

Beta-Site Amyloid Precursor Protein Cleaving Enzyme 1 Inhibition Impairs Synaptic Plasticity via Seizure Protein 6

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ABSTRACT

BACKGROUND: Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is a promising drug target for the treatment of Alzheimer's disease. Prolonged BACE1 inhibition interferes with structural and functional synaptic plasticity in mice, most likely by altering the metabolism of BACE1 substrates. Seizure protein 6 (SEZ6) is predominantly cleaved by BACE1, and *Sez6* knockout mice share some phenotypes with BACE1 inhibitor-treated mice. We investigated whether SEZ6 is involved in BACE1 inhibition-induced structural and functional synaptic alterations.

METHODS: The function of NB-360, a novel blood-brain barrier penetrant and orally available BACE1 inhibitor, was verified by immunoblotting. In vivo microscopy was applied to monitor the impact of long-term pharmacological BACE1 inhibition on dendritic spines in the cerebral cortex of constitutive and conditional *Sez6* knockout mice. Finally, synaptic functions were characterized using electrophysiological field recordings in hippocampal slices.

RESULTS: BACE1 enzymatic activity was strongly suppressed by NB-360. Prolonged NB-360 treatment caused a reversible spine density reduction in wild-type mice, but it did not affect *Sez6*^{-/-} mice. Knocking out *Sez6* in a small subset of mature neurons also prevented the structural postsynaptic changes induced by BACE1 inhibition. Hippocampal long-term potentiation was decreased in both chronic BACE1 inhibitor-treated wild-type mice and vehicle-treated *Sez6*^{-/-} mice. However, chronic NB-360 treatment did not alter long-term potentiation in CA1 neurons of *Sez6*^{-/-} mice.

CONCLUSIONS: Our results suggest that SEZ6 plays an important role in maintaining normal dendritic spine dynamics. Furthermore, SEZ6 is involved in BACE1 inhibition-induced structural and functional synaptic alterations.

Keywords: Alzheimer's disease, BACE1 inhibition, Dendritic spines, In vivo two-photon imaging, Long-term potentiation, *Sez6*

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Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder among the elderly. No effective medical treatment or preventive approach exists. Two of the typical neuropathological hallmarks of AD are neuritic plaques, formed by the aggregation of β -amyloid (A β) peptides, and neurofibrillary tangles, composed of hyperphosphorylated tau (1,2). Because overt accumulation of A β induces tau phosphorylation, A β is considered the causative factor for neurodegeneration (1–3). A β is produced by sequential proteolytic cleavage of amyloid beta precursor protein (APP) via beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) and γ -secretase complex in the amyloidogenic pathway. BACE1 initiates β -amyloidogenic processing of APP, which makes BACE1 a major drug target for AD therapies. Currently, several promising BACE1 inhibitors are undergoing phase 2 or 3 clinical trials (4–6).

Although *Bace1* knockout mice are viable and fertile, they have subtle neurological abnormalities such as impaired

memory, reduced dendritic spine density in the hippocampus, and decreased myelination (7–9). At least some of these abnormalities may be developmental deficits. The inhibition of BACE1 in adult mice has been reported to decrease postsynaptic spine density (10), but whether BACE1 inhibitor treatment would cause similar deficits in humans remains unknown. Furthermore, it is also unclear which substrates are responsible for BACE1 inhibition-induced alterations of postsynaptic spines. Because synaptic loss correlates with cognitive impairments as reported in AD patients and AD mouse models (11,12), it is necessary to identify the molecular mechanism by which BACE1 inhibition impairs synaptic function.

Seizure protein 6 (SEZ6) is a type I transmembrane protein expressed in neurons. The full-length SEZ6 (flSEZ6) is nearly exclusively cleaved by BACE1, generating a secreted soluble ectodomain (soluble SEZ6 [sSEZ6]) and a transmembrane

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C-terminal fragment (SEZ6-CTF) (13–15). Later, this SEZ6-CTF will be further cleaved by γ -secretase, generating intracellular domain (16). SEZ6 mainly locates in the somatodendritic compartment, specifically in the dendritic plasma membrane, synaptosomes, and postsynaptic density fractions (13,17–19), as well as in recycling endosomes (T. Palumaa *et al.*, Nov 2012, Students of Brain Research Symposium, abstract), suggesting that SEZ6 may modulate synaptic function. Similar to BACE1 inhibitor-treated mice (10), *Sez6*^{-/-} mice have reduced dendritic spine density and spatial memory deficit (17). Constitutive *Sez6*^{-/-} mice also show poor motor coordination (17), which is observed in BACE1 inhibitor-treated mice (20). Recent whole-exome sequencing studies identified SEZ6 variants in human patients with severe intellectual disability and childhood onset schizophrenia (21,22).

To shed light on the mechanism of BACE1 inhibition-induced synaptic alteration, we used long-term in vivo two-photon imaging to investigate the structural postsynaptic plasticity in constitutive and conditional *Sez6* knockout mice on BACE1 inhibition. In addition, ex vivo electrophysiological recordings were used to study the functional synaptic plasticity in BACE1 inhibitor-treated *Sez6* knockout mice. Our data indicate that SEZ6 is important for maintaining normal dendritic spine dynamics and synaptic functions. Moreover, SEZ6 is critically involved in BACE1 inhibition-induced synaptic deficits in adult mice. Our findings imply that soluble SEZ6 might be used as a biomarker in BACE1 inhibitor-treated AD patients to adjust inhibitor dosage to achieve optimal clinical efficacy and safety.

METHODS AND MATERIALS

Mice and Drug Administration

C57BL/6J mice were purchased from Charles River Laboratories (Sulzfeld, Germany). *Bace1*^{-/-} (23), *SlickV* (*Thy1-creER^{T2}, -eYFP*) (24), and *GFP-M* (*Thy1-eGFP*) (25) transgenic mice were obtained from Jackson Laboratory (Bar Harbor, ME). *Sez6* knockout (*Sez6*^{-/-}) and floxed *Sez6* (*Sez6*^{LoxP/LoxP}) mice were kindly provided by Jenny Gunnarsen (17). *Sez6*^{-/-}:*GFP-M* and *Sez6*^{LoxP/LoxP}:*SlickV* lines were generated by interbreeding. All animals were bred in the animal housing facility of the Center for Neuropathology and Prion Research of the Ludwig Maximilian University Munich under pathogen-free conditions. Food and water were provided ad libitum (21 ± 1°C at 12/12-hour light/dark cycle). The health condition of each animal was monitored on a daily basis, and body weight was measured three times a week. Mice (3–4 months old) of both sexes were used for in vivo two-photon imaging and electrophysiological recordings. All animal procedures were performed in accordance with a protocol approved by the Ludwig Maximilian University of Munich and the Government of Upper Bavaria.

