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Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model

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In Alzheimer disease, increased β-secretase (BACE1) activity has been associated with neurodegeneration and accumulation of amyloid precursor protein (APP) products. Thus, inactivation of BACE1 could be important in the treatment of Alzheimer disease. In this study, we found that lowering BACE1 levels using lentiviral vectors expressing siRNAs targeting BACE1 reduced amyloid production and the neurodegenerative and behavioral deficits in APP transgenic mice, a model of Alzheimer disease. Our results suggest that lentiviral vector delivery of BACE1 siRNA can specifically reduce the cleavage of APP and neurodegeneration *in vivo* and indicate that this approach could have potential therapeutic value for treatment of Alzheimer disease.

Alzheimer disease continues to be the leading cause of dementia among the aging population in the US, but no definitive treatments are currently available. The precise mechanisms leading to Alzheimer disease are not completely understood, although recent studies suggest that alterations in the processing and clearance of proteolytic products, such as C-terminal fragments (CTFs) and amyloid-β peptides (Aβ), of the amyloid precursor protein (APP) have a key role in the pathogenesis of Alzheimer disease[1–3]. APP CTFs and Aβ peptides are generated by sequential cleavage of the transmembrane APP at the β and γ locations. The initial cleavage that generates CTFs is produced by BACE, an aspartyl protease[4,5] and is followed by cleavage by a second protease known as γ-secretase[6]. Unlike the BACE that specifically cleaves APP, γ-secretase cleavage is less specific and produces major fragments of 40 ($A\beta_{1–40}$), 42 ($A\beta_{1–42}$) or 43 ($A\beta_{1–43}$) amino acids[7,8].

Thus, β- and γ-secretase activities are required for the production of the Aβ peptides. γ-secretase activity is also involved in the processing of other substrates such as Notch[9]. Furthermore, genetic ablation of γ-secretase is embryonic lethal[10]. It thus follows that inhibition of this protease activity could have serious side effects. On the other hand, the activity of BACE1 seems to be redundant, as BACE knockout mice are viable and show no deleterious side effects[11]. Furthermore, crossing these mice with transgenic mice expressing human APP with familial Alzheimer disease mutations results in a complete lack of amyloid pathology[12]. In contrast, in Alzheimer disease, BACE1 expression and generation of APP fragments is increased[13–15]. Therefore, strategies that could reduce BACE1 activity have great therapeutic potential for the treatment of Alzheimer disease[16].

Because the active site of BACE1 is more open and less hydrophobic than in other proteases, it has been difficult to generate small molecules by traditional medicinal chemistry[17]. Therefore, alternative approaches to suppressing BACE1 such as the use of small interfering RNAs (siRNAs) are being considered[18]. We have previously shown that lentiviral vectors expressing siRNAs (lenti-siRNAs) can efficiently silence expression of a target protein in a variety of dividing and nondividing cell types[19]. The ability of lentiviral vectors to stably transduce nondividing cells such as neurons[20] makes them suitable for long-term expression of siRNAs in the brain. This led us to the idea that this system could be used to knock down genes that are involved in progression of Alzheimer disease: specifically, BACE1. In the present study we tested the hypothesis that delivery of lentiviral vectors expressing siRNAs specific for BACE1 can reduce the Alzheimer-like neuropathology and accumulation of Aβ and APP CTFs in the mouse model of Alzheimer disease. These results suggest that lentiviral vector delivery of BACE1 siRNA can specifically reduce the cleavage of APP and neurodegeneration *in vivo* and indicate the potential therapeutic value of this approach for treating Alzheimer disease.

RESULTS

Lenti-siBACE delivery results in decreased BACE1 expression

We chose seven different siRNA target sequences from the open reading frame of human BACE1, constructed siRNA expression cassettes driven by the H1 promoter and cloned these into lentiviral vectors as previously described[19]. For selection, human embryonic kidney (HEK293T) cells were transfected with a lentiviral construct expressing mutant APP and presenilin-1 (HEK293T-AIP) in order to increase levels of substrate for the endogenous BACE1. Cotransfection of these cells with plasmids expressing the seven different siRNA constructs showed that three of the siRNAs (siBACE1-1, siBACE1-2 and siBACE1-6; **Fig. 1a**) reduced the levels of both endogenous BACE1 and levels of secreted $A\beta_{1–42}$ peptide (data not shown). Both siBACE1-1 and

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siBACE1-6 sequences are identical in the human, mouse and rat orthologs of BACE1 and therefore were chosen for further analysis. To confirm the ability of the siBACE1-1 and siBACE1-6 lentiviral constructs to knock down high levels of BACE1, HEK293T cells were transfected with human and mouse BACE1 cDNAs to overexpress BACE1 and siBACE1-1, siBACE1-6 or an siRNA specific for mouse Glut-4 (siGlut4) as a control. siBACE1-1 (data not shown) and siBACE1-6 (**Fig. 1b**) reduced expression of BACE1 protein by over 90%, as shown by western blot. However, siBACE1-6 was more efficient in reducing mouse BACE1 (data not shown); therefore, all of the subsequent *in vitro* and *in vivo* experiments were carried out with the BACE1-6 siRNA lentiviral construct (lenti-siBACE1-6). To further validate the effects of siBACE1-6, HEK293T-AIP cells were transduced with lenti-siBACE1-6 or lenti-siGlut4. In this system, only lenti-siBACE1-6 effectively reduced $A\beta_{1-42}$ compared with controls (**Fig. 1c**). As shown by western blot, the BACE1 antibody specifically recognized both mouse and human BACE1 in brain homogenates as well as in transfected HEK293T cells (**Fig. 1d**).

