Specific Inhibition of β-Secretase Processing of the Alzheimer Disease Amyloid Precursor Protein

Graphical Abstract

Highlights
- The AD-linked protease BACE1 cleaves APP to produce toxic β-amyloid peptides
- BACE1 also cleaves the non-amyloid substrates NRG1 and L1
- BACE1 cleavage of NRG1 and L1 is endocytosis-independent, unlike the cleavage of APP
- The endosomally targeted BACE1 inhibitor spares NRG1 and L1 but inhibits APP processing

Authors
Saoussen Ben Halima, Sabyashachi Mishra, K. Muruga Poopathi Raja, ..., Christian Haass, Amedeo Caflisch, Lawrence Rajendran

Correspondence
rajendran@bli.uzh.ch

In Brief
Ben Halima et al. demonstrate the feasibility of designing drugs targeting the Alzheimer-related enzyme BACE1 without affecting its physiological function. Using structural, biochemical, and cellular approaches, they show that BACE1 inhibitors can be designed to specifically inhibit its disease-causing activity, enhancing their potential as therapeutics without undesired side effects.
Specific Inhibition of β-Secretase Processing of the Alzheimer Disease Amyloid Precursor Protein

Saaoussen Ben Halima,1,2,3 Sabyashachi Mishra,4,12 K. Muruga Poopathi Raja,5 Michael Willem,6 Antonio Baici,4 Kai Simons,7 Oliver Brustle,6,9,10 Philipp Koch,8 Christian Haass,6,9,11 Amedeo Califisch,4 and Lawrence Rajendran1,2,3,*

1Systems and Cell Biology of Neurodegeneration, Institute of Regenerative Medicine, University of Zurich, Wagistrasse 12, 8092 Schlern, Switzerland
2Graduate Program in Neuroscience, Neuroscience Center Zurich, 8057 Zurich, Switzerland
3Graduate Program of the Zurich Center for Integrative Human Physiology, University of Zurich, 8057 Zurich, Switzerland
4Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland
5Department of Physical Chemistry, School of Chemistry, Madurai Kamaraj University, Tamil Nadu 625002, Madurai, India
6Biomedical Center, Ludwig-Maximilians-University, 81337 Munich, Germany
7Max Planck Institute of Molecular Cell Biology and Genetics, 01337 Dresden, Germany
8Institute of Reconstructive Neurobiology, University of Bonn, 53127 Bonn, Germany
9German Center for Neurodegenerative Diseases, 53175 Bonn, Germany
10Life & Brain, 53127 Bonn, Germany
11Munich Cluster for Systems Neurology (SyNergy), 81377 Munich, Germany
12Present address: Department of Chemistry, Indian Institute of Technology of Kharagpur, West Bengal 721302, Kharagpur, India
*Correspondence: rajendran@bli.uzh.ch http://dx.doi.org/10.1016/j.celrep.2016.01.076 This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

SUMMARY

Development of disease-modifying therapeutics is urgently needed for treating Alzheimer disease (AD). AD is characterized by toxic β-amyloid (Aβ) peptides produced by β- and γ-secretase-mediated cleavage of the amyloid precursor protein (APP). β-secretase inhibitors reduce Aβ levels, but mechanism-based side effects arise because they also inhibit β-cleavage of non-amyloid substrates like Neuregulin. We report that β-secretase has a higher affinity for Neuregulin than it does for APP. Kinetic studies demonstrate that the affinities and catalytic efficiencies of β-secretase are higher toward non-amyloid substrates like Neuregulin. We report that β-secretase has a higher affinity for Neuregulin than it does for APP. Kinetic studies demonstrate that the affinities and catalytic efficiencies of β-secretase are higher toward non-amyloid substrates than toward APP. We show that non-amyloid substrates are processed by β-secretase in an endocytosis-independent manner. Exploiting this compartmentalization of substrates, we specifically target the endosomal β-secretase by an endosomally targeted β-secretase inhibitor, which blocked cleavage of APP but not non-amyloid substrates in many cell systems, including induced pluripotent stem cell (iPSC)-derived neurons. β-secretase inhibitors can be designed to specifically inhibit the Alzheimer process, enhancing their potential as AD therapeutics without undesired side effects.

INTRODUCTION

Alzheimer disease (AD) is associated with extracellular deposits of β-amyloid (Aβ) peptide (De Strooper, 2010; Hardy and Higgins, 1992; Tanzi, 2005), which is generated by proteolytic processing of the amyloid precursor protein (APP) by the β-secretase BACE1 (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999) and γ-secretase. Mutations within the β-cleavage sites of APP can increase the risk for familial forms of AD (Citron et al., 1992; Thinakaran et al., 1996; Zhou et al., 2011) or confer protection against cognitive decline in the elderly (Jonson et al., 2012), causatively linking BACE1 to AD. Moreover, because of the failure in recent clinical trials of γ-secretase inhibition, mainly because of mechanism-based side effects in humans, BACE1 is considered the preferred drug target (Vassar and Kandalepas, 2011). BACE1 is an attractive therapeutic target for AD. However, complete abolishment of BACE1 activity is associated with specific behavioral and physiological alterations in mice (Cai et al., 2012; Cheret et al., 2013; Hitt et al., 2012; Kim et al., 2007; Lahiri et al., 2014; Li and Sudhof, 2004). These alterations may arise from the failure to process some non-amyloid BACE1 substrates such as NRG1, which is involved in axonal myelination of neurons (Hu et al., 2006, 2010; Ma et al., 2007; Willem et al., 2006); immunoglobulin (Ig)-containing β1 NgR1 (IgNgR1[β1]) (Cheret et al., 2013); the j2 subunit voltage-gated sodium channel (Nav1[2]) (Kim et al., 2007; Wong et al., 2005); and axon guidance molecules, including CHL1 (Rajapaksha et al., 2011) and L1 (Zhou et al., 2012), Jagged (Hu et al., 2013), β-galactoside ζ2, 6-sialytransferease (ST6Gal1) (Kitazume et al., 2005; Sugimoto et al., 2007), APLP1 and APLP2 (Li and Sudhof, 2004), lipoprotein receptor-related protein (LRP) (von Arnim et al., 2005), interleukin 1 receptor II (IL-1R2, but not tumor necrosis factor α [TNF-α] (Kuhn et al., 2007, 2012), and vascular endothelial growth factor receptor 1 (VEGFR1) (Cai et al., 2012). Therefore, a general BACE1 inhibitor might block cleavage of non-amyloid substrates, decreasing its value as an AD therapeutic drug (Cai et al., 2012; Cheret et al., 2013; Hitt et al., 2012; Kim et al., 2007; Lahiri et al., 2014; Li and Sudhof, 2004).
In the cell, proteins are distributed to multiple subcellular locations (Mellman and Nelson, 2008). Spatial distribution of proteins is crucial for spatial cellular functions (Rajendran and Simons, 2005). In polarized cells such as neurons, cellular distribution of proteins allows temporal and spatial control of diverse cellular functions. Many cellular proteins show multiple subcellular locations, such as BACE1. BACE1 is located in the trans-Golgi network (TGN) (Yan et al., 2001), plasma membrane (Walter et al., 2001a, 2001b), and early endosomes (Rajendran et al., 2006) and in polarized cells such as neurons because these cells also display compartmentalization of BACE1 in axons versus somatodendritic compartments (Buggia-Prévet et al., 2014; Vassar et al., 2014). BACE1 cleaves APP in early endosomes after endocytosis (Rajendran et al., 2006). Both the enzyme and the substrate undergo endocytosis, presumably through different routes, and meet in early endosomes for processing (Rajendran et al., 2006; Sannerud et al., 2011; Schneider et al., 2008). Currently it is unknown whether non-amyloid substrates such as NRG1 or L1 are processed in the endosomal compartment or other compartments. Selectively inhibiting the activity of BACE1 in a particular subcellular compartment, early endosomes, where it cleaves APP, could be an effective therapeutic strategy provided the other substrates are cleaved in non-endosomal compartments. Therefore, in this work, we explored the compartmentalization of BACE1 substrate processing to address the feasibility of targeting BACE1 for inhibition in the subcellular compartment, where it cleaves the APP substrate.

We studied the expression profiles of BACE1 substrates. Indeed, RT-PCR analysis of many BACE1 substrates, including APP and NRG1, revealed their expression throughout the mouse lifespan (Figure S1A), suggesting that general BACE1 inhibition could affect the processing of both amyloid and non-amyloid substrates at all stages. Whether an inhibitor could be developed to specifically target APP cleavage and, thereby, minimize non-specific side effects is currently unclear. We explored the possibility of specifically inhibiting B-cleavage of APP, but not that of non-amyloid substrates, by assessing its distinct structural, biochemical and, cellular requirements.

RESULTS

Molecular Dynamics Simulations Suggest that NRG1 Is a Better Substrate Than WT APP for BACE1

To characterize BACE1 interaction with non-amyloid versus amyloid substrates, we designed eight-residue P4-P4’ peptides as substrates based on the BACE1 binding regions from wild-type (WT) APP, NRG1, and P-selectin glycoprotein ligand 1 (PSGL1, [SELPGL1]) for structural and biochemical analyses (Figure S1B; Hu et al., 2006; Lichtenhainer et al., 2003; Willem et al., 2006). The corresponding peptide sequence derived from the Swedish mutant of the amyloid precursor protein (swAPP), a familial mutation that causes early-onset AD (Citron et al., 1992) and differs by only two residues from WT APP and binds BACE1 better, was used as a positive control (Hong et al., 2000). The corresponding substrate analog inhibitors were derived by replacing the scissile peptide bond with an isostere moiety, which renders the peptide bond non-cleavable.

