Somatostatin-evoked Aβ catabolism in the brain: Mechanistic involvement of α-endsosulfine-K$_{ATP}$ channel pathway

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INTRODUCTION

Alzheimer’s disease (AD) is characterized by the deposition of amyloid β peptide (Aβ) in the brain. The neuropeptide somatostatin (SST) regulates Aβ catabolism by enhancing neprilysin (NEP)-catalyzed proteolytic degradation. However, the mechanism by which SST regulates NEP activity remains unclear. Here, we identified α-endsosulfine (ENSAs), an endogenous ligand of the ATP-sensitive potassium (K$_{ATP}$) channel, as a negative regulator of NEP downstream of SST signaling. The expression of ENSA is significantly increased in AD mouse models and in patients with AD. In addition, NEP directly contributes to the degradation of ENSA, suggesting a substrate-dependent feedback loop regulating NEP activity. We also discovered the specific K$_{ATP}$ channel subtype that modulates NEP activity, resulting in the Aβ levels altered in the brain. Pharmacological intervention targeting the particular K$_{ATP}$ channel attenuated Aβ deposition, with impaired memory function rescued via the NEP activation in our AD mouse model. Our findings provide a mechanism explaining the molecular link between K$_{ATP}$ channel and NEP activation, and give new insights into alternative strategies to prevent AD.

MATERIALS AND METHODS

Animals

All animal experiments were conducted according to guidelines of the RIKEN Center for Brain Science. Sst$_1$KO and Sst$_2$KO mice were kindly provided by Oklahoma Medical Research Foundation as described previously [22]. Sst$_1$KO mice were purchased from Jackson laboratory. Sst$_1$KO and Sst$_2$KO mice were generated as described previously [22]. Mme KO mice were used as negative controls [24]. Abcc8 KO mice were generated as described previously [25], and kindly provided by Department of Pharmacology, Tübingen University. Kncj8KO and Kncj11KO mice were generated as described previously [26, 27], and kindly gift from Center for Animal Resources and Development, Kumamoto University and RIKEN BioResource Research Center. C57BL/6J and ICR mice were used as zygote donors and foster mothers. C57BL/6J mice were also used for backcrossing with EnsA KO mice. App$^{NL-F}$ mice harbor the humanized sequence of Aβ,

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and the Swedish (KM670/671NL) and Iberian (I716F) mutations, while App<sup>NC-GT</sup>-mice harbor the Arctic (E693G) mutation in addition to the humanized sequence of Aβ, and Swedish (KM670/671NL) and Iberian (I716F) mutations as previously described [28]. Male mice were used in all experiments.

**Antibodies**

Antibodies used in this research are listed in Supplementary Table S1. The specificity of ENSA antibody was confirmed using the Enzo KO mouse.

**Primary neurons**

Neurons from the cerebral cortex, hippocampus and basal ganglia regions of brains from embryonic day (E) 16–18 C57BL/6Ncr mice were isolated and cultured. Briefly, brains were excised and placed in culture plates (FALCON) containing neurobasal medium (Thermo Fisher Scientific). The aforementioned brain regions were excised by scalpel and treated with 5 ml of 0.25% trypsin solution (Nacalai tesque 32777-44) at 37 °C for 15 min. 250 µl of 1% Dnase I was added by pipette and mixed. Subsequently, centrifugation was performed at 1500 rpm for 5 min and 5 ml of Hank’s buffered salt solution containing 250 µl of 1% Dnase I was added to the pellet and incubated in a water bath at 37 °C for 5 min. An additional 10 ml of Hank’s buffered salt solution was added to the mixture and centrifuged at 1500 rpm for a further 5 min. The resulting pellet was added to neurobasal medium with B27 Plus Supplement (Thermo Fisher Scientific 17504-044) and 25 µM glutamine (Thermo Fisher Scientific 05030-149). The cells were filtered using a cell strainer with 100 µm nylon mesh (Falcon 2360), and seeded on 6- or 96-well plates (Falcon 353046 or Corning 356640). Cortical/hippocampal and basal ganglia neurons were mixed in a 9:1 ratio as co-cultured neurons.

