**ORIGINAL ARTICLE**

**Herpes simplex virus RNAi and neprilysin gene transfer vectors reduce accumulation of Alzheimer’s disease-related amyloid-β peptide in vivo**

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Accumulation of insoluble aggregates of amyloid-β peptide (Aβ), a cleavage product of amyloid precursor protein (APP), is thought to be central to the pathogenesis of Alzheimer’s disease (AD). Consequently, downregulation of APP, or enhanced clearance of Aβ, represent possible therapeutic strategies for AD. We generated replication-defective herpes simplex virus (HSV) vectors that inhibit Aβ accumulation, both in vitro and in vivo. In cell culture, HSV vectors expressing either (i) short hairpin RNA directed to the APP transcript (HSV-APP/shRNA), or (ii) neprilysin, an endopeptidase that degrades Aβ (HSV-neprilysin), substantially inhibited accumulation of Aβ. To determine whether these vectors showed similar activity in vivo, we developed a novel mouse model, in which overexpression of a mutant form of APP in the hippocampus, using a lentiviral vector (LV-APPsw), resulted in rapid Aβ accumulation. Co-inoculation of LV-APPsw with each of the HSV vectors showed that either HSV-APP/shRNA or HSV-neprilysin inhibited Aβ accumulation in this model, whereas an HSV control vector did not. These studies demonstrate the utility of HSV vectors for reducing Aβ accumulation in the brain, thus providing useful tools to clarify the role of Aβ in AD that may facilitate the development of novel therapies for this important disease.

**Keywords:** herpes simplex virus; Alzheimer’s disease; amyloid-β protein; siRNA; neprilysin; lentivirus

**Introduction**

Alzheimer’s disease (AD) is the most common type of dementia, and the most prevalent neurodegenerative disease, affecting more than 4 million individuals in the United States alone. Although significant improvement has been made in clinical diagnosis and care for AD patients, treatments that prevent clinical progression have not yet been identified. Amyloid-β peptide (Aβ) is generated from the amyloid precursor protein (APP) by the combined cleavage activity of β- and γ-secretases. Amyloid-β peptide is a major component of neuritic plaques that accumulate in the brains of AD patients and together with neurofibrillary tangles represent the hallmark pathological features of the disease. Studies performed to investigate Aβ accumulation suggest that overproduction and/or ineffective clearance of the peptide contributes to the disease development.¹,² Thus, downregulation of APP gene expression or upregulation of Aβ clearance may prevent or slow the disease process. Recently, inhibitors of β- and γ-secretases have been developed.³ However, these enzymes have additional functions, other than APP processing, and the long-term effects of inhibiting these processes are unknown. For example the γ-secretase complex is involved in post-translational processing of the Notch receptor,⁴ which has crucial physiological roles in the brain, raising the concern that long-term inhibition of γ-secretase may adversely affect normal brain function. Amyloid-β peptide might also be eliminated by antibody-directed scavenging of plaque-forming proteins through opsonization. Immunotherapy against Aβ has been shown to reduce the establishment of plaques in an AD transgenic mouse model,⁵ suggesting a promising approach for clinical evaluation. However, clinical studies demonstrated serious adverse events related to the development of meningoencephalitis in the vaccination groups, which forced termination of phase II trials.⁶ Another approach to treatment might involve gene therapy strategies to reduce the synthesis of Aβ or prevent its accumulation. The recent development of short interfering RNA (siRNA) technology, and its successful application in a number of experimental systems, suggests that targeting APP mRNA could represent a viable strategy for downregulating APP synthesis. Alternatively, methods to increase expression of natural membrane-bound cellular proteases, such as neprilysin, could be used to degrade extracellular Aβ.