To this end, we first performed explicit solvent molecular dynamics (MD) simulations of the BACE1(substrate complexes (Figures 1A and 1B; Figure S1C) and BACE1(substrate analog inhibitors (Figure S2A) based on the BACE1-OM99 inhibitor complex (Hong et al., 2000). The ensemble-averaged interaction energies and their electrostatic and van der Waals contributions were determined. We found that the overall plasticity of the BACE1 protein was essentially the same in all MD runs irrespective of the substrate to which it was bound (Figure S2). As anticipated, the interaction energy for BACE1 with swAPP was more favorable than with WT APP (Barman et al., 2011; Hong et al., 2000; Figure 1B).

Unexpectedly, MD simulations indicated that BACE1 interacts more favorably with the non-amyloid substrate NRG1 than with either amyloid substrate (Figure 1B). Furthermore, a higher affinity of BACE1 for NRG1 than WT APP and swAPP was also observed in MD simulations carried out with the substrate analog inhibitors (Figure S2). The range of values of the electrostatic contribution for the four different substrates was about an order of magnitude larger than the van der Waals range (400 kcal/mol versus 40 kcal/mol, respectively) and therefore dominated the variation in the total interaction energy (which is the sum of these two terms). The individual contributions of each of the P4-P4’ residues of the substrates showed that the Glu residues at (P2) and (P2’) of NRG1 are involved in more favorable interactions with BACE1 than the corresponding residues in WT APP and PSGL1 (Figures 1A and 1C; Figure S1C). We observed that this could be due to salt bridge interactions of the P2 and P2’ Glu residues in NRG1 with Arg235 and Arg128 of BACE1, respectively (Figure 1A). This observation suggested that the replacement of the acidic Glu to a basic amino acid in the substrate would be unfavorable because this will inhibit the formation of the electrostatic interaction between the substrate and the enzyme. Indeed, in WT APP, a Lys residue is present at the (P2) position, which creates an unfavorable interaction with BACE1, presumably because of the proximity of the side chain of Arg235 (Figure 1A).

To ascertain the individual contributions of the P2 and P2’ Glu side chains of NRG1, simulations were carried out with mutants containing Lys instead of Glu at both sites or only at (P2). The interaction energy of the single (P2) mutant was weakened almost as much as for the double mutant, which suggests that the Glu at (P2) site alone contributes significantly to the interaction with BACE1 and much more than the Glu at (P2’) (Figure 1C). Further, we replaced the Lys at (P2) in WT APP with Glu and found that this substitution conferred a higher affinity for BACE1 (Figure 1B). Therefore, position (P2) strongly affects the substrate affinity for BACE1 through the formation of favorable (NRG1) or unfavorable (WT APP) interactions (Figure 1C). These results also revealed additional information: it is the presence of the (P2) Lys that renders WT APP a weaker substrate rather than the acquisition of the dipeptide segment Asn-Leu at (P2)-(P1), which makes the Swedish mutant of APP a better substrate (Figure 1C; Figure S2; Barman et al., 2011; Hong et al., 2000). Interestingly, another non-amyloid substrate, L1, also harbors a similar acidic amino acid (Glu) and may potentially interact with the Arg235 in BACE1 (Zhou et al., 2012).

Therefore, MD simulations of BACE1 binding to amyloid as well as the non-amyloid substrate NRG1 uncovered the
importance of the critical acidic residue (P2) Glu of NRG1 in conferring a stable interaction with BACE1.

In Vitro Fluorescence Experiments Demonstrate that NRG1 Is a Better Substrate Than APP

To experimentally test the predictions of MD simulations and to evaluate the potency of amyloid and non-amyloid substrate analog inhibitors, we used the cell-free fluorescence resonance energy transfer (FRET)-based BACE1 activity assay (Ermolieff et al., 2000). We quantified the effect of the different substrate analog inhibitors on BACE1 cleavage of a fluorophore quencher-labeled, WT-APP-based peptide FRET reporter (Figure 2A; Figure S3). As a positive control for inhibition, we used the BACE1 inhibitor C3. We found that the NRG1-derived inhibitor reduced BACE1 cleavage with comparable efficiency to that of an swAPP-derived inhibitor (Figure 2A), confirming the simulation predictions. In contrast, the WT APP-derived inhibitor was less effective at reducing BACE1 activity, and the PSGL1-based inhibitor resembled that of control condition with no inhibition of the fluorescence readouts (Figure 2A). Similar results were obtained with a fluorophore quencher-labeled, NRG1-based peptide FRET reporter (Figure 2B). Therefore, at least in vitro, BACE1 binds NRG1 with a higher affinity than WT APP.

The (P2) Glu Residue in the Binding Site of NRG1 Confers High-Affinity Binding to BACE1

To understand whether BACE1 not only binds but also cleaves NRG1 better than WT APP, we determined the hydrolytic

Figure 1. Molecular Dynamics Simulation Predicts that NRG1 Is a Better Substrate Than WT APP for BACE1
(A) The active site of BACE1 with the octapeptide substrates of WT APP, NRG1, and swAPP. The snapshot for each BACE1-substrate complex shown is the representative structure of the most populated conformer, which was obtained by clustering all MD snapshots by root-mean-square deviation and a cutoff of 0.8 Å. All Cα atoms of BACE1, except for the loops A, C, D, and F, were used in the structural fitting prior to the clustering. The flap is shown as a ribbon and the side chains of BACE1 and substrate as sticks. The carbon atoms of substrate are shown in magenta for clarity.
(B) Time series of interaction energy between BACE1 and the four substrates (swAPP, WT APP, NRG1, and PSGL1 are shown in black, red, green, and blue, respectively) and the K(P2)E mutant of WT APP (maroon). The order of the stabilizing interaction was as follows: NRG1 > WT APP K(P2)E mutant > swAPP > WT APP > PSGL1.
(C) Time series of the interaction of BACE1 with NRG1 and its three mutants. The stabilizing interaction by P2 (Glu) is more significant than that of P2’ (Glu). The order of the stabilizing interaction was as follows: NRG1 (black) > NRG1 G(P4)E (red) > NRG1 E(P2)K (green) > NRG1 E(P2K) E(P2K) (blue).
See also Figures S1, S2, and S3.
A. wtAPP-based FRET reporter

- S+E
- S+E+C3
- S+E+swAPP inhibitor
- S+E+NRG1 inhibitor
- S+E+wtAPP inhibitor
- S+E+SELPLG inhibitor

B. NRG1-based FRET reporter

- S+E
- S+E+C3
- S+E+swAPP inhibitor
- S+E+NRG1 inhibitor
- S+E+wtAPP inhibitor
- S+E+SELPLG inhibitor

C. Enzyme Kinetics

<table>
<thead>
<tr>
<th></th>
<th>V (μM s⁻¹)</th>
<th>Kₘ (μM)</th>
<th>Kᵥₐt (S⁻¹)</th>
<th>Kᵥₐt/Kₘ (M⁻¹ S⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>(5.0 ± 0.1) x 10⁻⁴</td>
<td>36 ± 3.6</td>
<td>(2.08 ± 0.04) x 10⁻⁷</td>
<td>57.8 ± 5.9</td>
</tr>
<tr>
<td>swAPP</td>
<td>(6.0 ± 0.5) x 10⁻³</td>
<td>5.2 ± 1.2</td>
<td>(2.5 ± 0.2) x 10⁻²</td>
<td>4800 ± 1200</td>
</tr>
<tr>
<td>NRG1</td>
<td>(2.7 ± 0.1) x 10⁻³</td>
<td>19.6 ± 2.8</td>
<td>(1.12 ± 0.04) x 10⁻⁷</td>
<td>570 ± 80</td>
</tr>
</tbody>
</table>

D. wtNRG1

- E + wtNRG1
- E + wtNRG1 + C3

E. wtAPP

- E + wtAPP
- E + wtAPP + C3

F. Aβ

- Normalized RFU values (normalized by cell viability and μg of protein)

G. NRG1 mutants

- NRG1-FL
- NRG1-CTFs

H. L1/ mutant L1/ wtAPP-based FRET reporters

- E = wtL1
- E = wtL1 + C3
- E = wtL1E1087K
- E = wtL1E1087K + C3
- E = wtAPP
- E = wtAPP + C3

(legend on next page)
efficiency of BACE1 for the amyloid and non-amyloid substrates and used swAPP as a positive control. We calculated $K_M$ and $k_{cat}$ values to determine the affinity and cleavage efficiencies, respectively, with specificity defined by $k_{cat}/K_M$ values. The affinity of BACE1 for NRG1 was twice that for WT APP ($K_M$ of 19.6 ± 2.8 μM and 36.6 ± 3.6 μM, respectively). As predicted, swAPP displayed a low $K_M$ value of 5.2 ± 1.2 μM. BACE1 cleaved WT APP with the lowest specificity, represented by a $k_{cat}/K_M$ value of 57.8 ± 5.9 M$^{-1}$ s$^{-1}$, whereas it cleaved NRG1 with a specificity of 570 ± 80 M$^{-1}$ s$^{-1}$, which is 10-fold higher than WT APP. In contrast, swAPP was cleaved with the highest specificity of 4,800 ± 1,200 M$^{-1}$ s$^{-1}$ (Figure 2C; Figure S4). Finally, compared with WT APP, the catalytic efficiency of BACE1 was approximately five times higher for NRG1 and ten times higher for swAPP. Therefore, NRG1 is a better substrate for BACE1 than WT APP.

Because MD simulations suggested that NRG1 binds BACE1 with higher affinity, and because this is most likely due to the (P2) Glu residue in NRG1, we wanted to verify this experimentally. We replaced the (P2) Glu residue of NRG1 with a Lys residue (NRG1 Glu residue in NRG1, we wanted to verify this experimentally. We replaced the (P2) Glu residue of NRG1 with a Lys residue (NRG1 Glu residue in NRG1 with the basic residue Lys but not with another acidic residue, Asp, dramatically decreased its cleavage by BACE1 in cells (Figure 2G). C3 inhibited processing of all mutants (Figure 2G), demonstrating that their cleavages are BACE-dependent. Therefore, these data unequivocally demonstrate that the Glu residue in the cleavage site of NRG1 plays a critical role in the affinity and cleavage efficiency of BACE1 both in vitro and in cells.