BACE1 inhibitor NB-360 (26) was kindly provided by Novartis Institutes for BioMedical Research (Basel, Switzerland). The inhibitor was supplied in food chow (250 mg/kg). *SlickV* mice coexpress the tamoxifen-inducible CreER^{T2} recombinase in enhanced yellow fluorescent protein (eYFP)-positive neurons. *Sez6* deletion was induced in CreER^{T2}/eYFP-positive neurons in *Sez6*^{LoxP/LoxP}:*SlickV* mice by oral gavage of tamoxifen for 5 consecutive days (0.25 mg/g body weight in a 1:10 ethanol/corn oil mixture; Sigma-Aldrich, St. Louis, MO) (27).

Supplementary Methods

For a detailed description of protein extraction, immunoblotting, cranial window implantation, in vivo two-photon imaging, immunohistochemistry experiments, and electrophysiological recordings, see the Supplement.

RESULTS

Brain Levels of BACE1 Cleavage Products Decrease After NB-360 Treatment

In this study, we used the BACE1 inhibitor NB-360 developed by Novartis Pharma AG (Basel, Switzerland). NB-360 is a small molecule compound (molecular structure shown in Supplemental Figure S1A) that efficiently crosses the blood-brain barrier (26). To minimize the stress caused by repeated drug administration to experimental animals, the inhibitor (250 mg/kg) was added to the mouse diet. Mice consumed 4.6 ± 0.1 g food pellets per day (*N* = 44). Body weight loss and health impairments were not observed during and after prolonged treatment (data not shown). Interestingly, most of the NB-360-treated mice developed hair depigmentation (Supplemental Figure S1B) because the BACE1 inhibitor NB-360 also inhibits BACE2, which is involved in melanogenesis (10,26,28,29).

To confirm the efficiency of NB-360 inhibition, we analyzed the protein levels of the BACE1 substrates, APP and SEZ6, by immunoblotting whole-brain homogenates from NB-360-treated mice and quantifying the protein levels of fSEZ6 as well as the BACE1-cleaved products sAPP β (soluble APP beta), β -CTF (C-terminal fragment of APP), and sSEZ6 (15,16). *Bace1*^{-/-} (*Bace1* knockout) mice were used as a control (Figure 1). Quantification of immunoblot signals revealed that fSEZ6 was significantly increased in *Bace1*^{-/-} and NB-360-treated wild-type (WT) mice compared with vehicle-treated WT mice. The BACE1 cleavage products sSEZ6, sAPP β , and β -CTF were significantly decreased in NB-360-treated WT and *Bace1*^{-/-} mice, confirming the reduced enzymatic activity of BACE1. In summary, these results confirm that NB-360 is a potent inhibitor of BACE1.

BACE1 Inhibition Does Not Affect Dendritic Spine Plasticity in *Sez6*^{-/-} Mice

It has been shown that the BACE1 inhibitors SCH1682496 (Merck/Schering-Plough Pharmaceuticals, North Wales, PA) and LY2811376 (Eli Lilly and Company, Indianapolis, IN) reduce BACE1 activity in the cerebral cortex, but they interfere with structural synaptic plasticity (10). To determine whether NB-360 has similar impacts on dendritic spine plasticity, as well as whether SEZ6 is involved in the BACE1 inhibition-induced spine alterations, we imaged layer I dendritic tufts of cortical layer V pyramidal neurons in inhibitor-treated *Sez6*^{+/+}:*GFP-M* and *Sez6*^{-/-}:*GFP-M* mice using long-term in vivo two-photon microscopy (Figure 2A). After baseline recordings at days 0 and 7, BACE1 inhibitor was applied for 21 days (as highlighted in gray), whereas vehicle was administered over the whole experimental period. It is noteworthy that before treatment dendritic spine density in *Sez6*^{-/-}:*GFP-M* mice was reduced by 15.9 ± 9.4% compared with *Sez6*^{+/+}:*GFP-M* mice. In line with our previous study (10), BACE1 inhibitor

