work shows for the first time the proteomic alterations that result from chronic levetiracetam treatment in an AD mouse model without the caveat of APP overexpression and provides a potential mechanism of action for the documented therapeutic effect of levetiracetam. Our findings demonstrate that chronic levetiracetam treatment selectively normalizes levels of presynaptic endocytosis proteins and is capable of lowering Aβ12 levels by altering APP processing.

Several supporting lines of evidence implicate dysregulation of endocytosis and presynaptic endocytic proteins in AD, thus supporting why normalization of this process reduces amyloidogenic APP processing and ultimately Aβ12 production. Much of the previous evidence gathered on Aβ toxicity implicates the postsynaptic membrane as the primary site of toxicity.27–30 However, the localization and processing of APP mainly occurs at presynaptic terminals, and it has been previously shown that APP interacts with SVs.31,32 Additionally, genome-wide association studies over the last decade have identified several AD-associated variants of endocytosis-related genes including PICALM, BIN1, and SORL1.33–35 PICALM, which is a recruiter of adaptor complex 2 (AP-2) and is required for clathrin-mediated endocytosis, was the most significantly modulated protein in our datasets. How modulation of PICALM affects APP processing is not well understood. Some evidence supports an inverse relationship between PICALM levels and Aβ12 pathology. For example, APPsw/0 × PICALM+/mice displayed hippocampal and cortical Aβ loads 4-fold higher compared to APPsw/0 × PICALM+/- controls.36 In addition, it has been shown that AP-2 is required for APP endocytosis and has the ability to alter APP processing by promoting BACE1 trafficking.37 These studies proposed that AP-2 functions at the presynapse to sort BACE1, leading to a regulation of its degradation during neuronal activity.37 This would explain why rescuing levels of endocytosis proteins, such as PICALM and AP-2, could result in a shift toward the nonamyloidogenic pathway of APP cleavage. Furthermore, additional proteins functioning in endocytosis, that were also normalized in our datasets, showed reduced levels in postmortem AD brains (e.g., AP180 and Dynamin1).38,39 Taken all together, there is substantial evidence that suggests that endocytosis and intracellular sorting determines how APP is processed. As we have previously identified an Aβ-dependent impairment in degradation at axon terminals, we propose that an upregulation in endocytosis could be beneficial as it could boost impaired SV cycling, leading to removal of APP from membranes where it is
susceptible to secretase cleavage. Our data supports the concept that levetiracetam lowers Aβ42 levels by normalizing the abundance of presynaptic endocytosis machinery that corresponds to a shift in APP processing toward the non-amyloidogenic pathway.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acs.jproteome.1c00180.

Table S1. Summary of relative abundances for significantly differentially regulated proteins across APP KI genotypes from cortical extracts. Related to Figure 1 Table S2. Significantly over-represented cellular components from a GO analysis using the query of proteins that were significantly differentially regulated in each data set against the reference of all proteins identified. Related to Figure 1 Table S3. Summary of significantly altered proteins identified by comparing vehicle groups AppNL-F/PLNL-F/AppNL or AppNL-F/PLNL-F/AppNL and the proteins that either remain or no longer are significantly altered after LEV. Related to Figure 3 and Figure S2 Table S4. Significantly over-represented cellular components from a GO analysis using the query of proteins that were no longer significantly differentially regulated after LEV against the reference of all proteins identified. Related to Figure 3 and Figure S2 (PDF)

Figure S1. TMT-MS experiments have similar data quality (XLSX)

Figure S2. Differentially regulated proteins in AppNL-F/PLNL-F levetiracetam versus vehicle cohorts (XLSX)

Figure S3. Chronic levetiracetam administration alters APP CTF production in AppNL-F/PLNL-F (XLSX)

Western blot full images related to Figure 4 (XLSX)

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N.R.R. and J.N.S. designed the experiments; N.R.R. performed all experiments; and N.R.R. and J.N.S. wrote the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the NIH, R01AG061787, R01AG061865, and R21NS107761 to J.N.S; N.R.R. is supported by Mechanisms of Aging and Dementia T32AG20506 as well as the Cure Alzheimer’s Fund and a pilot award from the CNADC of Northwestern Medicine to J.N.S.

Notes
The authors declare no competing financial interest. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeffrey N Savas (jeffrey.savas@northwestern.edu). The raw MS data have been deposited in MassIVE online database (MSV000087285) and upon acceptance will be available in the ProteomExchange online database.

ACKNOWLEDGMENTS
We thank Tim Hark for contributing to thioflavin S imaging and tissue collection. We thank the NU Center for Advanced Microscopy, which is generously supported by NCI CCSG P30 CA66553 awarded to the Robert H Lurie Comprehensive Cancer Center.

ABBREVIATIONS
Aβ, amyloid-beta; APP, amyloid precursor protein; AD, Alzheimer’s disease; App KI, APP knock-in; β-CTF/α-CTF, beta/alpha carboxyl-terminal fragment; GO, gene ontology; LEV, levetiracetam; MS, mass spectrometry; SV, synaptic vesicle; SV2A, synaptic vesicle glycoprotein 2A; TMT, tandem mass tag; VEH, vehicle

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