### BACE1 Displays Higher Cleavage Efficiencies toward Another Non-amyloid Substrate, L1

The non-amyloid substrate of BACE1, L1, also has a Glu residue in the BACE1 cleavage site (Zhou et al., 2012) and, therefore could confer a higher affinity to BACE1 binding and cleavage efficiency. To test this, we evaluated the BACE1 cleavage kinetics of L1 with and without the critical Glu residue mutated (Figure 2H). Indeed, similar to NRG1, wild-type L1 was processed with higher cleavage efficiency than WT APP (Figure 2H). Interestingly, mutating the Glu residue to Lys nearly abolished the cleavage by BACE1 (Figure 2H). As expected, the cell-permeable inhibitor C3 abolished BACE1 cleavage of all substrates. Therefore, BACE1 has a higher affinity and catalytic efficiency toward the non-amyloid substrates such as NRG1 and L1 than toward WT APP.

### β-Cleavage of the Non-amyloid Substrates NRG1 and L1 Does Not Require Endocytosis

Despite being a poor substrate (Grüninger-Leitch et al., 2002; Sauder et al., 2000), and even in the presence of the higher-affinity substrates, APP is cleaved in the cellular context and also in vivo. We hypothesized that BACE1 cleaves different substrates in distinct subcellular compartments. For instance, previous work has shown that swAPP, which has a higher affinity for BACE1, is cleaved by β-secretase in the biosynthetic compartments (Haass et al., 1995; Thinakaran et al., 1996), whereas...
cleavage of WT APP occurs in endosomes (Carey et al., 2005; Koo and Squazzo, 1994; Rajendran and Annaert, 2012; Rajendran et al., 2006; Udayar et al., 2013). Because our findings show that NRG1 also has a higher affinity for BACE1 than WT APP, we hypothesized that NRG1 might also be processed independently of endocytosis. To determine whether β-cleavage of NRG1 is similar or different to that of WT APP in terms of its requirement of endocytosis, we perturbed membrane trafficking pathways and assessed the β-cleavage of NRG1. Expression of dominant-negative dynamin (Dyn K44A), a mutant of the GTPase involved in fission of the endocytic vesicles that inhibits dynamin-dependent endocytosis, did not inhibit the cleavage of NRG1 (Figure 3A). Similarly, pharmacological inhibition of clathrin-dependent/clathrin-independent endocytosis using Pitstop2 (Dutta et al., 2012; Stahlschmidt et al., 2014; von Kleist et al., 2011), an inhibitor that inhibits clathrin-mediated endocytosis and clathrin-independent endocytosis, did not inhibit the cleavage of NRG1 (Figure 3A). However, both treatments significantly inhibited β-cleavage of APP and Aβ production (Figure 3B). Pitstop2 had a more pronounced effect on Aβ than secreted APPβ (sAPPβ), probably because of the inhibition of γ-secretase internalization as well as inhibition of BACE1 endocytosis. Control experiments showed that, indeed, treatment of cells with Pitstop2 (Figure 3C) or cells expressing Dynamin K44A inhibited endocytosis of Transferrin (Figures 3C and 3D) and epidermal growth factor (EGF) (Figure 3C), two clathrin/dynamin-dependent cargoes. Therefore, unlike APP, NRG1 does not require endocytosis for cleavage by BACE1.

Consistent with these observations and similar to NRG1, L1 was also cleaved by BACE1 in an endocytosis-independent manner (Figure 3E) suggesting that unlike APP, the other physiologically relevant substrates may not require dynamin/clathrin endocytosis for their β-cleavages and providing further support to the idea that BACE1 cleaves these higher affinity substrates in an endocytosis-independent manner.

The Endosomally Targeted, Sterol-Linked BACE1 Inhibitor Inhibits β-Cleavage of NRG1 and L1

Exploiting the observation that different membrane trafficking pathways differentially regulate β-cleavage of APP and NRG1 or L1, we tested whether inhibiting BACE1 activity specifically in the endosomal compartment would inhibit β-cleavage of APP and, thereby, spare the cleavage of the non-amyloid substrates NRG1 and L1. We found that the cell-permeable pan inhibitor C3 substantially inhibited β-cleavage of NRG1 in cells, whereas an endosomally targeted, sterol-linked BACE1 inhibitor (Rajendran et al., 2008) showed no significant inhibition of NRG1 β-cleavage (Figures 4A and 4B). However, both treatments inhibited β-cleavage of WT APP and Aβ production (Figure 4C). Similar results were obtained in primary mouse neuronal cultures, where an endosomally targeted, sterol-linked inhibitor of β-secretase spared cleavage of NRG1 but not of APP (Figure S5) under an endogenous BACE1 expression level.

Similar to NRG1, we then tested whether BACE1-mediated cleavage of L1, which also did not require endocytosis, could be spared by using the endosomally targeted, sterol-linked BACE1 inhibitor. Treatment with the endosomally targeted, sterol-linked BACE1 inhibitor did not affect β-cleavage of L1, whereas treatment with the general cell-permeable BACE1 inhibitor C3 abolished almost all BACE1 processing (Figures 4D and 4E). In control experiments performed with APP, β-cleavage of WT APP was inhibited completely by both the endosomally targeted, sterol-linked BACE1 inhibitor and the general cell-permeable inhibitor (Figure 4C).

The Endosomally Targeted, Sterol-Linked BACE1 Inhibitor Inhibits NRG1 Processing When Subcellular Compartmentalization Is Compromised

To test whether the endosomally targeted, sterol-linked BACE1 inhibitor can indeed inhibit NRG1 processing when the integrity of subcellular compartmentalization is compromised, we tested the effect of both BACE1 inhibitors: the endosomally targeted, sterol-linked inhibitor and the cell-permeable pan inhibitor C3 on solubilized membranes and assessed inhibition of NRG1 cleavage. Under these conditions, where cellular compartmentalization is compromised, both the endosomally targeted, sterol-linked BACE1 inhibitor and C3 significantly blocked BACE1 cleavage of NRG1 (Figure 5A). These results suggest that subcellular compartmentalization of different substrates in endosomal and non-endosomal compartments contributes to the differential processing of these substrates by BACE1.

Among the non-endosomal compartments that harbor β-secretase activity, the TGN and the plasma membrane have been suggested to be places for β-secretase activity (Li and Südhof, 2004; Prabhu et al., 2012). To investigate whether the processing of NRG1 by BACE1 occurs at the cell surface, we used a cell-impermeable BACE1 inhibitor, GL189 (Capell et al., 2002), and tested its inhibitory potential on BACE1 cleavage of NRG1. This inhibitor is a substrate analog transition state inhibitor that has been demonstrated to bind to active BACE1 in solubilized membrane fraction assays (Capell et al., 2002). Therefore, we hypothesized that, if active BACE1 is present at the cell surface that is competent to cleave NRG1, then this cell-impermeable transition state inhibitor should inhibit BACE1 cleavage of NRG1. However, treatment of cells expressing NRG1 with GL189 did not inhibit the processing of NRG1 by BACE1 (Figure 5B). However, the control C3, the cell-permeable BACE1 inhibitor, almost abolished the processing of NRG1 by BACE1 (Figure 5B). Similarly, endocytosis inhibition also did not inhibit BACE1 processing of NRG1. However, addition of C3 under endocytosis inhibition inhibits NRG1 cleavage, demonstrating that NRG1 cleavage occurs neither at the plasma membrane nor in endosomes (Figure 5B). As additional controls, we also checked whether BACE1 cleavage of APP was affected under similar conditions and found that treatment of cells with the cell-impermeable inhibitor GL-189 had no effect on β-cleavage of APP. However, treatment with the endocytosis inhibitor or cell-permeable inhibitor or both inhibited β-cleavage, showing conclusively that β-cleavage of APP does not occur at the plasma membrane but is dependent on endocytosis. Taken together, these results indicate that BACE1 cleaves NRG1 neither at the plasma membrane nor in endosomes but, most likely, at the TGN, the only other low-pH, non-endo-lysosomal organelle that is conducive for BACE1 activity.
Figure 3. β-Cleavage of the Non-amyloid Substrates NRG1 and L1 Does Not Require Endocytosis

(A) β-Cleavage of NRG1 is independent of dynamin/clathrin-mediated endocytosis. HEK293 stably expressing BACE1 cells were co-transfected with either NRG1β1 type III and GFP or NRG1β1 type III and dynamin dominant-negative mutant (Dyn K44A) or transfected with NRG1β1 type III and GFP and treated with Pitstop2. Western blot analysis of lysates to detect full-length (FL) NRG1 with Sc-348 antibody and NRG1-β-CTF using the neo-epitope antibody 4F10. Note the stabilization of NRG1 full-length and NRG1-β-CTF upon Dyn K44A co-transfection.

(B) HEK293 stably expressing BACE1 cells were co-transfected with DynK44A and WT APP or transfected with WT APP and treated with Pitstop2, Aβ40, and sAPPβ were measured from cell media using an ECL assay (Bali et al., 2012), with p values of 0.006 for DynK44A (Aβ40) and 0.002 for Pitstop2 (Aβ40).

(C) Treatment of cells with clathrin/dynamin inhibitors inhibits endocytosis of Transferrin and EGF. HeLa-GalT-GFP cells were treated with the solvent control (DMSO) or with Pitstop2 and incubated with Transferrin (red) and EGF (magenta). Scale bars, 10 μm. Confocal Leica SP8, 63× 1.5 zoom.