**Nephrilysin activity**

Nephrilysin activity measurements were performed on primary neurons after 15–28 days of in vitro (DIV15–28) culture as previously described [29]. Somatostatin (Peptide Institute AF-721), T232 (Tocris 3493), recombinant ENSA (abcam ab92932), recombinant NSG-1 (Creative BioMart 26810), and recombinant human ENSA (Sigma S8758), 10 nM phosphoramidon (Peptide Institute 4082), and 0.2 M MES buffer (pH6.5) with or without Thiorphan (Sigma T6031) for 1 h were added to neurobasal medium with B27 Plus Supplement (Thermo Fisher Scientific 17504-044) and 25 µM glutamine (Thermo Fisher Scientific 05030-149). Following this, 0.1 mM phosphoramidon (Peptide Institute 4082) and 0.2 M MES buffer (pH6.5) with or without Thiorphan (Sigma T6031) for 1 h were added to neurobasal medium. The supernatants were centrifuged at 70,000 rpm at 4 °C for 30 min. 5 ml of Hank’s buffered salt solution containing 250 µl of 1% Dnase I was added to the pellet and incubated in a water bath at 37 °C for 5 min. An additional 10 ml of Hank’s buffered salt solution was added to the mixture and centrifuged at 1500 rpm for a further 5 min. The resulting pellet was added to neurobasal medium with B27 Plus Supplement (Thermo Fisher Scientific 17504-044) and 25 µM glutamine (Thermo Fisher Scientific 05030-149). The cells were filtered using a cell strainer with 100 µm nylon mesh (Falcon 2360), and seeded on 6- or 96-well plates (Falcon 353046 or Corning 356640). Cortical/hippocampal and basal ganglia neurons were mixed in a 9:1 ratio as co-cultured neurons.

**Preparation of membrane fractions from brain tissue**

Brain tissues were homogenized in Tris-buffer (50 mM Tris pH 7.6, 0.15 M NaCl and oComplete protease inhibitor cocktail (Roche Diagnostics 11697948001)) using a Multi-bead shaker MB (Yasu-Kikai). Samples were rotated at 4 °C for 1 h and centrifuged at 15000 rpm for 30 min. Supernatants were collected as lysates and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF or nitrocellulose membranes. For detection of ENSA and CTF-APP, membranes were boiled in PBS for 5 min, treated with ECL prime blocking buffer (GE healthcare RPN418) for 1 h and incubated with antibody at 4 °C. Signal intensity was quantified using MultiNa (Shimadzu) to evaluate the efficiency of the CRISPR-mediated deletion of the Enso gene. Gasser sequencing analyses were conducted using a DNA sequencer (ABI 3730xl).

**Microinjection of mouse zygotes**

The SpCas9 mRNA (60 ng/µl) and sgRNAs (30 ng/µl) were injected into the cytoplasm of C57BL/6 J zygotes. After incubation at 37 °C for 24 h, embryos developed to the 2-cell-stage were transplanted into host ICR mice.

**Off-target analysis**

Off-target sites that accepted up to three mismatches were determined by COSMIC (https://crispr.bme.gatech.edu/) [33], listed in Supplementary Table S5. Target sites were amplified from tail genomic DNA by PCR using the Ex Taq Polymerase kit (Takara R001A) with primers listed in Supplementary Table S6. Target sequencing was performed using a DNA sequencer (ABI 3730xl).

**Western blot analysis**

Mouse brains were homogenized with lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 20 µg/µl Proteinase K) and PCR performed using the specific primer set listed in Supplementary Table S7. PCR products were analyzed by MultiNa (Shimadzu) to evaluate the efficiency of the CRISPR-mediated deletion of the Enso gene. Sanger sequencing analyses were conducted using a DNA sequencer (ABI 3730xl).