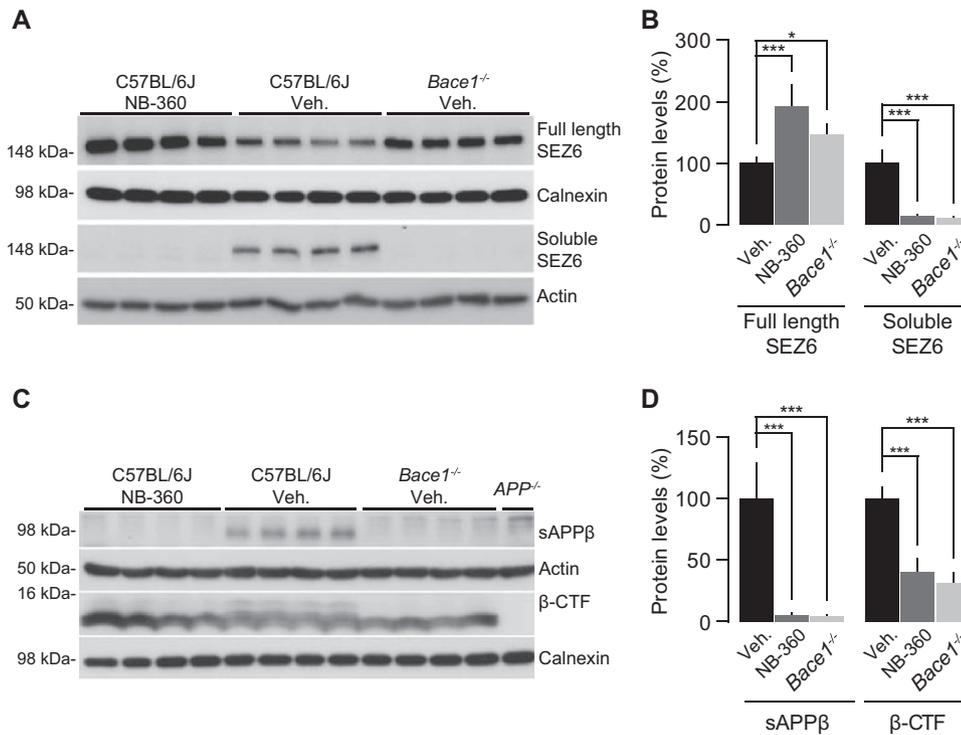


Figure 1. NB-360 is a potent beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibitor. **(A)** Membrane extracts and soluble fractions from NB-360 (supplied as food pellets, 250 mg/kg, for 21 days) or vehicle-treated C57BL/6J and *Bace1*^{-/-} mice whole-brain homogenates were probed for seizure protein 6 (SEZ6) by immunoblot. Actin and calnexin served as loading controls. **(B)** Quantitative analysis of panel **(A)**. **(C)** Same samples were probed for soluble APP beta (sAPPβ) and C-terminal fragment of APP (β-CTF). *APP*^{-/-} lane was used to validate antibody specificity. **(D)** Quantitative analysis of panel **(C)**. Animals per group: *n* = 4. One-way analysis of variance, full-length SEZ6: $F_{2,9} = 15.70$, $p < .01$; soluble SEZ6: $F_{2,9} = 67.86$, $p < .001$; sAPPβ: $F_{2,9} = 44.62$, $p < .001$; APP β-CTF: $F_{2,9} = 44.05$, $p < .001$; followed by Bonferroni's post hoc test, * $p < .05$, *** $p < .001$. Error bars represent SEM. APP, amyloid beta precursor protein; Veh., vehicle.

administration reduced total spine density in *Sez6*^{+/+}:*GFP-M* mice (Figure 2B, filled circles); however, NB-360 did not affect total spine density in *Sez6*^{-/-}:*GFP-M* mice (Figure 2B, open circles). Interestingly, by the last day of NB-360 administration, the total dendritic spine density in *Sez6*^{+/+}:*GFP-M* mice was reduced by 15.6 ± 8.9%, reaching a similar density as in *Sez6*^{-/-}:*GFP-M* mice (Figure 2B, upper). To emphasize the differential effects of inhibitor treatment in both groups, we normalized the total dendritic spine density to the pretreatment period of each mouse (Figure 2B, lower). To analyze the structural plasticity of dendritic spines, we quantified the density of the persistent spines (present for ≥ 7 days), newly gained spines, and lost spines. Inhibitor treatment reduced the densities of persistent spines and gained spines in *Sez6*^{+/+}:*GFP-M* mice. These effects were reversible and recovered shortly after withdrawal of the inhibitor (Figure 2C, D). In contrast, such effects were not detected in inhibitor-treated *Sez6*^{-/-}:*GFP-M* mice (Figure 2C, D). Similarly, BACE1 inhibition did not alter the density of lost spines in *Sez6*^{-/-}:*GFP-M* mice (Figure 2E). In conclusion, BACE1 inhibition altered structural postsynaptic plasticity in *Sez6*^{+/+}:*GFP-M* mice but not in *Sez6*^{-/-}:*GFP-M* mice. These data suggest that BACE1-mediated shedding of SEZ6 plays an important role in maintaining dendritic spine density under physiological conditions.

BACE1 Inhibition Does Not Affect Dendritic Spine Plasticity in Pyramidal Neurons Lacking SEZ6

To further investigate the impact of *Sez6* deletion on mature neurons, we crossed *Sez6*^{LoxP/LoxP} mice (K. Munro et al., Ph.D., unpublished data, May 2014) with *SlickV* mice (24). In *SlickV* mice, the tamoxifen-inducible recombinase CreER^{T2}

and eYFP are coexpressed in a small subset of cortical and hippocampal neurons. By applying tamoxifen to *Sez6*^{LoxP/LoxP}:*SlickV* mice, nuclear translocated CreER^{T2} removes *Sez6* exon 1, which is flanked by two LoxP sites (Supplemental Figure S2A). After 5 days of tamoxifen treatment, deletion of the *Sez6* occurs exclusively in eYFP/CreER^{T2}-positive neurons of these mice (*Sez6*^{cKO/cKO}:*SlickV*; Supplemental Figure S2B) (27). Because this cell-specific gene editing occurs in only a small subset of neurons, the majority of neurons, which are negative for eYFP and CreER^{T2}, are not affected (24,27). Therefore, any observed effects on eYFP/CreER^{T2}-positive neurons are mainly caused by the cell autonomous deletion of the *Sez6* gene.

We performed sequential imaging of layer I dendritic tufts of cerebral cortex layer V pyramidal neurons in 3-month-old *Sez6*^{LoxP/LoxP}:*SlickV* mice (Figure 3A). Tamoxifen (0.25 mg/g body weight in a 1:10 ethanol/corn oil mixture) was applied by oral gavage at imaging day 8 for 5 days (as highlighted in purple) to induce a conditional *Sez6* knockout (*Sez6*^{cKO/cKO}:*SlickV*). We have shown previously that tamoxifen treatment does not affect dendritic spine density (27). A small but significant decrease in dendritic spine density of cortical layer V pyramidal cells was observed in *Sez6* conditional knockout (cKO) neurons (Figure 3A, B). To assess whether this slight reduction in spine density also occurred in other brain regions, we analyzed apical and basal dendrites of hippocampal CA1 eYFP/CreER^{T2}-positive neurons by confocal microscopy (Figure 3C). Dendritic spine density of both apical and basal dendrites was reduced in *Sez6* cKO neurons in *Sez6*^{cKO/cKO}:*SlickV* mice compared with *Sez6*^{LoxP/LoxP}:*SlickV* mice (Figure 3D). Because this gene editing occurs in only a very small neuronal population, our observation suggests that loss