Next, high-titer lentiviral vectors expressing siBACE1-6 or siGlut4 (as a control) were prepared and intracranially injected into the hippocampus of nontransgenic and APP transgenic mice, a model of Alzheimer disease. As both constructs contain an enhanced GFP (eGFP) marker, we confirmed the level and distribution of the siRNA vector expression by direct visualization with a fluorescent microscope (data not shown) or by immunocytochemistry using an antibody against eGFP (**Fig. 2a–d**). Both vectors were expressed at comparable levels in the hippocampus of nontransgenic (**Fig. 2a,b**) and APP transgenic mice (**Fig. 2c,d**). The highest expression was detected throughout the dentate gyrus and stratus polymorphus, with abundant distribution in the cell bodies and dendrites and to a lesser extent in the axons (**Fig. 2a–d**).

To evaluate the effects of the lentiviral vectors on BACE1 expression in mice, we performed immunocytochemical analysis with an antibody against BACE1. BACE1 immunoreactivity was detected in the dentate gyrus and stratus polymorphus of the hippocampus (**Fig. 2e–h**) and in pyramidal neurons in layers 2, 3 and 5 of the neocortex (**Fig. 2i–l**). Compared with the nontransgenic controls treated with the lenti-siGlut4 (**Fig. 2e,i**), the APP transgenic mice treated with the lenti-siGlut4 demonstrated a moderate increase in BACE1 immunoreactivity in the hippocampus (**Fig. 2g,m**) and neocortex (**Fig. 2k,n**). In contrast, both nontransgenic and APP transgenic mice treated with the lenti-siBACE1-6 vector showed a significant decrease in the levels of BACE1 immunoreactivity in the hippocampus (**Fig. 2f,h,m**), where the injections were made (**Fig. 2o**), but not in the neocortex (**Fig. 2j,l,n**), which received no injection.

To confirm the effects of the lenti-siRNA vectors on BACE1 expression in the hippocampus, we performed fluorescent labeling (**Fig. 3**). Laser scanning confocal microscopy (LSCM) analysis showed that in mice treated with the lenti-siBACE1-6, the areas of the hippocampus with the highest eGFP activity corresponded with areas with decreased BACE1 expression (**Fig. 3a–c**). In contrast, mice treated with the lenti-siGlut4 (**Fig. 3d–f**) or a saline-injected vehicle (**Fig. 3g–i**) showed unchanged levels of BACE1 immunolabeling.

siBACE1 reduces Alzheimer-like alterations in transgenic mice

To investigate the effects of reducing BACE1 expression with lenti-siBACE1-6 vector on APP processing and amyloid pathogenesis in the APP transgenic mice, we used western blot analysis for CTFs, an enzyme-linked immunosorbent assay (ELISA), and immunocytochemical analyses for Aβ (**Fig. 4**). Western blot analysis showed that treatment with the lenti-siBACE1-6 reduced APP CTFs

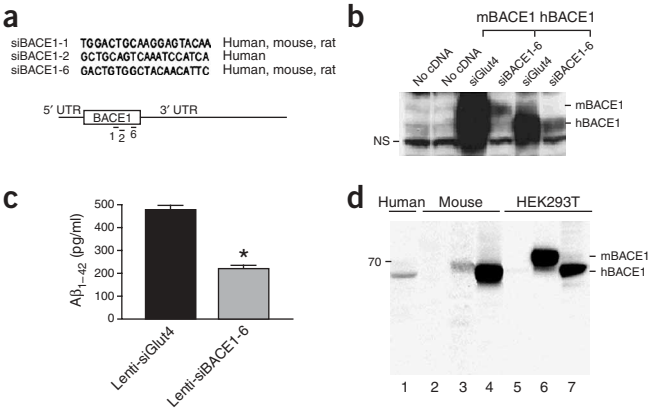

Figure 1 Construct design and *in vitro* testing of lenti-siRNA constructs. (**a**) Diagrammatic representation of human BACE1 cDNA showing effective siBACE1-1, siBACE1-2 and siBACE1-6 sequences and locations. Also shown is homology to human, mouse and rat sequences. (**b**) Confirmation by western blot analysis of silencing of overexpressed human and mouse BACE1. HEK293T cells were transfected with human (h) BACE1 or mouse (m) BACE1 expression plasmids and co-transfected with siBACE1-6 plasmids. Expression of siGlut4 was used as a negative control. Non-specific band (NS) was used as loading control. (**c**) Validation of silencing of endogenous BACE1. HEK293T-APP cells were transduced with lentivirus vector particles expressing siBACE1-6 or siGlut4. Media from transduced cells was analyzed for levels of secreted $A\beta_{1-42}$ using an ELISA assay. Levels of $A\beta_{1-42}$ reflect activity of endogenous BACE1. (**d**) Western blot analysis of the BACE1 antibody's ability to recognize human and murine BACE1. The BACE1 antibody recognized both the mouse and human forms of BACE1. Lanes 1–4 are frontal cortex homogenates from human and mouse brains. Lane 1 is from a neurologically unimpaired aged human control case, lane 2 from a BACE1-deficient mouse, lane 3 from a nontransgenic mouse and lane 4 from hBACE1 transgenic mouse. Lanes 5–7 are lysates from HEK293T cells transfected with a plasmid vector expressing eGFP, mBACE1 and hBACE1, respectively. $^*P < 0.05$ compared with lenti-siGlut4–treated controls (Student's *t*-test).

compared with the lenti-siGlut4 control (**Fig. 4a**). Furthermore, by ELISA, we detected lower levels of $A\beta_{1-42}$ in the hippocampus of APP transgenic mice treated with the lenti-siBACE1-6 vector (**Fig. 4b**) compared with mice treated with the lenti-siGlut4 vector control (**Fig. 4b**). Consistent with these results, confocal microscopy of Aβ immunoreactivity in the hippocampus (**Fig. 4c–f**) and neocortex (**Fig. 4g–j**) showed that in nontransgenic controls, only background levels of Aβ were detected (**Fig. 4c,d,g,h,k,l**). Compared with APP transgenic mice treated with lenti-siGlut4 (**Fig. 4e,i**), mice treated with lenti-siBACE1-6 vector (**Fig. 4f,j**) showed a significant reduction in the percentage of area occupied by Aβ-immunoreactive plaques in the hippocampus (injection site, **Fig. 4f,k**), but not in other brain regions such as the neocortex (**Fig. 4j,l**), where no lentiviral vectors were delivered.