**Co-incubation of ENSA and NEP**

In total, 25 ng/µl of ENSA were co-incubated with 2.5 ng/µl NEP, 0–500 nM Aβ<sub>40</sub> (Peptide Institute 4420-s) and arctic Aβ<sub>40</sub> (Peptide Institute AF-721), 0.1 M thiouracil, 1 mM phosphoramidon, and 10 mM EDTA at 37 °C for 24 h in 0.2 M MES buffer pH 6.5.

**Immunohistochemical analysis**

After deparaffinization of paraffin-embedded mouse brain sections, antigen retrieval was performed by autoclaving at 121 °C for 5 min. Sections were then treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol to inactivate endogenous peroxidases. Next, sections were rinsed several times with TNN buffer (0.1 M Tris pH 7.5, 0.15 M NaCl, 0.05% Tween20) for 30 min (TSA Biotin System kit), and incubated overnight at 4 °C with primary antibody diluted in TNN buffer (0.1 M Tris pH 7.5, 0.15 M NaCl). Sections were rinsed several times and incubated for 1 h at room temperature with biotinylated secondary antibody (Vector Laboratories). Next, sections were incubated with HRP-conjugated avidin for 30 min and tyramide-enhanced FITC or rhodamine for 10 min. Finally, sections were treated with DAPI (Cell Imaging Solution) and mounted using MOUNTAVERSE (Thermo Fischer Scientific). Immunoreactive bands were visualized by Immunohistochemistry (Fujifilm).
Fig. 1 Identification of ENSA as a NEP regulator in vitro. A-C. NEP activity after treatment of co-cultured cells with 1 µM somatostatin or TT232 for 24 h. A Cortical/hippocampal (Ctx&Hip) neurons (n = 12 wells per treatment), (B) co-cultured neurons (n = 10 wells per treatment), and (C) basal ganglia neurons (n = 8 or 9 wells per treatment) were used. D-F NEP activity in co-cultured neurons after the replacement of the culture medium with conditioned media from (E) Ctx&Hip and (F) basal ganglia neurons treated with 1 µM somatostatin for 0–6 h. n = 6–10 wells per treatment in co-cultured neurons. G-K NEP activity of co-cultured neurons after replacement of the culture medium with separated conditioned media from Ctx&Hip neurons treated with SST or TT232. H, I 10 and (J and K) 30kDa centrifugal filters were used for the separation (n = 7–10 for each group). NEP activity in co-cultured neurons after incubation with (L) ENSA, (M) NSG-1 and (N) NUCKS-1 recombinant proteins for 24 h. n = 8–10 wells per treatment in co-cultured neurons. Data represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA with Dunnett’s post-hoc test).
**Enzyme-linked immunosorbent assay**