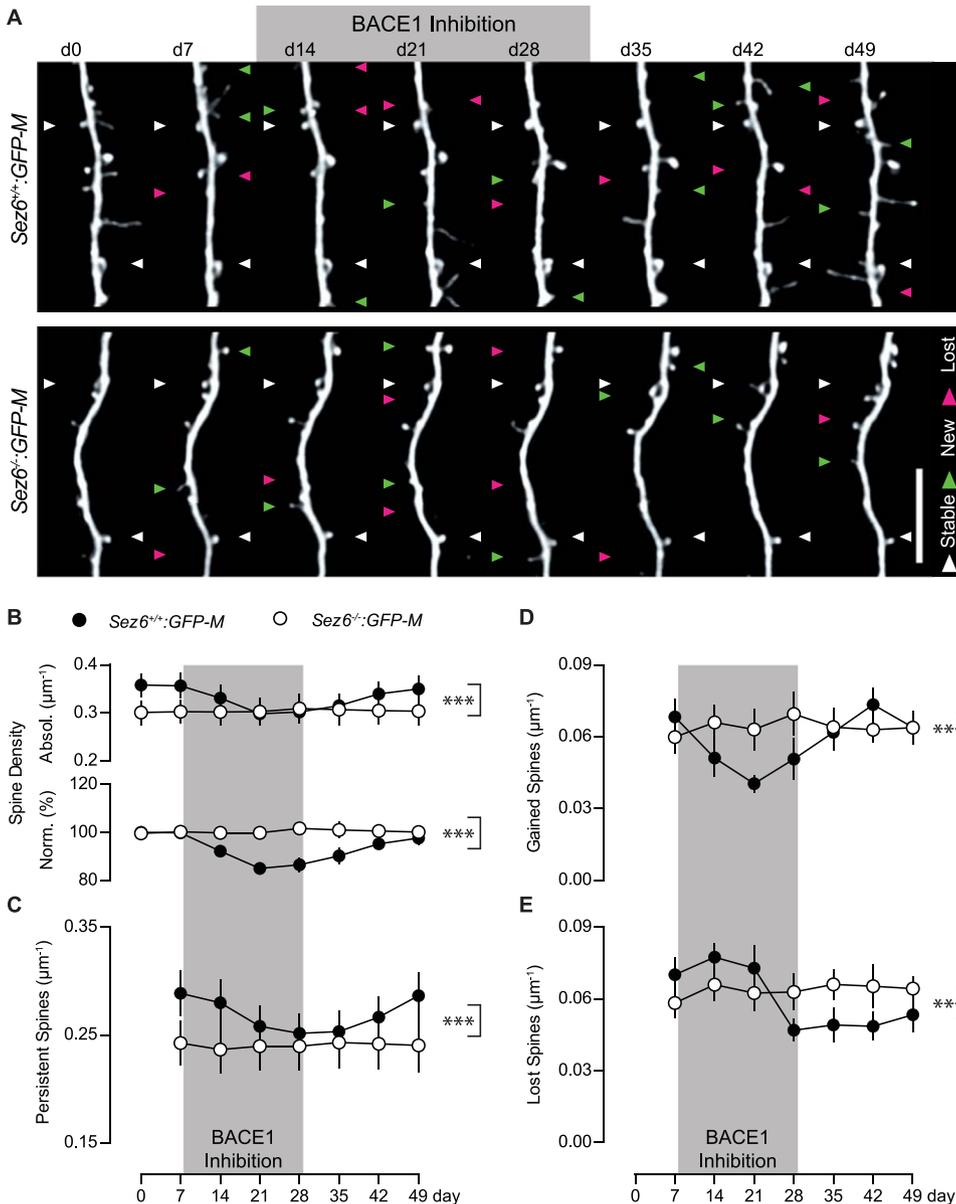


Figure 2. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibition does not alter structural synaptic plasticity in *Sez6*^{-/-} mice. **(A)** Time series micrographs of enhanced green fluorescent protein-labeled apical dendrites of layer 5 neurons in the cerebral cortex. The dendrites from *Sez6*^{+/+}:GFP and *Sez6*^{-/-}:GFP mice were repeatedly imaged over consecutive time points by in vivo two-photon microscopy. BACE1 inhibitor treatment started at day 8 and continued over 21 days (highlighted in gray). Vehicle was applied over the whole imaging period. White arrowheads mark persistent spines (present ≥ 7 days). Gained and lost spines are labeled with green and red arrowheads, respectively. Scale bar = 10 μm. **(B)** Quantitative analysis of the effect of BACE1 inhibition on spine density over time in *Sez6*^{+/+}:GFP and *Sez6*^{-/-}:GFP mice (top: genotype × days interaction two-way analysis of variance: $F_{7,77} = 15.16, p < .001$). The relative spine density is normalized to the average of the first two time points of each animal (bottom: interaction: $F_{7,77} = 12.28, p < .001$). **(C)** Persistent spines (interaction: $F_{6,66} = 13.75, p < .001$). **(D)** Gained spines (interaction: $F_{6,66} = 4.75, p < .001$). **(E)** Lost spines (interaction: $F_{6,66} = 6.74, p < .001$). Animals per group: $n = 6-7$. *** $p < .001$. Error bars represent SEM.

of SEZ6 induced dendritic spine reduction in a cell autonomous manner.

The observed lack of an NB-360 effect in *Sez6*^{-/-}:GFP-M mice could be the consequence of compensatory mechanisms taking place during development. To investigate this hypothesis, we imaged layer I dendritic tufts of cortical layer V pyramidal neurons in BACE1 inhibitor-treated adult *Sez6*^{cKO/cKO}:*SlickV* mice (Figure 4A). Conditional *Sez6* deletion was induced by treating 3-month-old *Sez6*^{LoxP/LoxP}:*SlickV* mice with tamoxifen for 5 days, followed by a 9-day interval before imaging commenced (as highlighted in purple). As a control, we used vehicle-treated *Sez6*^{LoxP/LoxP}:*SlickV* mice. NB-360 treatment reduced density of total and persistent dendritic spines in *Sez6* WT (*Sez6*^{LoxP/LoxP}:*SlickV*) mice, similar to inhibitor-treated *Sez6*^{+/+}:GFP-M mice. Both of these

parameters recovered shortly after withdrawing the inhibitor (Figure 4B, C, filled circles). However, BACE1 inhibition did not alter total and persistent spine density in *Sez6* cKO neurons of *Sez6*^{cKO/cKO}:*SlickV* mice (Figure 4B, C, open circles). Similar to *Sez6*^{-/-}:GFP-M mice, the density of gained and lost dendritic spines in *Sez6*^{cKO/cKO}:*SlickV* mice was not affected by NB-360 treatment (Figure 4D, E, open circles). In contrast, density of lost spines in *Sez6*^{LoxP/LoxP}:*SlickV* mice increased during NB-360 treatment. There was also a trend toward a reduced density of new gained spines, although it did not reach statistical significance ($p = .19$). Because *SlickV* mice have very sparse and weak eYFP labeling, the total number of analyzed dendrites is lower compared with GFP-M mice (2–6 vs. 8–10, respectively). Therefore, the statistical variation is high. Nevertheless, during NB-360 treatment both *Sez6*^{+/+}:

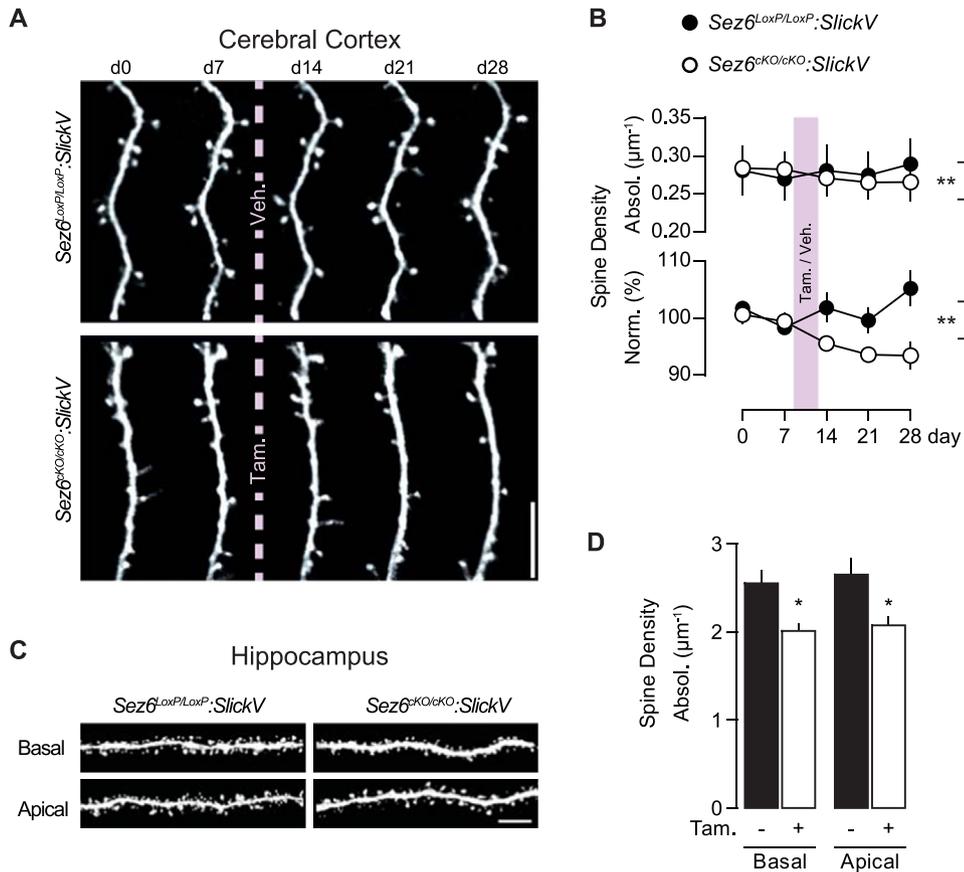


Figure 3. Knockout of *Sez6* reduces dendritic spine density in hippocampal and cortical neurons. **(A)** Time series micrographs of enhanced yellow fluorescent protein-labeled apical dendrites of layer 5 pyramidal neurons in the cerebral cortex. The dendrites from *Sez6^{LoxP/LoxP};SlickV* mice were repeatedly imaged over consecutive time points by in vivo two-photon microscopy. Tamoxifen or vehicle treatment started at day 8 and continued for 5 days (highlighted in pink). Scale bar = 10 μ m. **(B)** Quantitative analysis of the effect of *Sez6* knockout in mature cortical neurons (top: treatment \times days interaction two-way analysis of variance: $F_{4,40} = 4.21$, $p < .01$). The relative spine density was normalized to the average of the first two time points of each animal (bottom: interaction: $F_{4,40} = 4.69$, $p < .01$). Animals per group: $n = 6$. $**p < .01$. Error bars represent SEM. **(C)** Confocal micrographs of apical and basal dendrites of CA1 pyramidal neurons from tamoxifen (0.25 mg/g body weight) and vehicle-treated *Sez6^{LoxP/LoxP};SlickV* mice. Scale bar = 5 μ m. **(D)** Quantitative analysis. Animals per group: $n = 3$. Two-tailed Student's t test, $*p < .05$. Error bars represent SEM. Tam., tamoxifen; Veh., vehicle.

GFP-M and *Sez6^{LoxP/LoxP};SlickV* mice showed a reduced dendritic spine density, which is not altered in *Sez6^{-/-};GFP-M* and *Sez6^{cKO/cKO};SlickV* mice. These data further support the notion that SEZ6 mediates BACE1 inhibition-induced spine alterations in adult mice.

Inhibition of BACE1 Does Not Further Attenuate Long-term Potentiation in CA1 Neurons of *Sez6^{-/-}* Mice

Next, we investigated the role of SEZ6 in synaptic plasticity by analyzing high-frequency stimulation (HFS; 100 pulses/s)-induced hippocampal long-term potentiation (LTP) in *Sez6^{-/-}* mice and WT mice (Figure 5A, B). After 20 minutes of baseline recordings, the Schaffer collaterals were tetanized by HFS, followed by 60 minutes of continuous recording. HFS caused a pronounced posttetanic potentiation in vehicle-treated WT mice, whereas the magnitude of LTP in vehicle-treated *Sez6^{-/-}* mice was significantly reduced (Figure 5C).

To investigate whether NB-360 treatment would alter LTP in *Sez6^{-/-}* mice, we treated *Sez6^{-/-}* and WT mice with NB-360 for 21 days. On the last day of treatment, mice were sacrificed and acute hippocampal slices were prepared for field recordings. Our results showed that BACE1 inhibition did not further attenuate LTP in *Sez6^{-/-}* mice, whereas BACE1 inhibition caused a significant LTP reduction in WT mice.

In addition, to investigate whether the deficit in LTP induction in *Sez6^{-/-}* mice was caused by presynaptic

mechanisms, we monitored paired-pulse facilitation at different interstimulus intervals. The results showed no significant differences between WT and *Sez6^{-/-}* mice at interstimulus intervals of 35 and 50 ms. In addition, NB-360 treatment did not affect paired-pulse facilitation in either genotype (Figure 5D). Our findings revealed no obvious presynaptic alteration, implying that the LTP changes should be caused mainly by postsynaptic alterations.

DISCUSSION

As the most common form of senile dementia, AD is a great burden for patients, their families, society, and health care systems worldwide. To combat this devastating disease, several promising potential therapeutic approaches are currently being pursued: inhibiting A β production by targeting APP cleaving enzymes with small-molecule compounds (26,30,31), preventing A β aggregation (32), and enhancing clearance of A β or amyloid plaques by immunotherapy (33–35).