In order to assess the effects of lenti-siBACE1-6 on neurodegeneration, we used LCSM to measure the amount of microtubule-associated protein 2 (MAP2) and synaptophysin in APP transgenic mice. MAP2, a marker of dendritic integrity (**Fig. 5**), and synaptophysin, a marker of synaptic integrity (data not shown), were measured in the hippocampus (the site of injection; **Fig. 5a–d**) and neocortex (which did not receive an injection; **Fig. 5e–h**) of lenti-siGlut4– and lenti-siBACE1-6–treated mice. Nontransgenic mice treated with the lenti-siGlut4 or the lenti-siBACE1-6 vectors did not show obvious neurodegenerative alterations in the presynaptic terminals (data not shown) or dendrites (**Fig. 5a,b,e,f,i,j**). APP transgenic mice treated with the lenti-siBACE1-6

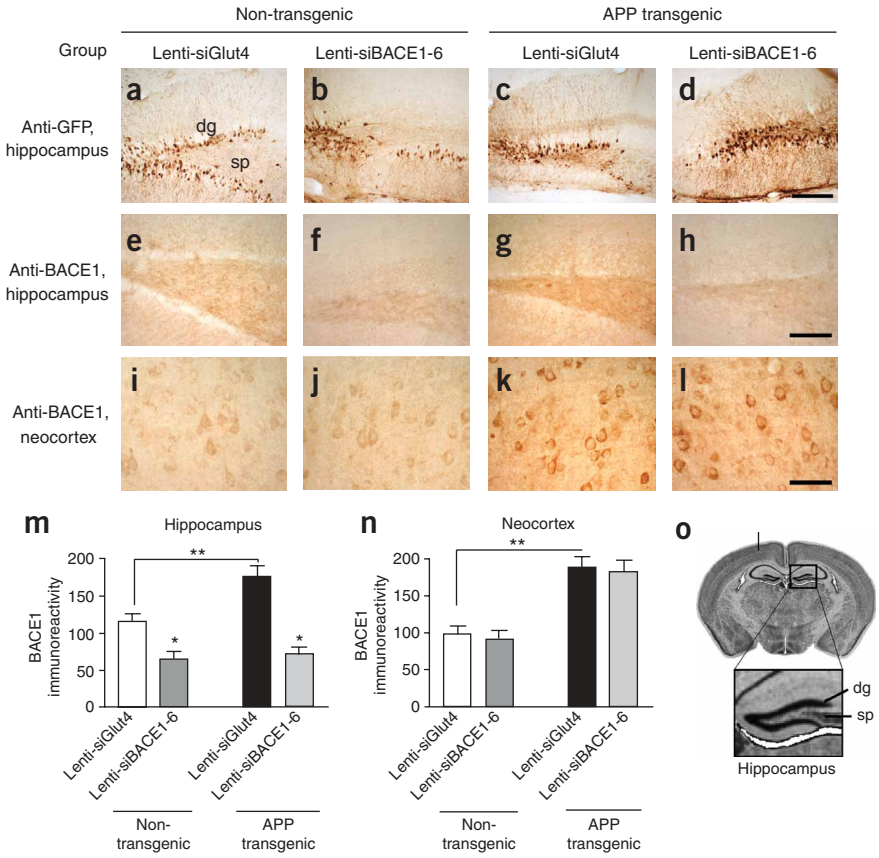

Figure 2 Characterization of the effects of lenti-siBACE1-6 expression in the brains of APP transgenic mice. (**a**–**d**) Anti-eGFP immunoreactivity in the hippocampus (the injection site) shows comparable and consistent expression of lenti-siRNA constructs in the dentate gyrus (dg) and stratus polymorphus (sp). (**e**) Anti-BACE1 immunoreactivity in the hippocampus of nontransgenic mice treated with lenti-siGlut4. (**f**) Reduced BACE1 immunostaining in the hippocampus of nontransgenic mice treated with lenti-siBACE1-6 vector. (**g**) Intense BACE1 immunoreactivity in the hippocampus of APP transgenic mice treated with lenti-siGlut4. (**h**) Reduced BACE1 expression in APP transgenic mice treated with lenti-siBACE1-6 vector. (**i**,**j**) Anti-BACE1 reacted with pyramidal cell bodies in the neocortex, which was not injected, of nontransgenic mice. (**k**,**l**) Increased BACE1 expression in APP transgenic animals in the neocortex, where no injections were placed. (**m**) Analysis of BACE1 immunoreactivity (optical density) in the hippocampus (the injection site). (**n**) Analysis of BACE1 immunoreactivity (optical density) in the neocortex (where there was no injection). (**o**) Anatomical representation of physical locations of the site of injection (hippocampus, including dentate gyrus and stratus polymorphus) and the control brain region (neocortex) that was not injected. $^{*}P < 0.05$, compared with lenti-siGlut4–treated controls (one-way ANOVA with post-hoc Tukey-Kramer). $^{**}P < 0.05$, compared with nontransgenic controls (one-way ANOVA with post-hoc Tukey-Kramer). Scale bars: **a**–**d**, 100 μm; **e**–**h**, 50 μm; **i**–**l**, 30 μm. Error bars: s.e.m.

showed MAP2-immunolabeled dendrites (**Fig. 5d**,**i**) and synaptophysin-immunoreactive terminals (data not shown) in the hippocampus comparable to nontransgenic controls. In contrast, APP transgenic mice treated with the lenti-siGlut4 vector control showed a significant decrease in MAP2 immunoreactivity (**Fig. 5c**,**i**) compared with nontransgenic controls. APP transgenic mice treated with the lenti-siBACE1-6 vector showed an amelioration of the neurodegenerative alterations in the hippocampus (**Fig. 5a–d**,**i**) but not in the neocortex, which did not receive an injection of lenti-siBACE1-6 vector (**Fig. 5e–h**,**j**).