(D) Dynamin K44A inhibits Transferrin endocytosis. HeLa cells were transfected with either control GFP (green, top) or Dynamin K44A GFP (bottom) and incubated with Transferrin (red).

(E) L1 β-cleavage is independent of dynamin/clathrin-mediated endocytosis. HEK293 stably expressing BACE1 were co-transfected with either L1 and GFP or L1 and Dyn K44A or transfected with L1 and GFP and treated with Pitstop2. Western blot analysis of the lysate detects full-length L1 and L1-β-CTF with PcytL1 antibody. Again, note the stabilization of L1 full-length and L1-β-CTF upon Dyn K44A co-transfection, similar to NRG1.
The Endosomally Targeted, Sterol-Linked BACE1 Inhibitor Inhibits Aβ Production and Cleavage of APP without Affecting Cleavage of NRG1 and L1

(A) The endosomally targeted, sterol-linked inhibitor spares NRG1 processing in cells. HEK293 stably expressing BACE1 cells were transfected with NRG1 (t type III and treated with DMSO as a control, with the cell-permeable BACE1 inhibitor C3, or with the endosomally targeted, sterol-linked BACE1 inhibitor. Western blot analysis of lysates detects full-length NRG1 with Sc-348 antibody and NRG1-[β]-CTF using the neo-epitope antibody 4F10. A representative western blot is shown.

(B) Semiquantification of western blots. Data were mean ± S.E. (n = 3). Student’s t test was used to calculate p values: C3-FL, 0.427; C3-[β]-CTF ([β]-cleaved C-terminal fragment), 0.0075; endosomally targeted, sterol-linked BACE1 inhibitor-FL, 0.882; endosomally targeted, sterol-linked BACE1 inhibitor-[β]-CTF, 0.488.

(C) The endosomally targeted, sterol-linked BACE1 inhibitor inhibited APP processing. HEK293 stably expressing BACE1 were transfected with WT APP and treated with DMSO as a control, with the cell-permeable BACE1 inhibitor C3, or with the endosomally targeted, sterol-linked BACE1 inhibitor for 12 hr. Aβ40 and sAPPβ were measured from cell media using an ECL assay. The values represent experimental triplicates. p values for the sterol-linked inhibitor for sAPPβ and Aβ40 are 0.0009 and 0.0021, respectively. Error bars indicate SD.

(D) The endosomally targeted, sterol-linked inhibitor spares L1 processing in cells. HEK293 stably expressing BACE1 cells were transfected with L1 and treated with DMSO as a control, with the cell-permeable BACE1 inhibitor C3, or with the endosomally targeted, sterol-linked BACE1 inhibitor. Western blot analysis of lysates with PcytL1 antibody detects full-length L1 and L1-[β]-CTF.

(E) Semiquantification of western blots. Data were mean ± SE (n = 3). Student’s t test was used to calculate the p values: C3-FL, 0.0169; C3-[β]-CTF, 0.0022; endosomally targeted, sterol-linked BACE1 inhibitor-FL, 0.4975; endosomally targeted, sterol-linked BACE1 inhibitor-[β]-CTF, 0.187.

The Endosomally Targeted, Sterol-Linked BACE1 Inhibitor Inhibits APP Processing without Affecting NRG1 Processing in iPSC-Derived Human Neurons

To validate the findings regarding the endosomally targeted, sterol-linked BACE1 inhibitor sparing NRG1 cleavage in a more relevant setting for potential treatment in AD patients, we treated neurons generated from human induced pluripotent stem cells (h-iPSCs) derived from healthy human donors (Figure 6A; Figure S6) with C3, the cell-permeable BACE1 inhibitor, or the endosomally targeted, sterol-linked BACE1 inhibitor and assayed for BACE1 processing of NRG1 and APP (as a control) in those cells. C3 BACE1 inhibitor treatment inhibited BACE1 processing of NRG1 (Figures 6B and 6C). Treatment with the endosomally targeted, sterol linked BACE1 inhibitor did not affect the processing of NRG1 by BACE1 (Figures 6B and 6C). However, upon C3 BACE1 inhibitor and endosomally targeted, sterol-linked inhibitor treatment, both Aβ40 and sAPPβ were reduced dramatically, with sAPPα levels increased (Figure 6D).

The Endosomally Targeted, Sterol-Linked BACE1 Inhibitor Is Targeted to Endocytic Compartments but Not to the Golgi

Consistent with these results, the fluorescently labeled, sterol-linked inhibitor trafficked to endosomal compartments (Figures 7A and 7B) but not to the trans-Golgi network, where high-affinity substrates could be cleaved (Haass et al., 1995; Thinakaran et al., 1996), as judged by the colocalization experiments (Figure 7C). When incubated on cells, the fluorescently labeled, sterol-linked inhibitor trafficked from the plasma membrane to EEA1-positive early endosomes (Figure 7A), the compartments shown previously to be important for β-cleavage of APP, and then was transported retrogradely to late endosomal/lysosomal compartments at longer periods of incubation, as demonstrated by colocalization with Lamp1 (Figure 7B). The endosomally targeted, sterol-linked inhibitor did not traffic to the TGN 30 min to 1 hr after uptake, as shown by the absence of localization between the inhibitor and GalT-GFP, a Golgi-resident protein.
Figure 5. The Endosomally Targeted, Sterol-Linked BACE1 Inhibitor Inhibits β-Cleavage of NRG1 When Subcellular Compartmentalization Is Compromised and β-Cleavage of NRG1 Does Not Occur at the Cell Surface

A) BACE1 activity and inhibition by C3, a cell permeable BACE inhibitor, and the endosomally targeted, sterol-linked BACE1 inhibitor in solubilized membranes. Shown is a western blot analysis of solubilized membrane from HEK293 cells stably expressing BACE1 and transfected with NRG1 β1 type III. The membrane BACE1 activity assay was carried out at 37°C and 0°C for the control. The cell-permeable C3 inhibitor and the endosomally targeted, sterol-linked BACE1 inhibitor were used to inhibit BACE1 activity toward NRG1 at 37°C. DMSO is used as a solvent control. Full-length and NRG1-CTFs are detected with Sc-348 antibody. NRG1-β-CTF is detected by 4F10 antibody (neo-epitope-specific antibody).

(B) Western blot analysis of cell expressing NRG1 β1 type III and treated with DMSO as a control, the cell-permeable BACE1 inhibitor C3, the cell impermeable transition state BACE1 inhibitor GL189, and the endocytosis inhibitor Pitstop2 or co-treated with Pitstop2 and C3. Full-length (NRG1-FL) and NRG1-CTFs are detected with Sc-348 antibody. NRG1-β-CTF is detected by 4F10 antibody (neo-epitope-specific antibody).

(C) β-Cleavage of APP does not occur at the cell surface. Supernatants of cells expressing APP were subjected to an ECL assay to measure sAPPβ to determine the effect of cell-permeable (C3), cell impermeable (GL-189), and endocytosis inhibitor (Pitstop2) on the β-cleavage of APP. Values were normalized to cell viability and to DMSO-treated cells.

(D) In vitro BACE1 FRET assay to assess the inhibitory potential of the cell non-permeable BACE1 inhibitor GL189 (red), the cell-permeable BACE1 inhibitor C3 (green), and DMSO (blue) as the control reaction. The y axis displays the relative fluorescence units, and the x axis displays time in seconds. The reaction is depicted from time = 200 s.

(Figure 7C). As a control, Shiga toxin, a TGN-dedicated cargo, promptly colocalized with GaIT-GFP (Figure 7D). This shows that the endosomally targeted, sterol-linked inhibitor confines itself to the endocytic pathway that is involved in Aβ production. Therefore, an endosomally targeted, sterol-linked BACE1 inhibitor specifically inhibited APP cleavage, which predominantly occurs in endosomal compartments, and spared the processing of other substrates (L1 and NRG1) that are cleaved independent of endocytosis. These results provide strong evidence that inhibition of BACE1 cleavage of APP is feasible without interfering with the cleavage of non-amyloid substrates, as shown here for NRG1 and L1.

Our results suggest that subcellular compartmentalization allows BACE1 to cleave APP in the endosomal compartment and other non-amyloid substrates in non-endosomal compartments. However, substrates could also be compartmentalized in different domains at the membrane level. Cell membranes are not homogenous in their lipid and protein distribution, and certain lipids, such as cholesterol and sphingolipid, tend to form dynamic nanoassemblies (Simons and Ikonen, 1997). Because the endosomally targeted, sterol-linked BACE1 inhibitor not only is endocytosis-competent but can also compartmentalize into lipid raft domains, we asked whether the specific inhibition is due to the fact that only APP cleavage was endocytosis-dependent or because only APP is partitioned into lipid rafts. Although our endocytosis inhibition experiment clearly pointed out that the subcellular compartmentalization of substrates is the reason, we tested this possibility if only APP was partitioned into lipid rafts and if this is the reason for specific inhibition with the endosomally targeted, sterol-linked BACE1 inhibitor. To this end, we studied the distribution of APP, BACE1, NRG1, and L1 in detergent-resistant microdomains (DRMs), a biochemical way of isolating such membrane domains. We isolated DRMs we identified using standard protocols (Rajendran et al., 2003; Figure S7). Western blot analysis of the lipid rafts revealed that APP and BACE1 strongly localize in those domains as reported by several groups (Figure S7). In compliance with our endocytosis inhibition data, we found that both NRG1 and L1 full-length and C-terminal fragment (CTF) were also localized in DRMs, similar to APP and BACE1. These results indicate that lipid raft localization is not the mechanism behind the sparing of BACE1 processing of NRG1 and L1 by the endosomally targeted, sterol-linked BACE1 inhibitor but, rather, compartmentalization into endosomal versus non-endosomal compartments.
Inhibition of BACE1 is being pursued intensively as a therapeutic target to treat AD, and BACE1 inhibitors are currently in clinical trials (Vassar et al., 2014). However, mechanism-based toxicity might arise from inhibition of BACE1. Chemical inhibition of BACE1 in adult animals has been shown to alter maintenance of muscle spindles (Cheret et al., 2013) and to impair synaptic functions (Filser et al., 2015). Therefore, it is essential to consider other strategies to design more selective BACE1 inhibitors that specifically inhibit APP cleavage and Ab production. In this work, we explored the differential subcompartmentalization of substrate processing as a strategy to increase the selectivity of BACE1 inhibitors. We show that the cleavage of high-affinity, non-amyloid substrates by BACE1 does not require dynamin/clathrin-mediated endocytosis, whereas processing of APP does. Our results point to the importance of the acidic residue in the BACE1 binding site of the substrate at the P2 position. Even before BACE1 was identified, Citron et al. (1995) predicted the importance of the P2 residue in APP, and we now show that, for non-amyloid substrates, the presence of an acidic residue confers high-affinity binding. This may be a mechanism used by BACE1 to be able to process its high- and low-affinity substrates in different compartments; namely, non-amyloid substrates in the non-endosomal compartments such as the TGN and APP in endosomes. An alternative explanation could be the protonation of the Glu residues in NRG1/L1 in low pH of the endosomes that inhibits its cleavage in endosomes, which could also explain why swAPP cleavage by BACE1, which does not have the Glu residue in this position, is still partly dependent on endocytosis.