RNA interference (RNAi; reviewed in Dykxhoorn et al.⁷) is a natural mechanism found in plants and animals that is believed to provide a host defense
response to intracellular pathogens and can be exploited to accomplish sequence-specific gene silencing. Briefly, long double-stranded RNA (dsRNA) (>200 nucleotides (nt)) is cleaved by Dicer, a RNase III family member, into siRNA of approximately 22 nt in length. Short interfering RNA is incorporated into a multicomponent complex, the RNA-inducing silencing complex, which mediates the endonucleolytic degradation of RNA that contains sequence complementary to the siRNA. Unlike long segments of dsRNA, short dsRNA species (<30 nt) do not induce a sequence-nonspecific interferon response in mammals, and can thus be deployed as gene targeting reagents without severe perturbation of cellular physiology. Although siRNA approaches have been highly effective in targeting gene expression in cell culture studies, efficient methods for the delivery of siRNAs in vivo have limited their application. Using viral vectors, expression of siRNA molecules can be accomplished through expression of short hairpin RNAs (shRNA) that self-anneal following transcription, to form 20–30 nt stretches of dsRNA. Retroviral and adeno-associated viral (AAV)-mediated delivery of shRNA has been demonstrated; expression of the targeted genes was reduced and it has been suggested that this approach may be useful for blocking expression of disease-causing dominant alleles.\\n
Neprilysin, a 750-amino-acid type II transmembrane protein, has been identified as a potent Aβ-degrading protease using biochemical and genetic approaches. It functions as a neutral endopeptidase, cleaving small peptides of 4–5 kDa, such as enkephalins, endorphins or peptides of 4–5 kDa, such as enkephalins, endorphins or leucine-enkephalin. Neprilysin plays a vital physiological role in Aβ metabolism. We have characterized the cDNA sequence of which results in the overproduction of Aβ-degrading enzyme using biochemical and genetic approaches. Neprilysin is expressed in neuronal cells, and has therefore been the subject of considerable interest as a potential gene therapy vector for neurological diseases. In order to prevent pathogenicity in vivo, we have produced HSV-derived vectors that are deleted for multiple essential immediate-early (IE) genes. These vectors do not replicate in vivo, but transiently express a minimal subset of viral genes before establishing a state that is similar to viral latency. We have characterized the cis-acting elements that allow long-term expression of the viral latency locus in vivo, shown that these can be used to drive long-term therapeutic transgene expression in models of neurological disease, in vivo, and have shown measurable changes in relevant pathologic end points in a number of neurological disease models.

In this study, we developed recombinant HSV gene therapy vectors to reduce the amount of Aβ peptide available for plaque formation. The first vector (HSV-APP/shRNA), which carries an shRNA expression cassette targeting the APP gene driven by the human H1 RNA polymerase III (pol-III) promoter, significantly lowered APP protein levels compared to controls in a multiplicity of infection (MOI)-dependent manner in vitro. The second vector (HSV-neprilysin), which expresses neprilysin, produced a fivelfold decrease in Aβ secretion compared to cells infected with a control virus. To test the utility of these vectors in vivo, we developed a novel mouse model using a lentiviral vector (LV-APPsv) to deliver a familial AD mutant form of the APP gene (the ‘Swedish mutation’). Both the HSV-APP/shRNA and HSV-neprilysin vectors efficiently reduce Aβ expression in the lentiviral mouse model of Aβ accumulation. These results demonstrate the potential utility for exploiting these vectors, both as novel gene therapeutics for AD and as tools to elucidate the role of APP and Aβ in the pathogenesis of AD.

Results

Expression of biologically active short interfering RNA from a replication-defective herpes simplex virus vector

To inhibit APP gene expression, we designed an shRNA sequence in which a synthetic 19-nt sequence derived from a region of the APP transcript (+126 to +144, with respect to the transcription start site) was separated by a short spacer sequence from the reverse complement of the same 19-nt sequence (Figure 1a, see Materials and methods). The synthetic oligonucleotide was inserted into an expression plasmid, downstream of the pol-III H1 RNA gene promoter, which has been shown to direct expression of high levels of various shRNAs. To test the shRNA for biological activity, we used a Chinese hamster ovary (CHO) cell subclone, 7W, which expresses the human APPisoform containing the K670N/M671L Swedish double-mutation, post-translational processing of which results in the overproduction of Aβ. CHO-7W cells were transiently transfected with the APP-shRNA plasmid (pAPP-shRNA). Western blot analysis 24 h post-transfection showed that cells transfected with pAPP-shRNA expressed lower amounts of APP compared with cells transfected with control vector (Figure 1b). The concentration of Aβ peptides released into the culture medium was measured using an Aβ-specific enzyme-linked immunosorbent assay (ELISA). pAPP-shRNA-transfected cells secreted significantly less (2-fold) Aβ compared with the mock-transfected cells (Figure 1c). We then inserted the APP-shRNA cassette into a replication-defective HSV vector. The parent vector QOZH (Figure 2a) is based on the d106 mutant made by Neal DeLuca. The two essential IE genes, ICP4 and ICP27, are deleted, and two of the non-essential IE genes, ICP22 and ICP47, have mutations in their promoters, which prevent their expression in cells that do not complement ICP4 and ICP27. This vector shows minimal cytotoxicity as a result of these alterations, but can be produced to high titer in a complementing cell line. An expression cassette for green fluorescent protein (GFP), which is located in place of the deleted ICP27 gene, readily facilitates identification of vector-transduced cells. The APP-shRNA cassette was inserted into the U41 locus of the QOZH backbone to produce the vector HSV-APP/shRNA. As the CHO-7W cell line lacks HSV receptors, we generated a 293T subclone that is...
stably transfected with an expression construct for wild-type APP751, in order to test the HSV-APP/shRNA vector for APP-targeting activity in vitro. Transduction of this cell line with HSV-APP/shRNA substantially reduced APP expression, in an MOI-dependent manner (Figure 2b). These data show that a recombinant HSV vector can deliver a biologically active shRNA, targeting APP gene expression in cultured cells.