To determine if the neuroprotective effects of the lenti-siBACE1-6 were associated with amelioration of the performance deficits in APP transgenic mice, animals were tested in the Morris water maze to examine their spatial learning and memory (**Fig. 6**). By day 3 of the cued component of this test (with the platform visible), all four groups achieved comparable escape latencies (**Fig. 6a**). When the platform was hidden, APP transgenic animals treated with lenti-siGlut4 showed deficits in their performance compared with nontransgenic mice treated with lenti-siGlut4 or lenti-siBACE1-6 (**Fig. 6b**). In contrast, APP transgenic mice treated with lenti-siBACE1-6 performed similarly to the nontransgenic controls (**Fig. 6a**). Similar effects were observed in the last day of the test, when the platform was removed from the water maze (the probe test; **Fig. 6b**).

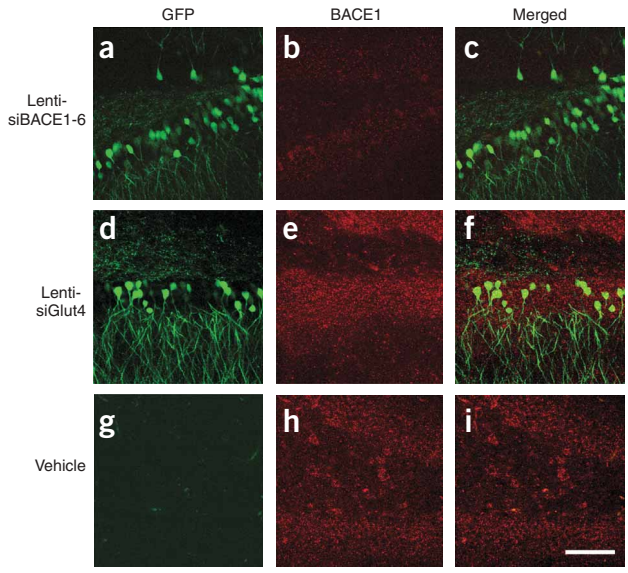

Figure 3 Immunolabeling patterns of BACE1 expression and the lenti-siRNA distribution. Sections from APP transgenic mice treated with the eGFP-tagged lenti-siRNA vectors (green) were co-immunolabeled with an antibody against BACE1 (red) and imaged with the LSCM. All sections are from the hippocampus of treated mice. (**a**–**c**) Lenti-siBACE1-6–treated mice. Areas within the hippocampus expressing the eGFP-tagged vector have reduced BACE1 immunolabeling. (**d**–**f**) Mice treated with the eGFP-tagged control lenti-siGlut4 show unchanged expression of BACE1 in the hippocampus. (**g**–**i**) Mice treated with a saline vehicle show unchanged expression of BACE1 in the hippocampus. Scale bar, 60 μm.