Currently, a lot of research effort is focused on BACE1, the rate-limiting enzyme in the amyloidogenic cascade (36). BACE1 activity can be blocked by small-molecule drugs (26,37,38). Further promising data are coming from mouse models; *Bace1^{-/-}* mice are viable and fertile (23), and when crossed with AD mouse models reduction of disease pathology is observed (39–41). However, the disappointing outcome of γ -secretase inhibitor trials calls for caution because

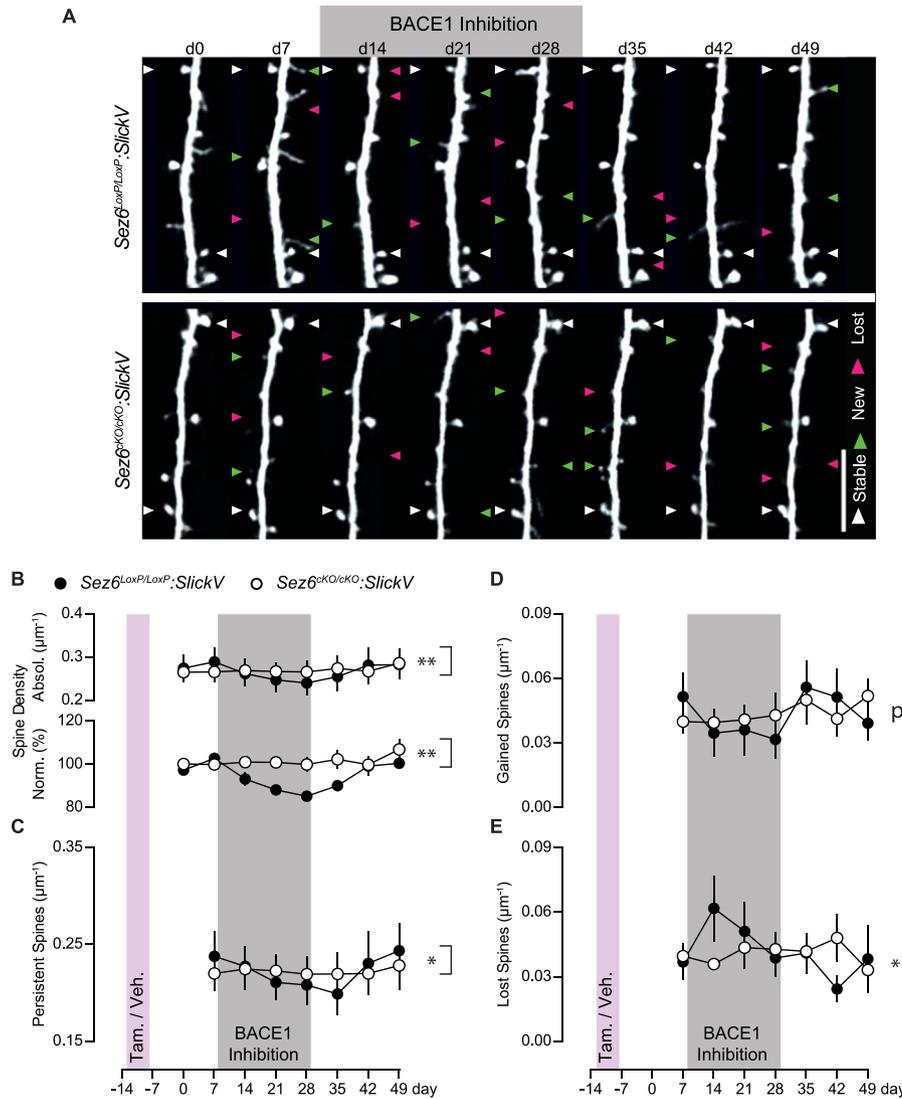


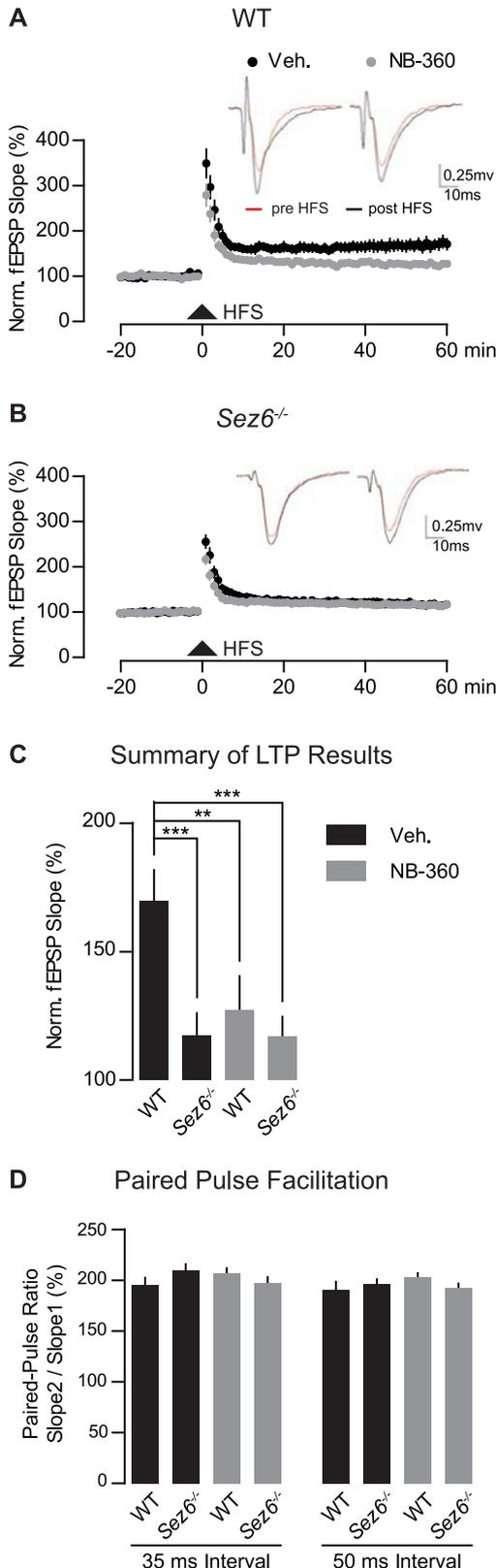
Figure 4. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibition does not alter structural synaptic plasticity in *Sez6* conditional knockout pyramidal neurons. **(A)** Time series micrographs of enhanced yellow fluorescent protein-labeled apical dendrites of layer 5 neurons in the cerebral cortex. Dendrites from *Sez6^{LoxP/LoxP};*SlickV** and *Sez6^{CKO/CKO};*SlickV** mice were repeatedly imaged over consecutive time points by in vivo two-photon microscopy. Tamoxifen was applied for 5 days, followed by a 9-day interval before imaging commenced (as highlighted in purple). BACE1 inhibitor treatment started at day 8 and was continued over 21 days (highlighted in gray). Scale bar = 10 μm. **(B)** Quantitative analysis of the effect of BACE1 inhibition on spine density over time in *Sez6^{LoxP/LoxP};*SlickV** and *Sez6^{CKO/CKO};*SlickV** mice (top: genotype × days interaction: $F_{7,70} = 3.58, p < .01$). The relative spine density was normalized to the average of the first two time points (bottom: interaction: $F_{6,60} = 4.16, p < .01$). **(C)** Persistent spines (interaction: $F_{6,60} = 2.71, p < .05$). **(D)** Gained spines (interaction: $F_{6,60} = 1.52, p = .19$). **(E)** Lost spines (interaction: $F_{6,60} = 2.73, p < .05$). Animals per group: $n = 6$. * $p < .05$, ** $p < .01$. Error bars represent SEM. Tam., tamoxifen; Veh., vehicle.