Mouse cortices were homogenized in TBS buffer (50 mM Tris pH 7.6, 150 mM NaCl, protease inhibitor cocktail) by a Multi-bead shocker (YASUI KIKAI), centrifuged at 70,000 rpm for 20 min, and supernatants collected as Tris-soluble fractions. Pellets were rinsed with TBS buffer following which 6 M guanidine-HCl solution was added and mixed with a Pellet Peste (KIMBLE). The mixture was then incubated for 1 h at room temperature. Next, samples were centrifuged at 70,000 rpm for 20 min and supernatants collected as guanidine-soluble fractions. Tris-soluble fractions and guanidine-soluble fractions were applied to 96-well plates. Aβ40 and Aβ42 levels were measured with the aid of an Aβ-ELISA kit (Wako 294–62501,294–62601).
Fig. 2 Elevation of NEP activity in Ensa KO mice. A. Immunostaining of NEP (Red) and VGAT (Green) from hippocampi of 3-month-old WT and Ensa KO mice. Scale bar is 100 µm in low magnification image and 50 µm in high-magnification image. B. Statistical analysis of NEP immunoreactivity (n = 5 for each group). LM: lacunosum-molecular layer, Omo: Outer molecular layer and MMO: middle molecular layer. C. Statistical analysis of colocalized NEP and VGAT signals (n = 5 for each group). D. NEP activity in membrane fractions from hippocampi of 3-month-old WT and Ensa KO mice (WT: n = 7, Ensa KO: n = 6). E, F. Aβ40 ELISA of hippocampi of 3-month-old WT and Ensa KO mice (WT: n = 5, Ensa KO: n = 6). F. Aβ42 ELISA of hippocampi of 3-month-old Mme KO mice and Ensa/Mme dKO (Mme KO: n = 4, Ensa/Mme dKO: n = 5). H and I. Immunostaining of Aβ (Green), NEP (Red) and DAPI (blue) from 18-month-old AppNL−/− and AppNL−/−/Ensa KO mice. Statistical analysis of amyloid plaque area in 18-month-old AppNL−/− and AppNL−/−/Ensa KO mice (n = 6 for each group). Scale bar is 100 µm. J. Aβ42 ELISA of Tris-HCl-buffered saline-soluble (Ts) hippocampal fractions from 18-month-old AppNL−/− and AppNL−/−/Ensa KO mice (AppNL−/−: n = 7, AppNL−/−/Ensa KO: n = 6). K. Aβ42 ELISA of guanidine-HCl-soluble (GuHCl) hippocampal fractions from AppNL−/− and AppNL−/−/Ensa KO mice (AppNL−/−: n = 7, AppNL−/−/Ensa KO: n = 6). L. Immunostaining of NEP (Red) and VGAT (Green) in hippocampi of 18-month-old AppNL−/− and AppNL−/−/Ensa KO mice. Scale bar is 100 µm in low-magnification image and 50 µm in high-magnification image. M. Statistical analysis of NEP immunoreactivity (n = 4 for each group). N. Statistical analysis of colocalized NEP and VGAT signals (n = 4 for each group). Data represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s or Welch’s t test).
TT232. We also used conditioned media from Sst1/Sst4 dKO mice as a negative control. We then searched for proteins absent or present only in the media of the SST- and TT232-treated WT neurons, but not in the media of Sst1/Sst4 dKO neurons. In this way, we identified three candidate proteins: (1) ENSA, (2) Neuron-specific protein family member 1 (NSG-1) and (3) Nuclear ubiquitous casein and cyclin-dependent substrate 1 (NUCKS-1) (Supplementary Tables S2 and S3). To determine which of the candidates is involved in the regulation of NEP activity, we analyzed the effects of corresponding recombinant proteins on
Fig. 3 Identification of ENSA as a substrate for NEP. A Immunoblotting of ENSA incubated with or without NEP and mentioned inhibitors for 24 h at 37 °C. Thio: Thiorphan, Phos: Phosphoramidon. B Specific peak of full-length of ENSA after incubation with or without NEP and thiorphan. C Specific peak of cleaved ENSA after incubation with or without NEP and thiorphan. D Sequence of full-length of ENSA. Arrows indicate cleavage site by NEP. E, F Immunoblotting of ENSA from cortices and hippocampi of 6-month-old WT and Mme KO mice. Values indicated in the graph show ENSA band intensities normalized to that of β-actin (n = 5 for each group). G–I Immunoblotting of (H) NEP and (I) ENSA from hippocampi of 3-month-old WT mice after overexpression of active or inactive mutant NEP by SFV gene expression system. Values indicated in the graph show NEP and ENSA band intensities normalized to that of β-actin (n = 4 for each group). J Aβ42 ELISA of Tris-HCl-buffered saline-soluble fractions containing 1% Triton-X from hippocampi of WT mice after overexpression of active or inactive mutant NEP by the SFV gene expression system (n = 4 for each group). K Immunostaining of ENSA (Green), NEP (Red) and DAPI (Blue) in CA3 from 3-month-old WT, ENSA KO and Mme KO mice. Scale bar is 50 μm in low-magnification image and 10 μm in high-magnification image. White arrows indicate colocalized signals. Data represent the mean ± SEM. *P < 0.05, **P < 0.0001 (Student’s t or Welch’s t test).

NEP activity in co-cultured neurons. Only the recombinant ENSA decreased NEP activity in co-cultured neurons from WT and Sst4/ Sst4 dKO mice in a dose-dependent manner (Fig. 1L–N, and Supplementary Fig. S1F).