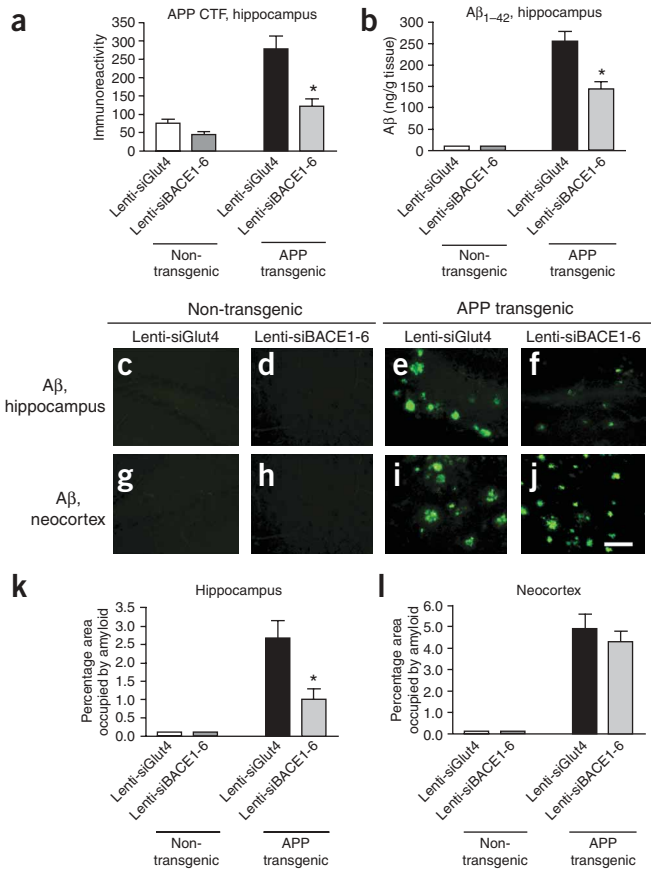

Figure 4 Effects of the lenti-siBACE1-6 treatment on APP processing and amyloid deposition. (**a**) Levels of APP CTFs (as determined by western blot) were significantly reduced in the hippocampus of the APP transgenic lenti-siBACE1-6 mice compared with the APP transgenic lenti-siGlut4 group. (**b**) Levels of $A\beta_{1-42}$ (as determined by ELISA) were significantly reduced in the hippocampus of APP transgenic lenti-siBACE1-6 animals compared with the APP transgenic lenti-siGlut4 group. (**c**,**d**) Images from the hippocampus of nontransgenic mice showing the absence of amyloid deposits. (**e**) APP transgenic mice treated with the lenti-siGlut4 show abundant amyloid deposits in the hippocampus. (**f**) APP transgenic mice treated with the lenti-siBACE1-6 show reduced accumulation of amyloid deposits in the hippocampus. (**g**,**h**) Neocortex of nontransgenic mice with hippocampal injections of the lenti-siRNA vectors. Amyloid deposits were absent. There was no injection in neocortex. (**i**) APP transgenic mice from the lenti-siGlut4–treated group show abundant amyloid deposits in the neocortex. (**j**) APP transgenic mice with hippocampal injections of lenti-siBACE1-6 show comparable numbers of amyloid deposits in the neocortex, which was not injected. (**k**) Image analysis of the percent area of the hippocampus occupied by amyloid deposits. In the APP transgenic lenti-siBACE1-6 group, there was a significant reduction compared with the APP transgenic lenti-siGlut4 group. (**l**) In the neocortex, which was not injected, no differences were observed between the APP transgenic groups. $^*P < 0.05$ compared with lenti-siGlut4–treated controls (one-way ANOVA with post-hoc Tukey-Kramer). Scale bar, 80 μm. Error bars: s.e.m.

DISCUSSION

The rapid development of gene delivery approaches holds promise for establishing new treatments for neurodegenerative disorders such as Alzheimer disease[21]. For example, we have previously shown that gene transfer of the Aβ-degrading enzyme neprilysin using a lentiviral vector reduces neurodegeneration and amyloid deposition in APP transgenic mice[22]. Alternative approaches for reducing APP degradation products, such as suppressing BACE1 expression, have also been considered[23]. Here, we have shown that lentiviral vector–mediated expression of an siRNA specific to BACE1 knocks down the expression of the target protein both *in vitro* and *in vivo*. Furthermore, reduced BACE1 levels result in decreased production of BACE-dependent APP proteolytic products, reduction in amyloid burden and amelioration of the dendritic and synaptic pathology in the hippocampus of APP transgenic mice. These results are consistent with a recent *in vitro* study showing that lipofectamine-mediated delivery of siBACE in APP-transfected neuronal cell lines and primary neuronal cultures from APP transgenic mice results in reduced APP CTFs and Aβ production and is protective from the neurotoxic effects of peroxide[24]. Thus, it is

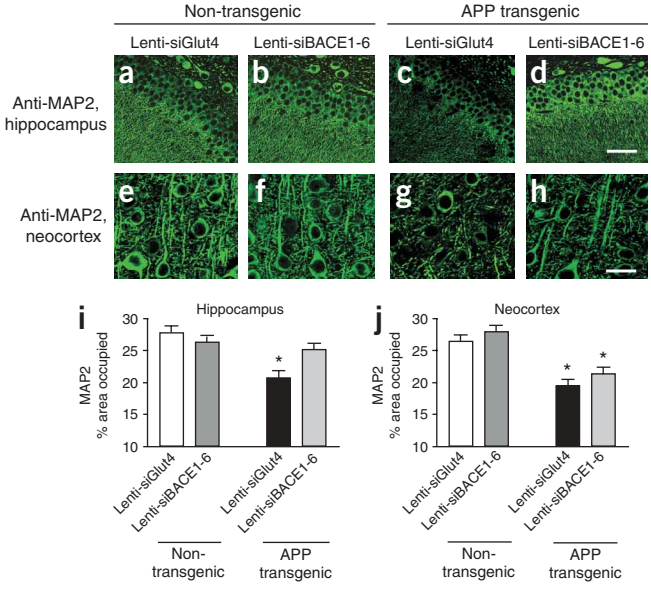

Figure 5 Effects of lenti-siBACE1-6 on neuronal integrity. (**a**,**b**) Images from the hippocampus (dentate gyrus) of nontransgenic mice showing abundant MAP2-immunoreactive dendrites in the molecular layer. (**c**) APP transgenic mice treated with lenti-siGlut4 show decreased MAP2-immunoreactive dendrites compared with nontransgenic controls. (**d**) APP transgenic mice treated with lenti-siBACE1-6 show a significant increase in MAP2-immunoreactive dendrites compared with transgenic mice treated with lenti-siGlut4 and similar expression of MAP2 as in nontransgenic controls. (**e**,**f**) Images from the neocortex of nontransgenic mice with hippocampal injections of the lenti-siRNA vectors show abundant MAP2-immunoreactive dendrites. Neocortex was not injected. (**g**) APP transgenic mice with hippocampal injections of lenti-siGlut4 show decreased MAP2-immunoreactive dendrites in the neocortex compared with nontransgenic controls. (**h**) APP transgenic mice with hippocampal injections of lenti-siBACE1-6 have MAP2-immunoreactive dendrites in the neocortex, which was not injected. Phenotype is similar to dendrites in the APP transgenic mice with hippocampal injections of lenti-siGlut4. (**i**) Image analysis of the percent area of the hippocampus occupied by MAP2-immunoreactive dendrites. In the APP transgenic lenti-siBACE1-6 group, there was a significant increase compared with the APP transgenic lenti-siGlut4 group. (**j**) Image analysis of the percent area of the neocortex occupied by MAP2-immunoreactive dendrites. In the neocortex, which was not injected, no differences were observed between the APP transgenic groups. $^*P < 0.05$ (one-way ANOVA with post-hoc Tukey-Kramer). Scale bars, 15 μm. Error bars: s.e.m.