of on-target interference with Notch and other signaling pathways (42,43). Therefore, it is crucial to understand the physiological role of BACE1 and its substrates. This is underpinned by our recent in vivo study in which we reported that treatment with high-dose BACE1 inhibitor affects structural and functional synaptic plasticity in mice (10).

In our current study, we hypothesized that BACE1 inhibition-induced structural and functional synaptic alterations are caused by disruption of the SEZ6 function. This hypothesis is based on the fact that SEZ6 is predominantly and initially processed by BACE1 (15,16) and that *Sez6^{-/-}* mice display certain similar deficits comparable to BACE1-inhibited mice (10,20) and *Bace1* knockout mice (44). These deficits include reduced dendritic spine density, poor motor coordination, and diminished performance in hippocampal-dependent behavioral tests (17). We targeted BACE1 with NB-360, a novel inhibitor developed by Novartis (26), which almost completely blocked BACE1 activity in our study, similar to the previously reported high efficacy of BACE1 inhibitors SCH1682496 and

LY2811376 (10,37,45). To better understand the role of SEZ6 in BACE1 inhibition-induced effects, we used long-term in vivo two-photon microscopy and electrophysiological field recordings in NB-360-treated WT and *Sez6* knockout mice. Our main findings strongly support the role of SEZ6 in the BACE1 inhibition-induced synaptic alteration.

Treatment with NB-360 inhibitor did not induce spine density reduction in constitutive knockout (*Sez6^{-/-}*) mice. In contrast, in control WT mice, we could observe a reduction to the similar extent as in our previous study where we administered two other BACE1 inhibitors. Because three structurally different BACE1 inhibitors (NB-360, SCH1682496, and LY2811376) influenced spine plasticity in a similar manner, and because these inhibitor (SCH1682496 and LY2811376) effects were absent in treated *Bace1^{-/-}* mice (10), we are convinced that off-target effects are rather unlikely (46). One possibility is that the observed lack of an NB-360 effect in *Sez6^{-/-}* mice is masked by developmental deficits. Namely, it is known that *Sez6^{-/-}* mice have reduced spine density and



altered neurite branching during development (17). To rule out this possibility, we used conditional knockout *Sez6*^{cKO/cKO}:*SlickV* mice, in which *Sez6* deletion was induced in a small subset of eYFP/CreER^{T2}-positive neurons during adulthood. In eYFP/CreER^{T2}-positive neurons of *Sez6*^{cKO/cKO}:*SlickV* mice, dendritic spine density was lower, indicating that SEZ6 is not only critical for neuronal development but also important for maintaining the normal dendritic spine density in adult mice. However, the spine density reduction in *Sez6*^{cKO/cKO} neurons is smaller than the reduction seen in *Sez6*^{-/-}, which may be attributed to a general increase of dendritic spine stability during adulthood (47,48). Importantly, conditional knockout of *Sez6* in a subset of neurons also prevented NB-360-induced synaptic changes, further confirming the critical role of SEZ6 in dendritic spine plasticity.

Another advantage of the conditional knockout model is that only a small subset of eYFP/CreER^{T2}-positive *Sez6*^{cKO/cKO} neurons is exposed to a relatively normal extracellular environment (Figure 6); that is, the majority of neighboring neurons are not affected (27). NB-360 treatment should decrease sSEZ6 production in these unaffected neurons, lowering the extracellular concentration of the sSEZ6. Interestingly, according to our long-term in vivo imaging, such an altered environment did not change dendritic spine density in *Sez6*^{cKO/cKO} neurons. Taken together, these data suggest that flSEZ6 or SEZ6-CTF, each of which is absent from a subset of neurons after *Sez6* deletion, might be important for regulation of dendritic spine density (Figure 6). However, based on our measurements, we cannot exclude the possibility that sSEZ6 may still be involved in this process.

Can NB-360 treatment cause functional impairments in WT mice, and if so are these impairments mediated by SEZ6 protein? To gain insight into potential electrophysiological alterations, we performed hippocampal field recordings using acute brain slices from age-matched WT and *Sez6*^{-/-} mice treated for 21 days with NB-360. *Sez6*^{-/-} mice show attenuated Schaffer collateral-CA1 LTP, which is consistent with previous data showing defects in hippocampus-dependent memory (17). Long-term BACE1 inhibition does not attenuate the weakened LTP any further, indicating that SEZ6 is involved in BACE1 inhibition-induced reduction in functional synaptic plasticity observed in WT mice. Because hippocampal dendrites undergo spinogenesis after LTP induction (49,50), the

Figure 5. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibition has no effect on hippocampal long-term potentiation (LTP) in *Sez6* knockout mice. (A, B) NB-360 treatment attenuated LTP in wild-type (WT) brain slices (A) but not in *Sez6*^{-/-} brain slices (B). Representative traces of evoked field excitatory postsynaptic potential (fEPSP) acquired before (red lines) and after (black lines) high-frequency stimulation (HFS) of Schaffer collaterals are shown. (C) Summary graph of LTP magnitudes calculated 50 to 60 minutes after HFS from graphs in panels (A) and (B) (genotype × treatment interaction: two-way analysis of variance [ANOVA]: $F_{1,24} = 9.57$, $p < .01$, followed by Bonferroni's post hoc test: $**p < .01$, $***p < .001$). Animals per group: $n = 7$. (D) Paired-pulse ratio in hippocampal slices of BACE1 inhibitor-treated WT and *Sez6*^{-/-} mice (35-ms interval: two-way ANOVA interaction: $F_{1,23} = 3.753$, $p = .07$; 50-ms interval: two-way ANOVA interaction: $F_{1,23} = 1.917$, $p = .18$). Animals per group: $n = 5-8$. Error bars represent SEM. Veh., vehicle.