One other possibility is that APP is cleaved in the endosomal compartment that is regulated by clathrin and/or dynamin, whereas other substrates are cleaved in a still unidentified endosomal compartment that is independent of clathrin/dynamin. However, our observations point out the existence of distinct subcellular compartments where APP and other physiological substrates are cleaved by BACE1. Exploiting this differential subcellular compartmentalization of BACE1 processing, we specifically targeted WT APP processing with an endosomally targeted, sterol-linked BACE1 inhibitor. Although the design of the inhibitor to target endosomal BACE1 has been described previously (Rajendran et al., 2008), our data show the utility of this inhibitor as a tool to distinguish BACE1 cleavage of APP.

**DISCUSSION**

Inhibition of BACE1 is being pursued intensively as a therapeutic target to treat AD, and BACE1 inhibitors are currently in clinical trials (Vassar et al., 2014). However, mechanism-based toxicity might arise from inhibition of BACE1. Chemical inhibition of BACE1 in adult animals has been shown to alter maintenance of muscle spindles (Cheret et al., 2013) and to impair synaptic functions (Filser et al., 2015). Therefore, it is essential to consider other strategies to design more selective BACE1 inhibitors that specifically inhibit APP cleavage and Ab production. In this work, we explored the differential subcompartmentalization of substrate processing as a strategy to increase the selectivity of BACE1 inhibitors. We show that the cleavage of high-affinity, non-amyloid substrates by BACE1 does not require dynamin/clathrin-mediated endocytosis, whereas processing of APP does. Our results point to the importance of the acidic residue in the BACE1 binding site of the substrate at the P2 position. Even before BACE1 was identified, Citron et al. (1995) predicted the importance of the P2 residue in APP, and we now show that, for non-amyloid substrates, the presence of an acidic residue confers high-affinity binding. This may be a mechanism used by BACE1 to be able to process its high- and low-affinity substrates in different compartments; namely, non-amyloid substrates in the non-endosomal compartments such as the TGN and APP in endosomes. An alternative explanation could be the protonation of the Glu residues in NRG1/L1 in low pH of the endosomes that inhibits its cleavage in endosomes, which could also explain why swAPP cleavage by BACE1, which does not have the Glu residue in this position, is still partly dependent on endocytosis.

One other possibility is that APP is cleaved in the endosomal compartment that is regulated by clathrin and/or dynamin, whereas other substrates are cleaved in a still unidentified endosomal compartment that is independent of clathrin/dynamin. However, our observations point out the existence of distinct subcellular compartments where APP and other physiological substrates are cleaved by BACE1. Exploiting this differential subcellular compartmentalization of BACE1 processing, we specifically targeted WT APP processing with an endosomally targeted, sterol-linked BACE1 inhibitor. Although the design of the inhibitor to target endosomal BACE1 has been described previously (Rajendran et al., 2008), our data show the utility of this inhibitor as a tool to distinguish BACE1 cleavage of APP.
and non-amyloid substrates because of the subcellular compartmentalization of these cleavages. An alternative possibility for the mode of action of the endosomally targeted, sterol-linked BACE1 inhibitor is that the endosomally targeted inhibitor, because of its sterol linkage, targets APP cleavage that occurs in lipid rafts of endosomes (Ehehalt et al., 2003) and, therefore, spares non-amyloid substrates that could be localized in non-raft domains in the endosomes. However, for this to occur, NRG1 processing must be endocytosis-dependent, and its processing should occur in non-raft domains of endosomes. By using Pitstop2, an inhibitor of clathrin-dependent and -independent endocytosis (Dutta et al., 2012; Stahlsmiedt et al., 2014; von Kleist et al., 2011), and also with dominant-negative dynamin, we clearly demonstrate that, although APP processing by BACE1 requires endocytosis, β-cleavage of the non-amyloid substrates NRG1 and L1 does not require endocytosis. This suggests that the NRG1/L1-sparing activity of the endosomally targeted, sterol-linked BACE1 inhibitor is most likely due to endosomal targeting and not due to differential localization of these non-amyloid substrates in non-raft domains of the endosomes. Moreover, in our experiments with isolated DRMs, all substrates are localized in DRMs as well. Therefore, our results point out that only APP β-cleavage occurs in endosomes and, therefore, encourages the possibility that endosomally targeted BACE1 therapeutic substances can reduce Aβ production without affecting other BACE1 substrate cleavages. Endosomally targeted BACE1 inhibitors are potential therapeutic substances for the specific treatment of AD without adverse effects.

The limitations of our study include the lack of translation in animal models because these are beyond the scope of this study, but our experiments on human iPSCs provides hope. Clearly, further research is needed to translate these findings in experimental animals and also patients. Regardless, our work demonstrates that inhibiting Aβ production without affecting BACE1 processing of other substrates is possible and of important clinical value for the specific treatment of AD.

**EXPERIMENTAL PROCEDURES**

**MD Simulations**

For the MD simulations, the initial structure of the BACE1-substrate complex was obtained from the crystal structure of the ectodomain of BACE1 bound to the OM99-2 inhibitor (Glu-Val-Leu-Asn-Leu-Ala-Asp-Glu-Phe), where α[CHOH-CH2] represents a hydroxyethylene isostere of the peptide bond (PDB: 1FKN) (Hong et al., 2000). The hydroxyethylene isostere was replaced by a carbonyl group to obtain the peptide bond and all MD simulations were carried out with peptide substrates. The OM99-2 inhibitor corresponds to the swAPP sequence with the Asp(P1)Ala mutation. The original form of the swAPP sequence was obtained by mutating Ala at P1 of OM99-2 to Asp. From the structure of the swAPP-substrate, the initial structures of WT APP (Glu-Val-Lys-Met-Asp-Ala-Glu-Phe), NRG1 (Gly-Ile-Glu-Phe-Met-Glu-Ala-Asp) were calculated. Similarly, we performed 60 ns of MD simulations for the four BACE-inhibitor complexes where the hydroxyethylene isostere of the peptide bond was retained (data not shown). Here, we considered the Ala(P1) residue of OM99-2 for a direct comparison with in vitro assays. In addition, a 120-ns MD run was performed for each of the complexes between BACE1 and the statin-based (hydroxyethylene isostere) inhibitors. The P4-P4' residues were extracted from the sequences of swAPP (OM99-2 inhibitor), WT APP, NRG1, and PSGL1, except for the Ala at P1, which was kept as in OM99-2 for all four inhibitors.

**Cell-free BACE1 Activity Assay**

The cell-free BACE1 activity assay was performed in a final volume of 100 μl in assay buffer: 50 mM sodium acetate (pH 4.5) and 0.1% Triton X-100. 0.24 μM of recombinant human BACE1-ectodomain (rhBACE1) (Sigma S4195) was mixed with 10 μM of each substrate in a flat-bottom black 96-wells plate (Nunc 237105). The assay was performed at 37 ± 1 °C with excitation and emission wavelengths set at 340 nm and 40 nm, respectively. For cell-free BACE1 activity assay with inhibitors, 0.24 μM of rhBACE1 was incubated with 10 μM of each inhibitor for 10 min at 37 °C in the assay buffer prior to the addition of each substrate.

**Km and Vmax Measurements and Calculations**

The specificity of the different substrates for rhBACE1 was kinetically assayed by measuring the Michaelis constant Km and the limiting rate Vmax from which the specificity constant Kcat/Km was calculated. Instability of the enzyme during assay was assessed with the Selwyn test (Selwyn, 1965). Fluorescence measurements were performed in a final volume of 100 μl using flat bottomed black 96-wells plate in a Tecan Infinite M1000Pro microplate reader thermostatted at 37 ± 1 °C with excitation and emission wavelengths of 340 nm and 400 nm, respectively. Readings were performed in kinetic mode for 5 min, during which time fluorescence increased linearly. For a constant 0.24 μM enzyme concentration, 5 or 6 substrate concentrations covering approximately the range 0.2 to 5 Km as determined in preliminary experiments, were used. Stock solutions of the substrates were prepared in pure DMSO, but the concentration of the organic solvent was kept constant at 1% v/v for all substrate concentrations. Only for the swAPP substrate, the final DMSO concentration was 2% v/v. The buffer was 50 mM sodium acetate (pH 4.5) with 0.1% Triton X-100 added. Km and V were calculated by non-linear regression fitting of the Michaelis-Menten equation.