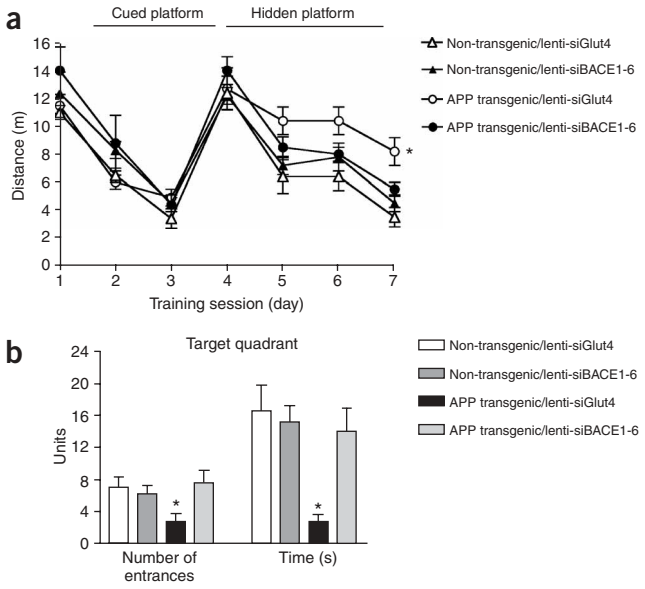

Figure 6 Behavioral analysis of transgenic mice treated with lenti-siBACE1-6 in the Morris water maze. (**a**) Spatial learning component of the water maze assay that includes 3 d of training with the visible platform (cued) and 4 d of testing with the submerged platform (hidden). (**b**) Memory portion of the test with the platform removed to evaluate the number of entrances and time spent swimming in the target quadrant where the platform was located. Compared with APP transgenic mice treated with lenti-siGlut4, transgenic mice treated with lenti-siBACE1-6 showed an improvement in learning and memory similar to the performance of nontransgenic controls. $*P < 0.05$ by repeated measures of one-way ANOVA. Error bars: s.e.m.

possible that siBACE treatment could improve behavioral deficits in APP transgenic mice, and, consistent with results that have been reported in APP transgenic mice crossed into a BACE1 null background[12], the present study demonstrates that APP transgenic mice treated with lenti-siBACE1-6 show an amelioration of performance deficits in the water maze. Notably, the lenti-siBACE1-6–treated animals perform similarly to nontransgenic controls. On the basis of these results, it has been proposed that the mechanisms through which reduced BACE1 expression might rescue the neurodegenerative phenotype in the APP transgenic mice might be mediated in part by the reduced production of Aβ species[12]. Considerable evidence now supports the notion that Aβ oligomers and protofibrils, rather than amyloid fibrils, might be responsible for the synaptic dysfunction and injury in APP transgenic mice and in patients with Alzheimer disease[25–28]. However, it is worth noting that BACE1 suppression reduces the generation not only of Aβ monomers (the precursor of the Aβ oligomers) but also of CTFs. Although most of the current research in Alzheimer disease is focused on the potential neurotoxic effects of Aβ, APP CTFs have also been shown to have deleterious effects. For example, in primary neuronal cultures, accumulation of the C99 fragment promotes apoptosis[29]. Transgenic mice overexpressing C99 in neurons show hippocampal degeneration as well as deficits in memory and long term potentiation[29–32]. The mechanisms through which APP CTFs might promote neurodegeneration are not completely clear, but some studies suggest that increased intracellular calcium might be involved[33].

The use of siRNA offers a unique opportunity for the development of gene therapy approaches for the treatment of neurodegenerative disorders[23]. For example, a recent study has shown that recombinant adeno-associated virus vector delivery of an siRNA that suppresses the mutant ataxin-1 gene rescues the phenotype in a transgenic mouse model of spinocerebellar ataxia[34]. Notably, the present study shows that lentiviral vector–mediated gene transfer of an siRNA rescues the neuropathological and neurodegenerative alterations in a transgenic model of Alzheimer disease. This is of substantial importance because increased levels of BACE1 and APP fragments have recently been identified in Alzheimer disease brains[14,15,35,36], raising the possibility that increased BACE1 activity may be causally involved in the pathogenesis of Alzheimer disease. For this reason, several new approaches have been devised to reduce BACE1 activity *in vivo*[16]. It is important that the focus in these studies be on lowering BACE1 activity, rather than abolishing it completely, because alternative substrates of BACE1 exist. For example, both *in vitro*[37] and *in vivo*[38], BACE1 cleaves ST6Gal I, a sialyltransferase whose cleavage product is secreted. Recently, it has been shown that BACE1 also cleaves β subunits of voltage-gated sodium channels[39]. As this enzyme has such diverse substrates, full elimination of BACE1 expression might have deleterious effects. Some promising results have been recently obtained in studies investigating *in vivo* BACE1 reduction by delivering small peptide inhibitors in APP transgenic mice using a carrier peptide molecule[40]. However, because the BACE1 active site is more open and less hydrophobic than in other proteases, the development of small molecule inhibitors is a serious challenge. Thus, alternative approaches toward suppressing BACE1 such as the one described in the present study might be of interest. In conclusion, our results suggest that lentiviral vector delivery of BACE1 siRNA can specifically reduce the cleavage rate of APP *in vivo*, indicating the potential therapeutic value of this approach for treating Alzheimer disease.