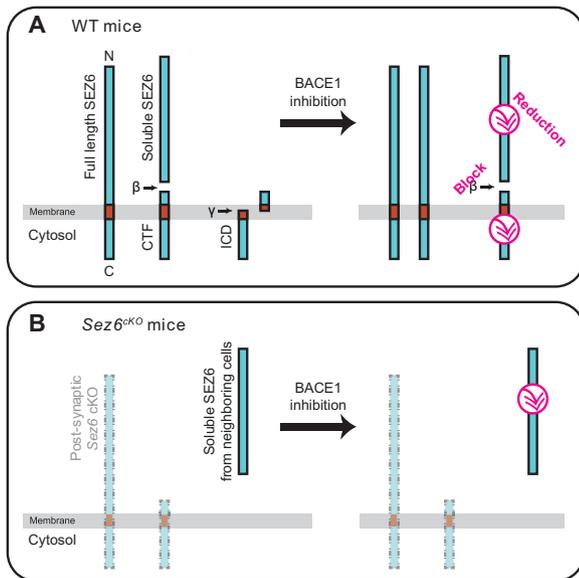


Figure 6. Schematic diagram of seizure protein 6 (SEZ6) proteolytic processing. **(A)** Under physiological conditions (left), beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) cleaves the type I transmembrane protein full-length SEZ6 (flSEZ6) to generate soluble SEZ6 (sSEZ6) and transmembrane C-terminal fragment (SEZ6-CTF). Subsequently, γ -secretase cleaves the SEZ6-CTF, thereby generating intracellular domain (ICD). During BACE1 inhibition (right), flSEZ6 accumulates while sSEZ6 and SEZ6-CTF levels decrease. **(B)** In conditional *Sez6^{CKO/CKO}* *SlickV* knockout mice (left), *Sez6* is deleted in a very small subset of neurons. Under this condition, the extracellular sSEZ6 is supplied by the majority of neighboring CreER^{T2}-negative neurons. BACE1 inhibition (right) sSEZ6 levels decrease as well.

observed LTP attenuation in *Sez6^{-/-}* mice may be a consequence of impaired structural dendritic spine plasticity. This is consistent with the overall decrease in spine density that we detected. In our experiments, LTP is induced by HFS. It was previously shown that *Sez6* messenger RNA levels are increased after strong neuronal activity (14). This increase in *Sez6* expression is dependent on *N*-methyl-D-aspartate receptor activation (51), which may imply that SEZ6 is functionally involved in LTP maintenance.

Because SEZ6 predominantly locates at the somatodendritic compartment (17), we hypothesized that SEZ6 is mainly involved in postsynaptic transmission. *Sez6* knockout did not affect paired-pulse facilitation in our study, supporting a postsynaptic role for SEZ6. Surprisingly, presynaptic deficits were also not observed in NB-360-treated WT mice, although BACE1 accumulates in presynaptic terminals (52,53) and *Bace1^{-/-}* mouse neurons display a presynaptic dysfunction at the mossy fiber terminals (54,55). This may be due to differences in the developmental trajectory of *Bace1* knockout-induced phenotypes compared with BACE1 inhibitor-treated adult mice. In addition, different results might have been obtained by studying different brain regions [stratum radiatum of CA1 in our study vs. mossy fiber terminals in another study (54)].

What other BACE1 substrates may be involved in the functional deficits we observed? Interestingly, recent studies reported that BACE1 inhibition results in a moderate increase of soluble APP alpha (sAPP α) (26) and A η - α , a novel proteolytic

fragment of APP (56). It was shown that sAPP α and A η - α have opposite effects on hippocampal LTP. Overexpression of sAPP α rescued LTP reduction in an AD mouse model (57), whereas application of A η - α -supplemented media attenuated hippocampal LTP in WT mice (56). However, we did not observe the LTP alteration in slices from NB-360-treated *Sez6^{-/-}* mice. Because both sAPP α and A η - α are present in our acute slice preparation, the opposing effects of these peptides could cancel out each other. Alternatively, because both peptides are extracellular soluble proteins, they might be washed out during the incubation period.

Finally, a profound understanding of BACE1 mechanisms of action and the physiological role of BACE1 substrates has additional important implications for clinical studies and potential therapies. A recent report described an endosomally targeted sterol-linked BACE1 inhibitor that inhibits A β production without affecting β -cleavage of neuregulin 1 and neural cell adhesion molecule L1 (58). Both APP and SEZ6 contain NPXY motifs for the endocytosis in their intracellular domains (59,60), indicating that SEZ6 also undergoes endocytosis (19) similar to APP (61). Therefore, this sterol-linked BACE1 inhibitor may also interfere with SEZ6 processing. This notion is important because then SEZ6 might serve as a useful indicator in preclinical testing of novel BACE1 inhibitors. By monitoring levels of SEZ6 and its proteolytic fragments, it would be possible to gain insight into BACE1 inhibitors' ability to reduce A β levels preferentially while preserving physiological functions of BACE1 substrates.

In conclusion, our findings confirm the disruptive effects of BACE1 inhibition on synaptic function and plasticity in WT mice. We also showed that SEZ6 is involved in dendritic spine dynamics and postsynaptic functions in mice. Finally, we showed that impaired processing of SEZ6 is one of the main reasons for the observed BACE1 inhibition-induced synaptic alterations in mice. Because a reduction in dendritic spine density is linked to cognitive decline, the clinical hallmark of AD (11), all treatments should be designed to avoid potential synaptic side effects. Fortunately, this BACE1 inhibition-induced synaptic interference can be potentially prevented by lowering the inhibitor dosage (10,46), supporting BACE1 inhibition as a valid therapeutic approach. Therefore, it is important to study potential BACE1 inhibition-induced synaptic alterations and the role of SEZ6 in healthy humans and AD patients. Finally, soluble SEZ6 is detectable in cerebrospinal fluid (62,63), suggesting that it might be considered as a biomarker to establish optimal dosage of BACE1 inhibitors to avoid potential treatment-induced synaptic impairments.

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KZ and JH designed this study. KZ performed all the experiments except Western blot, which was performed by XX under the supervision of CH. KZ, JMG, and JH interpreted the results. GR provided electrophysiological equipment and expertise. SC helped in electrophysiological recordings. JMG and SFL provided *Sez6^{-/-}* and *Sez6^{LoxP/LoxP}* mice. DRS and UN provided BACE1 inhibitor NB-360. MMD, PM, and SF wrote the animal

experiment proposal. KZ wrote the manuscript with major input from SF and PM as well as from all other authors.

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