**Quantitative Measurements of Aβ, sAPPα, and sAPPβ**

Human Aβ40, human sAPPα, human sAPPβ, and mouse Aβ40 were assayed from supernatants, after centrifugation at 1,000 rpm for 3 min, using the electrochemiluminescence (ECL) assay as described in Böringer et al., 2012.

**Primary Neuronal Culture, Transduction and Inhibitors Treatments**

Mixed cortical/hippocampal neurons were prepared from embryonic day 15 and 16 mice. In brief, dissociated cells were plated onto 6-well poly-D-lysine-coated...
dishes at a density of ~250,000/cm² and cultured in Neurobasal medium (Invitrogen) with B27 supplements (Invitrogen), 2 mM L-glutamine, and penicillin/streptomycin. After 5 days, in vitro neuronal cultures were incubated with lentiviral NRG1 (1:1 titer III or GFP particles for 8 hr. 48 hr after transduction, cells were treated with DMSO or inhibitors (C3, TAPI-1 and the endosomally-targeted sterol-linked BACE1 inhibitor) for 24 hr, as previously described.

**iPSC-Derived Neuronal Cultures and Treatment**
Human iPSC-derived long-term self-renewing neuroepithelial stem cells (i-NECs cells) (Mertens et al., 2013) were maintained in DMEM/F12, 2 mM L-glutamine, 1.6 g/l glucose, 0.1 mg/ml penicillin/streptomycin, N2 supplement (Life Technologies), B27 (1 μl/ml; Life Technologies), and fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF; both 10 ng/ml; Cell Guidance Systems) on tissue culture plates coated with poly-L-ornithine/laminin (both Sigma), and passaged every 3 or 4 days. Neuronal differentiation was induced by withdrawal of FGF2 and EGF in differentiation media (MACS Neuro Medium supplemented with MACS NeuroBrew-21 (1:50; Miltenyi Biotec) and DMEM/F12 supplemented with N2 mixed at a 1:1 ratio) that was exchanged every other day. C3 BACE1 inhibitor (5 μM), endosomally-targeted sterol-linked BACE1 inhibitor (100 nM), or DMSO solvent control were added to neuronal cultures differentiated for 4 weeks. Cultures were incubated for 22 hr before supernatant and protein lysates were collected.

**Statistical Analysis**
All data are shown as mean ± SD. Two-tailed Student’s t test was used for comparison of the means between two groups. *p < 0.05, **p < 0.005, and ***p < 0.0005.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.076.

**AUTHOR CONTRIBUTIONS**
L.R. designed the research. S.B.H. and M.W. performed the biochemistry and cell biological experiments. S.M. and A.C. analyzed the MD simulations. A.B. designed the research. S.B.H. and M.W. performed the biochemistry and cell biological experiments. S.M. and A.C. analyzed the MD simulations. A.B. contributed to the iPSC experiments. P.K. and O.B. contributed to the iPSC experiments.

**ACKNOWLEDGMENTS**
We thank T. Südhof, M. Schwab, A. Aguazzi, G. Jennings, H. Knoeiker, and U. Konietzko for their input. We gratefully acknowledge the help of Jado Technologies GmbH with synthesis of the sterol-linked BACE1 inhibitors. We thank E. Schwarz and S. Hoey for critical reading of the manuscript. We thank U. Konietzko for the APP-HA tag plasmids, B. de Strooper for the L1 plasmid, P. Altevogt for the anti-L1 antibody, L. Johannes for the fluorescently labeled U. Konietzko for the APP-HA tag plasmids, B. de Strooper for the L1 plasmid, E. Schwarz and S. Hoey for critical reading of the manuscript. We thank Z. Goodger and J. Ries for help with RT-PCR analysis, and H. Schwalbe for all authors participated in the editing of the paper. P.K. and O.B. contributed to the iPSC experiments.

**REFERENCES**


neuro2a (N2a) cells. Evidence that cleavage at the “beta-secretase” site occurs in the golgi apparatus. J. Biol. Chem. 277, 9390–9397.


Supplemental Information

Specific Inhibition of β-Secretase Processing
of the Alzheimer Disease Amyloid Precursor Protein

Saoussen Ben Halima, Sabyashachi Mishra, K. Muruga Poopathi Raja, Michael Willem, Antonio Baici, Kai Simons, Oliver Brüstle, Philipp Koch, Christian Haass, Amedeo Caflisch, and Lawrence Rajendran
Supplementary information including the methods is available at
www.cell.com/cellreports

Supplementary figure Legends

Supplementary Figure 1.

Molecular dynamic simulations of BACE1 and the substrates-analog. (A) Expression of BACE1 substrates at different ages in control (wild type) and transgenic mouse model of AD (Arc/SwAPP mutant). RT-PCR of cortex RNA extracted from healthy or AD mice, 1 male and 1 female mouse in each group. ∆Ct = Ct gene − Ct housekeeping. Normalized to 18S and GAPDH, ∆∆Ct = ∆Ct experimental time point - ∆Ct control. X axis: age and y axis: ∆∆Ct. (B) Structure and sequence of the octapeptide substrates of wtAPP, swAPP, NRG1, and PSGL1. (C) The active site of BACE1 with the octapeptide of PSGL1 substrate-analog. The snapshot for each BACE1-substrate complex shown is the representative structure of the most populated conformer which was obtained by clustering all MD snapshots by root mean square deviation and a cutoff of 0.8 Å. All Cα atoms of BACE1 except for the loops A, C, D, and F, were used in the structural fitting prior to the clustering. The flap is shown as ribbon while the side chains of BACE1 and substrate as sticks. The carbon atoms of substrate are shown in magenta for clarity. (D) Root mean square fluctuation of Cα atoms of BACE1, averaged over intervals of 2 ns after excluding the first 20 ns of each MD trajectory. Black, red, green, blue, and orange represent swAPP, wtAPP, NRG1, PSGL1, and apo complex of BACE1, respectively. Vertical dashed lines indicate the root mean square fluctuation of the catalytic aspartates and the flap residues. The similar RMSF values indicate that the overall plasticity of BACE1 is essentially identical when it is bound to any
of the four substrates. The small fluctuations in the flap region (residue 67 to 77) reflect the stable flap in the presence of substrates in the active site. On the other hand, in the apo form, BACE1 shows a comparatively larger fluctuation in the flap region. Similarly, the catalytic active site (near the regions of the two catalytic Asps - Asp32 and Asp228) also shows small fluctuations representing stable enzyme-substrate complexes. (E) The correlation of fluctuations of the eight substrate Cα atoms (shown by different colors in each panel) with the BACE1 Cα atoms. The correlation coefficient is obtained from the normalized covariance matrices of the corresponding Cα atom displacements which were then averaged over intervals of 2 ns after excluding the first 20 ns of each MD trajectory. The flap region and the two catalytic Asp residues (Asp32 and Asp228) are indicated by vertical dashed lines. The correlated motion of BACE1 and substrate for four complexes show similar correlation pattern where a moderately strong correlation is seen between substrate and the active site, in particular, in the region of two catalytic Asp’s (Asp32 and Asp228) and in the flap region which suggests that the four substrates considered here follow a similar binding pattern, and therefore validate further comparison between their binding affinity towards BACE1.

Supplementary Figure 2.

The interaction energy between BACE1 and NRG1, swAPP, PSGL1 and wtAPP derived inhibitors. (A) Time series of the total interaction energy (van der Waals plus electrostatic) between BACE1 and the four inhibitors, i.e., OM99-2 derivative of swAPP, wtAPP, NRG1 and PSGL1, in black, red, green, and blue respectively. Note, (P1’) for all four inhibitors is Ala. The order of stabilizing interaction between BACE1 and the inhibitors (NRG1 > swAPP > wtAPP > PSGL1) is similar to that between BACE1 and the corresponding substrates (Figure 1A). (B) Time series of interaction energy between BACE1 and each of
the eight residues of the substrate-analog inhibitors. The residue type is given by one-letter code with the same color as the time series. The Glu residues at P2 and P2’ of NRG1 have more favorable interactions than the residues in the other three inhibitors.

Supplementary Figure 3
Chemical structure and ESI-Mass spectra of BACE1 peptide inhibitors. (A) Chemical structures of BACE1 peptide inhibitors having non-coded gamma amino acid statine moieties. (B) ESI-Mass spectra of BACE1 peptide inhibitors a) wtAPP, b) swAPP, c) NRG1 and d) PSGL1. (C) Molecular Characterization of the substrate-derived statine-based inhibitors through electrospray ionization-mass spectrometry (ESI-MS).

Supplementary Figure 4.
Determination of kinetics parameters of NRG1, wtAPP and swAPP reactions with BACE1. The specificity of the different substrates for human recombinant BACE1 was determined by measuring the Michaelis constant $K_m$ and the limiting rate $V$. The specificity constant $K_{cat}/K_m$ was calculated from these parameters and the enzyme concentration. Progress curves measured at a fixed substrate concentration and three enzyme concentrations showed considerable loss of enzyme activity and did not pass the Selwyn test at reaction times larger than 15 min. This prevented the use of entire progress curves of substrate hydrolysis for calculating kinetic parameters and the initial rate method was used instead.

Supplementary Figure 5.
The endosomally-targeted sterol-linked BACE1 inhibitor does not inhibit NRG1 cleavage in primary neurons. (A) NRG1 β1 type III or GFP (as control for the transduction) was expressed in primary hippocampal/cortical neurons using Lentivirus-mediated transduction,
and cells were treated with the indicated inhibitors TAPI-1, BACE1 inhibitor C3, the endosomally-targeted sterol-linked BACE1 inhibitor, for 24h. Western blotting of Lysates with the Sc-348 antibody to detect full length NRG1 β1 type III and NRG1-CTFs and Conditioned medium was subjected to ECL assay to measure mouse Aβ40 (B). The values are experimental triplicates.