METHODS

Short hairpin RNA (shRNA) design and validation. Expression of siRNAs was achieved by transcription of shRNAs driven by an RNA polymerase III (Pol III) promoter. shRNA constructs were generated by synthesizing an 83-mer oligonucleotide containing (i) a 19-nucleotide sense strand and a 19-nucleotide antisense strand, separated by a nine-nucleotide loop (TTCAAGAGA; ref. 41; **Fig. 1a**); (ii) a stretch of five adenines as a template for the Pol III promoter termination signal; (iii) 20 nucleotides complimentary to the 3′ end of the Pol III H1 promoter and (iv) a 5′ end containing a unique *Xba*I restriction site. Briefly, the long oligonucleotide was used together with a T3 oligonucleotide (5′-CTCGAAATTAACCCTCACTAAAGGG-3′) to PCR-amplify a fragment containing the entire H1 promoter plus shRNA sequences; the resulting product was cut with *Xba*I and ligated to a *Nhe*I-digested lentivirus vector[19]. PCR conditions were as follows: an initial cycle of 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 50 s. The following shRNA oligonucleotides were used (bold: sense and antisense strands; underlined: loop): siBACE1-1 (5′-CTGTCTAGACAAAAA**TGGACTGCAAGGAGTACAA**TCTCTTGAA**TTGTACTCCTTGCAGTCCA** GGGGATCTGTGGTCTCATACA-3′), SiBACE1-2 (5′-CTGTCTAGACAAAAA **GCTGCAGTCAAATCCATCA**TCTCTTGAA**TGATGGATTTGACTGCAGC** GGGGATCTGTGGTCTCATACA-3′), siBACE1-6 (5′-CTGTCTAGACAAAAA **GACTGTGGCTACAACATTC**TCTCTTGAA**GAATGTTGTAGCCACAGTC** GGGGATCTGTGGTCTCATACA-3′) and siGlut4 (5′-CTGTCTAGACAAAAA **CTTCATTGTCGGCATGGGT**TCTCTTGAA**ACCCATGCCGACAATGAAG** GGGGATCTGTGGTCTCATACA-3′).

Validation of shRNA constructs was performed by calcium phosphate transfection to HEK293T cells, followed by protein extraction and western blot analysis or cell media collection and ELISA analysis. For the BACE1 detection assay, HEK293T cells were transfected with a combination of siRNA-expressing constructs and cDNA of human or mouse BACE1 expressed from pcDNA3.1 (Invitrogen). Western blot analysis was used to determine the level of BACE1 expression using rabbit anti-BACE1 (1:1,000; BD Pharmingen). For Aβ detection assays, HEK293T cells were transduced both with a plasmid encoding mutant APP, an internal ribosome entry site and presenilin-1

(pAPP-IRES-presenilin) and with siRNA-expressing constructs. An shRNA construct that silences the glucose transporter-4 (Glut4) was used as the vector control for *in vitro* and *in vivo* experiments. Expression of Aβ was measured as described below.

Production of lentiviral vectors. Vector plasmids were constructed for the production of third-generation lentiviruses expressing siRNA for BACE1-6 or Glut4. All vectors contained the eGFP coding sequence located in the middle of the lentiviral vector. This sequence is driven by a cytomegalovirus (CMV) promoter and terminates using the polyadenylation signal in the 3′ long terminal repeat (LTR). Downstream of the eGFP is a woodchuck hepatitis virus regulatory element (WPRE) that enhances the expression of the transgene. Recombinant lentiviruses were produced by transient transfection in HEK293T cells using the calcium phosphate method, as described previously[20,42,43]. Infectious lentiviruses were harvested at 48 and 72 h post-transfection and filtered through 0.22-μm-pore cellulose acetate filters as described. The infectious lentiviruses were concentrated by ultracentrifugation (2 h at 50,000*g*) and subsequently purified by ultracentrifugation on a 20% sucrose gradient (2 h at 46,000*g*) as described[20,42]. Vector concentrations were analyzed using an immunocapture p24-*gag* ELISA (Alliance; DuPont-NEN)[20] and by flow cytometry quantification of eGFP-positive transduced cells, as described previously[22].

Determination of Aβ levels by ELISA. For *in vitro* studies, HEK293T cells were transduced first with a lentiviral vector that expressed APP with the Swedish and London mutations (K670M / N671L / V717I) and mutant presenilin-1 (Δ9; multiplicity of infection (MOI) 10). One day later these cells (HEK293T-AIP) were transduced with lentiviral vectors expressing siRNAs specific for siBACE1-6 or siGlut4 at the same MOI. Three days post-transduction, the cell culture medium was changed and old medium was collected to be assayed 24 h later. Cell culture supernatant was measured for concentrations of $A\beta_{1-42}$ using a specific ELISA kit (Biosource International). The background from conditioned medium of untransduced 293T cells was subtracted from the sample values. *In vivo* studies were as previously described[44,45]: briefly, the hippocampus was dissected from each of the nontransgenic and APP transgenic mice and was homogenized in 5 M guanidine and centrifuged at 16,000*g* for 20 min at 4 °C. The supernatant was diluted tenfold in Dulbecco's PBS (pH 7.4) containing 5% bovine serum albumin and 0.03% Tween 20. Quantification of $A\beta_{1-42}$ in the diluted brain homogenates was performed with a commercially available sandwich-type ELISA (Biosource International); results were expressed in μg/g of protein of wet weight tissue.