We next analyzed ENSA levels in the brains of Sst4/ Sst4 dKO mice and SST knock out (Sst4 KO) mice and found that ENSA levels were significantly elevated in the cortex and hippocampal CA1 and CA3 regions in these animals (Supplementary Fig. 2D–G). Taken together, these results suggest that ENSA functions as a negative NEP regulator downstream of SST signaling.

Activation of NEP by genetic deficiency of ENSA in vivo

ENSA, an endogenous blocker of SUR1 which is a regulatory subunit of KATP channel, is highly expressed in brain, skeletal muscle and pancreas [39, 40]. Although we found that ENSA is a negative regulator of NEP in vitro, the function of ENSA in vivo is largely unknown. We therefore generated ENSA knock out (Ensa KO) mice using CRISPR/Cas9 technology. Dual adjacent single-guide RNAs (sgRNAs) were designed that targeted exon 1 of the Ensa gene including the initiation codons (Supplementary Fig. S3A). This strategy facilitates CRISPR-mediated genome targeting [41]. We injected sgRNA1-Ensa-Exon1 (30 ng/ml) and sgRNA2-Ensa-Exon1 (30 ng/ml) together with Streptococcus pyogenes Cas9 mRNA (60 ng/ml) into WT mouse zygotes. Sanger sequencing analysis and PCR-based genotyping indicated the deletion of exon 1, including the initiation codons (Supplementary Fig. S3B and C). Expression of ENSA in homozygous F2 mutant mice, generated by crossbreeding the heterozygous F1 mutant mice with each other, was fully deleted (Supplementary Fig. S3D and E). To assess the off-target effects of CRISPR/Cas9 in the founder, we searched for potential off-target sites using COSMID [33], with S3 candidate sites being identified (Supplementary Table S5). Of note, there was no off-target mutation on chromosome 3, which contains the Ensa gene. PCR-based genotyping and Sanger sequencing analyses for each candidate site revealed that founder had an off-target mutation in an intergenic region of chromosome 2 which was removed by backcrossing with WT mice (Supplementary Fig. S3F and G).

NEP, a pH-sensitive enzyme, efficiently degrades Aβ42 at the presynaptic region where the pH is neutral rather than inside secretory vesicles where the pH is acidic [15], without altering Aβ40 levels under different pH conditions. To determine whether a deficiency of ENSA affects the localization of NEP, we used immunohistochemical analyses, ENSA signals in the cerebral cortices of patients with AD (Supplementary Fig. 5G–J).

We next investigated whether the deficiency of ENSA affected the processing of Aβ production or expression of other Aβ-degrading enzymes. We performed Western blot analysis of full-length APP, its C-terminal fragments generated by ß-secretase (CTF-ß) and ß-secretase (CTF-ß), insulin-degrading enzyme (IDE), and endothelin converting enzyme 1 (ECE-1). No significant differences were observed in the expression levels of these proteins and ratio of the CTF-ß/α fragments (Supplementary Fig. S3H and I).

To examine the effect of ENSA deficiency on Aβ pathology, we next crossedbred Ensa KO mice with AppNL−/−, F mice. Knock-in (AppNL−/−) mice, AppNL−/− mice harbor two full-length AD-causing mutations (Swedish (KM670/671NL) and Beyreuther/Iberian (I716F)) in the endogenous App gene as well as humanized Aβ sequences, and develop amyloid pathology in the hippocampus and cortex around 6 months of age [28]. Immunohistochemical analyses using specific antibodies against the unmodified amino-terminus Aβ, N1D and modified amino-terminus of Aβ, N3(pE), respectively [42], revealed that both N1D- and N3(pE)—positive amyloid depositions in hippocampal molecular layer area were significantly reduced in AppNL−/−/Ensa KO mice, where NEP expression was elevated (Fig. 2H and I, and Supplementary Fig. S3J and K). Aβ ELISA on the hippocampi of AppNL−/−/Ensa KO mice also showed significant reduction of Aβ42 levels (Fig. 2J and K). We consistently found that NEP expression in the LM and OMO of AppNL−/−/Ensa KO mice was upregulated, particularly in the presynaptic region of OMO (Fig. 2L–N). Taken together, these observations suggest that ENSA is a negative regulator of NEP in vivo and that a deficiency of ENSA attenuates Aβ pathology by allowing NEP activity to be upregulated.