Supplementary Figure 6.
Characterization of iPSC-derived human neurons. Human induced pluripotent stem cell (iPSC)-derived neuroepithelial stem cells express the neural stem cell-associated transcription factors Sox2 and DACH1 as well as the intermediate filament NESTIN (left panel). Following growth factor withdrawal and differentiation for 4 weeks, these cultures form dense neuronal networks (right panel; phase contrast). Scale bars: 200 µm

Supplementary Figure 7.
Amyloid (APP) and non-amyloid BACE1 substrates (NRG1 and L1) have similar detergent insolubility /subcellular localizations in detergent resistant microdomains (DRMs)/ lipid rafts. Western blot analysis of cells expressing either NRG1 β1 type III, L1 or wtAPP subjected to 1% Triton solubility and subsequent separation on sucrose gradient fractionation. DRMs refer to lipid raft fractions and are flotillin-1 positive.
Supplementary Information on Methods

FRET reporters of amyloid and non-amyloid substrates of BACE1

All the FRET reporters (See below tables) were purchased from Bachem. All the FRET peptides contained (7-Methoxycoumarin-4-yl)acetic acid (Mca) and 2,4-Dinitrophenyl (DNP) to serve as fluorophore and a quencher, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sequence</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtAPP</td>
<td>(Mca-(Asn670,Leu671)- Amyloid Protein Precursor (APP) 770(667-676)-Lys(Dnp)-Arg-Ar-NH₃ trifluoroacetate salt )</td>
<td>M-2460</td>
</tr>
<tr>
<td>wtAPP K(P2)E</td>
<td>(Mca-(Asn670,Leu671)- Amyloid Protein Precursor (APP) 770(Κ670E)-Lys(Dnp)-Arg-Ar-NH₃ trifluoroacetate salt )</td>
<td>custom product</td>
</tr>
<tr>
<td>swAPP</td>
<td>(Mca-(Asn670,Leu671)- Amyloid Protein Precursor (APP) 770(667-676)-Lys(Dnp)-Arg-Ar-NH₃ trifluoroacetate salt )</td>
<td>M-2465</td>
</tr>
<tr>
<td>NRG1</td>
<td>Mca- Ser-Gly- Ile- glu- Phe-Met-Glu-Ala-Glu-Lys(Dnp)-Arg-Arg-NH3, amid trifluoroacetate salt</td>
<td>custom product</td>
</tr>
<tr>
<td>NRG1 E(P2)K</td>
<td>Mca-Gly- Ile-Lys- Phe-Met-Glu-Ala-Glu-Lys(DNP)-Arg-Arg-NH2 trifluoroacetate salt</td>
<td>custom product</td>
</tr>
<tr>
<td>PSGL1</td>
<td>Mca-Ser-Ala-SerAsn-Leu-Ser-Val-Asn-Tyr(Dnp)-arg-Arg-NH₂ trifluoroacetate salt.</td>
<td>custom product</td>
</tr>
</tbody>
</table>

Synthesis of substrate-analog inhibitors derived from BACE1 substrates

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Sequence</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtAPP</td>
<td>Glu- Val- Lys- Met- ϕ[(CHOH-CH2 ) - Asp- Ala- Glu- Phe</td>
<td>see method</td>
</tr>
<tr>
<td>swAPP</td>
<td>Glu-Val-Asn-Leu- ϕ[(CHOH-CH2 ) -Asp-Ala- Glu- Phe</td>
<td>see method</td>
</tr>
<tr>
<td>NRG1</td>
<td>Gly- Ile-Glu-Phe- ϕ[(CHOH-CH2 ) -Met-Glu-Ala-Glu</td>
<td>see method</td>
</tr>
<tr>
<td>PSGL1</td>
<td>Ala-Ser-Asn-Leu- ϕ[(CHOH-CH2 ) -Ser-Val-Asn-Tyr</td>
<td>see method</td>
</tr>
</tbody>
</table>

Transition-state mimic 8-residue peptide inhibitors (see above table) were designed based on the BACE1-binding region in the substrates. The scissile peptide bond is replaced with an isostere moiety to render the peptide bond non-cleavable. The BACE1 substrate-derived peptide inhibitors (Supplementary Fig. S3a) were synthesized by conventional manual solid-
phase peptide synthesis, employing standard Fmoc chemistry protocols (Chan and White, 2000). In brief, syntheses were done at 100 μM scale, the respective preloaded 2-chlorotrityl resins (0.5-0.8 mmol/g) were swollen in DCM/DMF and couplings were carried out using HBTU/DIEA/DMF. Five-fold excess of Fmoc-amino acids were used for each coupling. The deprotection of Fmoc group was achieved by 20% piperidine in DMF. The washings were done by DMF after each step in the coupling cycle and in the final cycle DCM and methanol washings were performed. The synthesised peptides were cleaved from dried resins by TFA-TIS-Water cocktail mixture (95:2.5:2.5) and precipitated in precooled TBME. The N- and C-termini free peptides were collected after centrifuges and TBME wash cycles. The crude peptides were subjected to HPLC purification by reverse phase C18 column, using acetonitrile-water (containing 0.1% TFA) as gradient solvent system. The fractions were collected, lyophilized and the molecular integrity is confirmed by ESI-MS analysis (Supplementary Fig. S3b). Supplementary Fig. S3C summarizes the molecular characterization by ESI-MS. The observed molecular masses [M+H]+ and [M+Na]+ are very well matches with calculated mass of these peptides, confirming their chemical integrity. All preloaded resins, Fmoc-amino acids, reagents and solvents were purchased from Merck-NovaBiochem, Germany and Fmoc-Statine was purchased from PolyPeptide Laboratories, France.

Fmoc - 9-Fluorenlymethoxycarbonyl
Stat - (3S,4S)-4-amino-3-hydroxy-6-methyl-heptanoic acid

DCM- Dichloromethane
DMF- Dimethylformamide
HBTU - O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
DIEA - Diisopropyl ethyl amine
TFA - Trifluoro acidic acid
Real-Time PCR

RNA was isolated from mouse cortical brain tissue at the ages of 1 day, 1 month, 3 months, 6 months, 9 months and 12 months from wild type (WT) and Tg (Arc/SwAPP) mice. Tissue samples were homogenized by titration in 1 ml Trizol using 18G, 22G and 26G gauge needles to sequentially dissociate the tissue. RNA was extracted following the Trizol protocol, according to manufacturer’s guidelines. RNAs were further purified using Qiagen RNeasy kit including on column DNAseI digestion. RNA was eluted into the RNA storage solution (AM7001; Ambion). RNA quality and concentration were determined by Agilent Bioanalyser and Nanodrop Spectrophotometer. Real-time PCR (RT-PCR) was carried out by reverse transcribing 5 μg of total RNA using first strand cDNA synthesis Kit (11904-018; Invitrogen) according to manufacturer’s instructions. Briefly, the cDNA was then diluted 1:5 in a TaqMan-Low density array (Applied Biosystems) and PCR was performed according to manufacturing protocol.

Cell Lines and Cultures

Human embryonic kidney cells (HEK293T), HEK 293 stably expressing BACE1 (kind gift from Prof. D. Selkoe), HeLa Kyoto, HeLa-GalT-GFP were cultured in low-glucose DMEM (Invitrogen) containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/mL) in a humidified 5% CO2 atmosphere.

Confocal Microscopy
Images were acquired on a Leica TCS/SP8 confocal microscope (Leica, Wetzlar, Germany) with a 63Å~ water immersion objective. A 543 nm HeNe laser was used to excite Cy3 (HYD window: 553–600 nm). A 633 nm HeNe laser was used to excite Cy5 (HYD window: 655–710nm).

**Transferrin and EGF receptor endocytosis**

Transferrin uptake experiments were performed as described in Kamiok et al., 2004 (1). Briefly, HeLa Kyoto cells or HEK cells were transfected with Dyn WT or Dyn K44A plasmids. 24h post transfection, cells were serum-starved in DMEM for 1 h, non transfected cells were treated with 35μM Pitstop2 (Abcam, ab120687) during the serum starvation step, and incubated with 25 μg/ml Alexa 546-conjugated transferrin or 100nM of Alexa 633-conjugated EGF receptor for 20 min at either 37 °C or 4 °C (control). After rinsing three times with PBS and reducing surface labeling using 50 mM deferoxamine mesylate-containing buffer (150 mM NaCl, 2 mM CaCl₂, and 25 mM sodium acetate/acetic acid, pH 4.5), cells were fixed with 4% paraformaldehyde (PFA), in PBS and subjected to fluorescence imaging.

**Shiga toxin and endosomally-targeted sterol-linked BACE1 inhibitor uptake**

HeLa-GalT-GFP and HeLa cells were plated tow days prior to treatment (4000 cells). The day of experiment cells were washed twice with serum free medium then incubated with 2ug/ml of Alexa 633-conjugated Shiga toxin or 2uM of rhodoamine -conjugated endosomally sterol-linked BACE1 inhibitor on ice for 45 min (2). Then cells were washed 3 times with full serum medium and incubated at 37°C for 0, 5, 20 30 and 1 hour. Cells were then washed tree time with PBS and fixed with 4% PFA, in PBS. For the colocalization of the sterol-linked BACE1 inhibitor with EEA1 and Lamp1, after PFA fixation, cells were
permeabilized with 0.02% Triton for 5 min and blocked with 2% BSA for 1 hour at room temperature. Anti-EEA1 (Abcam1/400) and anti-Lamp-1 (Santa cruz 1/100) were applied overnight at 4°C. After washing, secondary antibody 633-conjugated anti-mouse antibody 1/400 was applied for 1 hour at room temperature, cells were then washed and DNA stained with DAPI for 10 min. After mounting cells were subjected to fluorescence imaging.