Determination of BACE1 and APP CTFs by western blot. Brain homogenates from the brains of mice treated with lenti-siBACE1-6 or lenti-siGlut4 were separated into cytosolic and particulate fractions as previously described[46]. Cytosolic fractions were loaded onto a 10% Bis-Tris gel and transferred onto Immobilon membranes (Millipore); blots were probed with a monoclonal antibody against Aβ (6E10, 1:1,000; Signet Laboratories). Particulate fractions were loaded onto a 4–12% Bis-Tris gel and transferred onto Immobilon membranes; blots were probed with rabbit antibodies against BACE1 (1:1,000; ProSci) and APP CTFs (1:5,000; provided by E. Koo, University of California San Diego). Blots were then incubated with anti-mouse or anti-rabbit secondary antibodies (1:5,000) tagged with horseradish peroxidase (HRP). Further western blot analysis was performed to assess the specificity of the BACE1 antibody to recognize both human and murine variants of this molecule. For this purpose, western blot analysis was performed with brain homogenates from nontransgenic mice, human BACE1–transgenic[47] mice, BACE1-deficient mice[11] and lysates from HEK293T cells transfected with a plasmid expressing murine BACE1, human BACE1 or eGFP (control). Proteins were separated on a 10% Bis-Tris gel, transferred to Immobilon membranes and probed with the BACE1 antibody (1:1,000; ProSci), followed by incubation with a secondary anti-rabbit antibody (1:5,000). All blots were visualized with enhanced chemiluminescence (ECL, PerkinElmer) and analyzed on a VersaDoc gel imaging system (BioRad).

Transgenic mouse lines, intracerebral injections of lentiviral vectors and behavioral analysis. All experiments were performed following US National Institutes of Health guidelines for the humane treatment of animals, with approval from the animal subjects committee at the University of California San Diego. Briefly, as previously described[48], for the present study we used transgenic mice expressing high levels of double mutant (V717I and K670M/N671L) human APP751 under the control of the murine Thy1 regulatory sequences (line 41). This line of APP transgenic mice was selected because early on, they develop neurodegenerative alterations with memory impairment and amyloid plaques[49]. A total of 16 APP transgenic mice and 16 nontransgenic age-matched controls (10 months old) received unilateral injections of 2 μl of the lentiviral preparations (1.5×10^7 transduction units) into the hippocampus (using a 5 μl Hamilton syringe, 0.25 μl/min). For each group, a total of eight mice received the lenti-siBACE1-6 vector, and the other eight received the lenti-siGlut4 vector control. Briefly, as previously described[22], mice were placed under anesthesia on a Koft stereotaxic apparatus, and coordinates were determined by means of the Franklin and Paxinos atlas. The lentiviral vectors were delivered using a Hamilton syringe connected to a hydraulic system to inject the solution at a rate of 1 μl every 2 min. To allow diffusion of the solution into the brain tissue, the needle was left for an additional 5 min after the completion of the injection. Mice survived for 4 weeks after the lentiviral injection. Mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains were removed and post-fixed in phosphate-buffered 4% PFA (pH 7.4) at 4 °C for 48 h for neuropathological analysis.

For behavioral and biochemical analysis, an additional set of ten nontransgenic and ten APP transgenic mice (10 months old) received bilateral injections with 2 μl of the lentiviral preparations into the hippocampus. For each group, a total of five mice received the lenti-siBACE1-6 vector, and the other five received the lenti-siGlut4 vector control as described above. After 4 weeks, mice were tested for spatial learning and memory in the water maze as previously described[49]. Briefly, animals were trained in a visible platform (cued platform) for 3 d followed by 4 d of testing for learning and memory abilities with the platform submerged (hidden platform), and then a final test trial was performed with the platform removed (probe test). At the end of the water maze test, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. The right hemibrain was post-fixed in phosphate-buffered 4% paraformaldehyde (PFA) (pH 7.4) at 4 °C for 48 h for additional neuropathological analysis, and the left hemibrain was snap-frozen and stored at –70°C for subsequent protein analysis.

Immunocytochemical, neuropathological and confocal microscopy analyses. The fixed brains were serially sectioned at 40 μm with a vibratome (Leica) and sections were immunolabeled as previously described[22,49], with antibodies against Aβ (4G8, 1:2,000; Signet Laboratories), APP CTFs (1:5,000; CT-15, courtesy of E. Koo, University of California San Diego), MAP2 (1:50, Chemicon) or synaptophysin followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, and were imaged with the LSCM (BioRad MRC1024) as previously described[22,49]. The percent area of the neuropil occupied by Aβ-immunoreactive plaques was used to estimate amyloid load (nine fields from three sections of the neocortex and hippocampus), and the percent area of the neuropil occupied by MAP2-immunoreactive dendrites and synaptophysin-immunoreactive terminals was used to evaluate the integrity of the neuronal structure. To measure BACE1 expression, sections were immunolabeled with a rabbit polyclonal antibody (BACE1, 1:500, ProSci) and developed with diaminobenzidine (DAB). Sections were imaged with a digital microscope and analyzed with the Quantimet 570C (Leica). Additional immunolabeling experiments were performed to ascertain colocalization of the lentiviral vectors and BACE1 expression. For this purpose, vibratome sections were incubated with antibodies against BACE1 visualized with Tyramide Red (PerkinElmer), followed by analysis with the LSCM[22]. The lentiviral vectors were imaged in the FITC channel, as the constructs contain an eGFP tag. All sections were processed simultaneously under the same conditions, and experiments were performed twice in order to assess the reproducibility of results. Sections were imaged with a Zeiss 63× (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss) with an attached MRC1024 LSCM system (BioRad)[46].

Statistical analysis. All *in vivo* and *in vitro* experiments were conducted in triplicate on blind-coded samples. After the results were obtained, the code was broken and data were analyzed with the StatView 5.0 program (SAS). Differences among APP transgenic and nontransgenic mice treated with the

lentiviral vectors were assessed by one-way ANOVA followed by post-hoc Dunnett's or Tukey-Kramer as indicated. The null hypothesis was rejected at the 0.05 level. Results are expressed as mean ± s.e.m.

Accession codes. GenBank: BACE1, AF201468.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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