To explore the involvement of ENSA in the etiology of AD, we analyzed ENSA levels in an AD mouse model and in postmortem brain of patients with AD. Western blot analyses revealed that ENSA expression was significantly increased in the cortices and hippocampi of AppNL−/− mice at 24 months (Supplementary Fig. 5A–D). In immunohistochemical analyses, ENSA signals in the cerebral cortices and hippocampal CA1 and CA3 regions of AppNL−/− mice were also increased at 24 months (Supplementary Fig. 5E and F). Consistent with these observations, ENSA levels were markedly increased in the cortices of patients with AD (Supplementary Fig. 5G–J).

Feedback mechanism regulating NEP activity

SST, an endogenous regulator of NEP, is degraded by NEP in a substrate-dependent feedback manner [15, 43]. We hypothesized
that NEP might also directly degrade ENSA in a similar feedback manner. Co-incubation of recombinant ENSA with NEP resulted in a remarkable decrease in ENSA levels (Fig. 3A). Several NEP inhibitors such as thiorphan, phosphoramidon and EDTA attenuated this effect, indicating that NEP degrades ENSA in vitro (Fig. 3A). To identify the NEP-mediated cleavage sites in ENSA, we performed MALDI-TOF analysis after incubation of recombinant ENSA with NEP. Multiple ENSA fragments were detected in the NEP-treated sample, but not in a sample treated in the presence of thiorphan (Fig. 3B and C, and Supplementary Fig. S6). We
We also measured lower levels of NEP activity in the hippocampi of Kcnj11 KO mice than that of WT mice (Fig. 4N). These results suggest that the SUR1/Kir6.2 K<sub>ATP</sub> channel subtype is the main regulator of NEP action in the brain.

To determine the impact of the SUR1/Kir6.2 subtype on the etiology of AD, we analyzed the transcripts of each K<sub>ATP</sub> channel component in datasets from human postmortem brains, with or without AD, obtained from the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI). The GSE15222 dataset, which included 135 controls and 106 late-onset AD samples, revealed that ABCC8 gene expression was significantly reduced among the K<sub>ATP</sub> channel components in AD patients (Supplementary Fig. S8A, and Supplementary Table S10) [35]. Consistently, this reduction was reproduced in other cohort datasets (GSE95587 and GSE125583) (Supplementary Fig. S8B and C, and Supplementary Table S10) [36, 37]. Furthermore, Van Rooji et al., also indicated that robust reductions of both ABCC8 and KCNJ11 mRNA expression levels were observed in AD patients (Supplementary Table S10) [38]. According to gene expression analyses with differentiating Braak stages in both GSE95587 and GSE125583 datasets, reductions of ABCC8 and KCNJ11 mRNA levels were observed with the progression of AD (Supplementary Fig. SBD-G, and Supplementary Table S11).

Improvement of Aβ pathology and memory function by diazoxide in an AD mouse model