For immunocytochemical characterization of iPSC-derived neuronal cultures, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA, 10 min, RT), blocked in 0.1% Triton X-100 (Sigma) and 10% FCS in PBS, incubated with the primary antibodies (16 hours, 4°C), washed with PBS, counterstained with secondary antibodies (1 hour, RT) and DAPI and mounted with Mowiol.

Mutagenesis, cell transfection and inhibitor treatments

NRG1 β1 type III, and wtAPP mutants were obtained using the quick-change site-direct mutagenesis kit according to the manufacturer’s protocol (Stratagene 200519). HEK 293 stably expressing BACE1 cells were transfected using 1 µg of DNA and 2 µl of Lipofectamine 2000 (Invitrogen) -according to the manufacturer’s instructions for 24-well plate -with pcDNA or the needed plasmid. 24 hours post-transfection, cells were treated with DMSO, 5 µM of C3 (beta-secretase Inhibitor IV, 565788; Calbiochem) 20 µM TAPI-1 (579051; Calbiochem), 20 µM GL189 (565780; Calbiochem) or 500 nM endosomally-targeted sterol-linked BACE1 inhibitor for 12 hours. Endosomally-targeted sterol-linked BACE1 inhibitor was replenished every 4 hours. Lysates were used for western blotting and cells supernatants were used for ECL assays whenever is needed.

Cell viability
Cell viability was assessed using Alamar Blue assay (10 % v/v Alamar Blue) according to the manufacturer’s instructions (BUF012B; AbD Serotec). Absorbance was monitored at the end of the reaction (after 4 h) and spectroscopically read at wavelengths of 544nm and 590 nm (Spectra Max Gemini XS, Molecular devices).

**In vitro BACE1 solubilized membrane assay**

Four 10 cm dishes of HEK 293 stably expressing BACE1 cells were transfected with NRG1β1 Type III and were washed with PBS, scraped in 1 ml PBS and centrifuged for min at 8000 rpm. Cell pellets were resuspended in 700 µl buffer H (20 mM HEPES, 150 mM NaCl, 10% glycerol, 5 mM EDTA, pH 7.4) and the solution drawn 20 times through a 3 ml syringe with 20 gauge needle. Unbroken cells were removed by centrifugation at 4000 rpm for 5 min. In order to obtain P2 fractions, the supernatant was centrifuged at 55000 rpm for 1 h and 4°C. Membrane fractions were washed once in 300 µl incubation buffer (0.1 M Na Acetate pH 4.0, 10 µg/ml Leupeptin, 1 µg/ml Aprotinin, 1 mM PNT and 5 mM EDTA) and resuspended in 100 µl incubation buffer in absence or presence of 5 µM C3 or 500 nM sterol linked BACE1 inhibitor. After incubation for 4 h at either 0°C or 37°C, the samples were loaded on 4-12% BisTris SDS-PAGE gel and investigated by Western blot analysis.

**Isolation of detergent-resistant microdomains (DRMs)/ lipid rafts**

1 T75 flask of 80-90% confluent cells was lysed for 30 minutes in cold in TNE (10mM Tris HCl, pH 7.5, 150mM NaCl, and 5 mM EDTA) buffer with 1% Triton X-100, protease and phosphatase inhibitors. The solution after lysis was further homogenized with ten strokes in a loose fitting dounce homogenizer and centrifuged at 900 g for 10 minutes. 1ml of the cleared supernatant was mixed with 1ml of 85% sucrose in TNE buffer and layered at the bottom of a Beckman 12 ml centrifuge tube. The lysate was overlaid with 6 ml of 35% sucrose in TNE buffer and finally with 3.5ml of 5% sucrose in TNE buffer. The samples
were centrifuged in a SW41 rotor at 200,000 g for 20 h at 4°C. At the end of the run, 1ml fractions were collected from the top of the gradient. 50 μl from each of the fractions was boiled with SDS buffer and subjected to a reducing 4–12% gradient SDS PAGE and western blotting analysis.

**Sample preparation and Western blotting**

To obtain total cell lysate, cells were washed with ice-cold PBS twice, lysed for 30 min on ice in lysis buffer: 2% NP-40, 0.2% SDS in PBS, supplemented with protease inhibitors (Roche) and post-nuclear supernatant was obtained by centrifugation (20 min, 1200 x g, 4°C). Protein concentration was determined with BCA protein assay (Pierce) and equal amount of protein (30-50 μg) was subjected to SDS-PAGE analysis. Proteins were transferred onto nitrocellulose membrane (Protran; Whatman) and the indicated antibodies were used for immunodetection. Bound antibodies were detected with HRP-conjugated secondary antibodies using the chemiluminescence detection reagents ECL (GE Healthcare).

**Antibodies**

The following antibodies were used for immunoblotting: antibody to NRG1 C-terminus (pRb, 1:1000 Sc-348; Santa Cruz biotechnology), to NRG1-β-CTF (rat, 1:40, monoclonal neoepitope-specific antibodies, 4F10; from Dr. Michael Willem), to L1, anti C-terminal antibody (rabbit, 1:50000, PcytL1; from Prof. Peter-Hans Altevogt). HRP-conjugated secondary antibodies (rat, mouse and rabbit, 1:15000; GE Healthcare). Primary antibodies and concentrations for immunocytochemical characterization of iPSC-derived neuronal cultures were as follows: Sox2 (1:500, R&D Systems), Nestin (1:600, R&D Systems), Dach1 (1:100, Proteintech), beta-III tubulin (1:2000, Covance), Map2ab (1:250, Chemicon),
GFAP (1:1000, DakoCytomation). Secondary antibodies were Alexa488 anti-ms, Alexa555 anti-ms, Alexa488 anti-rb and Alexa555 anti-rb (all 1:1000, Life Technologies).

Supplementary References:

Figure S2:

Panel A: A graph showing the interaction energy (kcal/mol) over time (ns) for different conditions labeled as swAPP, wtAPP, NRG1, and PSGL1.

Panel B: Graphs depicting interaction energy (kcal/mol) over time for various helices labeled as P1: LMFL, P2: NKEN, P3: VVIS, P4: EEGA, and P1': AAAA, P2': AAEEV, P3': EEAN, P4': FFEEY.
<table>
<thead>
<tr>
<th>Substrate peptide</th>
<th>Calculated $[M]_{xyg}$ (Da)</th>
<th>Observed $[M+H]^+$ (Da)</th>
<th>Observed $[M+Na]^+$ (Da)</th>
<th>Observed $[M+2H]^+^2$ (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtAPP</td>
<td>978.15</td>
<td>979.37</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>swAPP</td>
<td>964.08</td>
<td>965.21</td>
<td>986.84</td>
<td>--</td>
</tr>
<tr>
<td>NRG1</td>
<td>902.99</td>
<td>904.43</td>
<td>925.94</td>
<td>--</td>
</tr>
<tr>
<td>PSGL</td>
<td>923.03</td>
<td>924.35</td>
<td>945.97</td>
<td>--</td>
</tr>
</tbody>
</table>
Ben Halima., Fig. S4
**A**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Control Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type III NRG1-β1</td>
<td>DMSO</td>
<td>Sterol-linked Inhibitor</td>
</tr>
<tr>
<td>NRG1-FL</td>
<td>TAPI-I</td>
<td></td>
</tr>
<tr>
<td>(mature)</td>
<td>C3</td>
<td></td>
</tr>
<tr>
<td>NRG1-CT</td>
<td>Sterol-linked Inhibitor</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

- **Aβ**
  - DMSO
  - TAPI-1
  - C3 Inhibitor
  - Sterol-linked Inhibitor

% Control

Ben Halima., Fig. S5
Ben Halima., Fig. S6
Ben Halima et al., Fig. S7
Inventory of Supplemental information

1. Supplementary Figure 1
Molecular dynamic simulations of BACE1 and the substrates-analog (Related to Main figure 1)

Figure S1 shows the details and provide the controls of the MD simulations between BACE1 and the substrates-analog conducted in Figure 1.

2. Supplementary Figure 2: The interaction energy between BACE1 and NRG1, swAPP, PSGL1 and wtAPP derived inhibitors. (Related to main figure 1)

Figure S2 shows the details and provide the controls of the MD simulations between BACE1 and the substrates derived inhibitors conducted simulations are conducted in Figure 1.

3. Supplementary Figure 3: Chemical structure and ESI-Mass spectra of BACE1 peptide inhibitors. (Related to main figures 2 and 3)

Figure S3 provides information about the substrates-derived inhibitors used in Figure 2 and 3. Also provides further mass spectrometry information about the substrates derived inhibitors used in Figure 1.

4. Supplementary Figure 4: Determination of kinetics parameters of NRG1, wtAPP and swAPP reactions with BACE1. (Related to main figures 2 and 4)

Figure S4 gives the kinetics parameters that were used for the calculations for Figure 2C.

5. Supplementary Figure 5: The endosomally-targeted sterol-linked BACE1 inhibitor does not inhibit NRG1 cleavage in primary neurons. (Related to Figure 4)

Figure S5 shows the validation of the endosomally-targeted sterol-linked BACE1 inhibitor in a different cellular type than in Figure 4. (Related to main figures 4 and 5)

6. Supplementary Figure 6: Characterization of h-iPSCs. (Related to main figure 6)

Figure S6 provides the characterization of the h-iPSCs used in the assay of Figure 6.

7. Supplementary Figure 8: Amyloid (APP) and non-amyloid BACE1 substrates (NRG1 and L1) have similar detergent insolubility /subcellular localizations in detergent resistant microdomains (DRMs)/ lipid rafts. (Related to Figure 7)

Figure S7 shows the subcellular localization of BACE1, APP, NRG1 and L1 to lipid rafts as suggested by the reviewer. (Related to main figure 7)