On the basis of these observations, we hypothesized that agonist stimulation of SUR1/Kir6.2 in brain might be beneficial for the prevention of AD via the NEP upregulation. To investigate whether diazoxide (Dz), a well-known K<sub>ATP</sub> channel activator with a high affinity for SUR1 [47], regulates NEP action, we incubated co-cultured primary neurons with Dz, and found that NEP is activated in a dose-dependent manner (Fig. 5A). As Dz has been reported cross the blood brain barrier [48–50], we treated WT mice by oral administration of Dz for 1 month. This treatment significantly increased NEP activity in the anterior cortex and hippocampus of these animals (Fig. 5B), with elevated levels of NEP expression also seen in the anterior cortex (Fig. 5C and D). In line with this, Dz significantly lowered Aβ<sub>42</sub> levels in the anterior cortex and hippocampus, where NEP was activated (Fig. 5E), whereas Dz treatment had no effect on Aβ<sub>42</sub> levels in the anterior cortex and hippocampus of Mme KO mice (Fig. 5F). These results suggest that Dz decreased Aβ<sub>42</sub> levels in a NEP-mediated manner. We next investigated the therapeutic effect of Dz on App<sup>NL</sup>-f mice by carrying out contextual fear-conditioning tests to assess memory function after 3 months of Dz treatment from the age of 15 months. Dz treatment recovered the freezing ratio of App<sup>NL</sup>-f mice to a level comparable to that of WT mice (Fig. 7G). We also performed open field maze tests to assess the anxiety phenotype as it has been shown that anxiety may affect performance in spatial memory tasks [51]. Dz treatment in WT and App<sup>NL</sup>-f mice did not alter the amount of time spent in the central region.
(Supplementary Fig. S9A), indicating that Dz had no effect on psychological status. Dz treatment also decreased both N1D- and N3(pE)—positive amyloid depositions in the cortex, subiculum and hippocampal molecular layer (Fig. 5H and I and Supplemental Fig. S9D and E). Aβ_{42} levels in the cortices and hippocampi of Dz-treated AppNL-F mice were also significantly reduced (Fig. 5J). Immunohistochemical analyses indicated an increase of NEP expression in the anterior cortex of these mice (Supplementary Fig. S9B and C). In addition, colocalized signals of NEP and VGAT were also increased in the presynaptic regions of the hippocampal
**Fig. 5** Improvement of Aβ pathology and memory function in AppNL-F mice via enhancement of NEP activity by Dz treatment. A NEP activity after treatment of co-cultured neurons for 24 h with different doses of diazoxide (Dz) (n = 9–10 for each group). B NEP activity in membrane fractions from anterior cortex (Cx), posterior Ctx and hippocampus (Hip) of 4-month-old WT mice treated with or without Dz (n = 6 for each group). C. D Immunoblotting of NEP in anterior cortex of 4-month-old WT mice treated with or without Dz. Values indicated in the graph Values indicated in the graph show NEP band intensities normalized to that of β-actin (n = 4 for each group). E Aβ42 ELISA of GuHCi fractions from anterior Ctx and Hip of 4-month-old WT mice with or without Dz (Dz (-) n = 6, Dz (+) n = 7). F Aβ42 ELISA of GuHCi fractions from anterior Ctx and Hip of 6-month-old MmKO mice with or without Dz (n = 8 for each group). Freezing ratio of 18-month-old WT and AppNL-F mice treated with or without Dz (WT Dz (-): n = 12, WT Dz (+): n = 13, AppNL-F Dz (-): n = 10, AppNL-F Dz (+): n = 11). H. Immunostaining of Aβ (Green) and NEP (Red) in Ctx, Subiculum and Molecular layer from 18-month-old AppNL-F with or without Dz (n = 7 for each group). Scale bar in cortical image = 500 µm and hippocampal image = 200 µm. J Aβ42 ELISA of GuHCi fractions from cortices and hippocampi of 18-month-old AppNL-F with or without Dz (n = 8 for each group). K Immunostaining of NEP (Red) and VGAT (Green) in hippocampi of 18-month-old AppNL-F with or without Dz (n = 8 for each group). L. Immunostaining of Aβ (Green) and NEP (Red) in Ctx, Subiculum and Molecular layer from 18-month-old AppNL-F with or without Dz (n = 7 for each group). LM: lacunosum-molecular layer, Omo: Outer molecular layer, Mm: Middle molecular layer. M Statistical analysis of colocalized signals of NEP and VGAT (n = 5 for each group). N. In (A), the data represent the mean ± SEM. *P < 0.05. **P < 0.01. ***P < 0.001 (one-way ANOVA with Dunnett’s post hoc test). In (B), (D), (E), (I), (J), (L), (M), the data represent the mean ± SEM. *P < 0.05, **P < 0.01, (Student’s t test). In (G) the data represent the mean ± SEM. On day 3, WT Dz (-) vs AppNL-F Dz (-) *P < 0.05. On day 4, WT Dz (-) vs AppNL-F Dz (-) *P < 0.05, WT Dz (-) vs AppNL-F Dz (-) **P < 0.01, AppNL-F Dz (-) vs AppNL-F Dz (+) *P < 0.05 (two-way ANOVA with Turkey’s multiple comparison test).

LM and OMo (Fig. 5K–M). Dz had no effect on behavior (Supplementary Fig. 59F and G) or Aβ pathology in AppNL-F/MmKO mice (Supplementary Fig. 59H and I). Taken together, these results suggest that Dz improves Aβ pathology and memory impairment in AppNL-F mice by upregulating NEP activity.

**DISCUSSION**

In the present study, we used in vitro and in vivo experimental paradigms to identify ENSA as a negative regulator of NEP activity downstream of SST signaling. To further understand the mechanism of NEP regulation downstream of SST, we have revealed that, of the multiple possible subtypes of KATP channels, SUR1/Kir6.2 regulates NEP activity. Finally, agonist stimulation of SUR1 by Dz prevented Aβ deposition via the upregulation of NEP, thereby improving memory function in AppNL-F mice.

SST mRNA levels were reported to decrease in brain with aging and in AD [16–21]. As such, ENSA, a downstream protein of SST signaling, may be related to the etiology of AD. Indeed, we showed elevation of ENSA levels in AppNL-F mice as well as in AD patients (Supplementary Fig. 5). Moreover, in vitro and in vivo experiments revealed that NEP degraded ENSA as a substrate, suggesting that NEP and ENSA form a negative feedback loop. This hypothesis is based on the fact that opioids and substance P, cell-specific ligands in monocytes and bone marrow cells, respectively [52, 53], regulate NEP via a feedback mechanism. It is possible that Aβ and ENSA compete against each other in the NEP-mediated degradation, additively exacerbating this feedback loop and inducing a vicious cycle.

A selective agonist of the KATP channel such as Dz could serve as a beneficial approach to break this vicious cycle given that it is used as a drug for antihypertensive and hypoglycemic properties, and has the potential in the preclinical setting to improve Aβ pathology and behavioral abnormalities in AD [48, 49]. A previous study showed that Dz treatment reduced the extracellular accumulation of Aβ in 3xTg mice which display both amyloid and tau pathology due to overexpression of mutated APP and MAPT genes on a mutant PSEN1 background [54, 55]. The mechanism by which Dz attenuated Aβ plaque deposition was, however, unclear. Our findings indicate that Dz reduced amyloid deposition in AppNL-F mice via the regulation of NEP activity in the anterior cortex and hippocampus. This regional selectivity of NEP activity by Dz may be dependent on the dopaminergic system in the brain. The KATP channel is highly expressed in dopaminergic neurons in the midbrain and regulates dopamine release. These neurons project to the frontal cortex and hippocampus [56–61]. Recently we confirmed that dopamine regulates NEP expression and/or activity in the frontal cortex and hippocampus regions in AppNL-F mice. To further elucidate the mechanism for the regulation of NEP activity, it will be necessary to investigate pathways downstream of SUR1/Kir6.2. Likewise, it is important to develop a more specific opener of SUR1/Kir6.2 to avoid off-target effects given that different KATP channel subtypes are expressed in vascular smooth muscle cells, cardiac muscle cells and pancreatic β-cells [62]. In addition to promoting NEP-mediated Aβ degradation, KATP channel agonists may have beneficial effects in AD. Dz treatment prevents Aβ-induced neurotoxicity induced by oxidative stress and inflammatory damage and also shows neuroprotective effects against apoptosis in vitro [63–67]. Compared to Aβ-targeting immunotherapies, synthetic agonists for the KATP channel are less expensive and would be more acceptable in aging societies around the world.

Taken together, we have demonstrated a new preventive approach at the preclinical stage of AD based on the function of ENSA. This negative regulator of NEP and KATP channel could be a new therapeutic target for lowering Aβ.

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AUTHOR CONTRIBUTIONS
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COMPETING INTERESTS
The authors declare no conflict of interest.

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