After 5 h, residual Aβb40 was measured in the medium by thiorphan 150. Next, cells were transduced with HSV-APP/shRNA at increasing MOI and, 2 days later, challenged with synthetic amyloid precursor protein-short hairpin RNA (APP-shRNA) and neprilysin expression plasmids reduce APP and amyloid-β peptide (Aβ) levels in vitro. (a) A schematic illustration depicting the genome of the HSV derivative QOZHG. The HSV-APP/shRNA and HSV-neprilysin vectors were generated by insertion of the expression cassettes from either pAPP-shRNA or pNeprilysin into the U41 locus of QOZHG. Deletions of viral immediate-early genes ICP4 and ICP27, and mutations in the promoters of ICP22 and ICP47, are shown. The presence of a green fluorescent protein (GFP) expression cassette at the ICP27 locus allowed efficient identification of transduced cells. (b) A 293T cell subclone, which expresses APPsw around the nucleus, in contrast to GFP, which is located in the surface membrane, as expected for a membrane-bound protein, whereas GFP expression from the same vector appeared in the cytoplasm (Figure 2d). To determine whether the neprilysin expressed from HSV-neprilysin showed functional Aβ protease activity, Vero cells were transduced with HSV-neprilysin at increasing MOI and, 2 days later, challenged with synthetic neprilysin. After 48 h, neprilysin expression was detected by Western blot analysis using an anti-APP primary antibody. Amyloid precursor protein expression was significantly reduced by HSV-APP/shRNA transduction; the magnitude of the effect was dependent on MOI. The fibroblast control confirms that equal amounts of protein were loaded in each lane. (e) Vero cells were transduced with either QOZHG or HSV-neprilysin. After 48 h, neprilysin expression was detected by Western blot analysis using anti-neprilysin antibody. A single band of expected size, whose intensity was MOI dependent, was detected after transduction with HSV-neprilysin, but not after transduction with QOZHG. (d) A murine neuronal cell line, N2A, was infected with HSV-neprilysin at MOI = 1 and examined microscopically 24 h later. The three panels show phase-contrast microscopy, fluorescence microscopy to detect GFP expression from the HSV-neprilysin vector and neprilysin expression, visualized by indirect immunofluorescence. To facilitate comparison between the images, the same transduced cell is marked with an arrowhead on each picture. Neprilysin expression appears localized at the cell membrane and around the nucleus, in contrast to GFP, which is located in the cytoplasm. (e) Vero cells were transduced with either QOZHG (MOI = 3) or HSV-neprilysin (MOI = 0.1, 1 or 3). After 48 h, 1000 pg/ml of synthetic Aβ1-40 peptide was added to the medium. After 5 h, residual Aβ40 was measured in the medium by enzyme-linked immunosorbent assay (ELISA). Cells transduced with HSV-neprilysin-degraded Aβ40 significantly more rapidly than cells transduced with QOZHG. The effect was MOI dependent and was abolished by addition of the specific neprilysin inhibitor thiorphan 150 μM.

Figure 2 Characterization of herpes simplex virus (HSV) vectors expressing amyloid precursor protein-short hairpin RNA (APP-shRNA) and neprilysin in vitro. (a) A schematic illustration depicting the genome of the HSV derivative QOZHG. The HSV-APP/shRNA and HSV-neprilysin vectors were generated by insertion of the expression cassettes from either pAPP-shRNA or pNeprilysin into the U41 locus of QOZHG. Deletions of viral immediate-early genes ICP4 and ICP27, and mutations in the promoters of ICP22 and ICP47, are shown. The presence of a green fluorescent protein (GFP) expression cassette at the ICP27 locus allowed efficient identification of transduced cells. (b) A 293T cell subclone, which expresses APPsw around the nucleus, in contrast to GFP, which is located in the surface membrane, as expected for a membrane-bound protein, whereas GFP expression from the same vector appeared in the cytoplasm (Figure 2d). To determine whether the neprilysin expressed from HSV-neprilysin showed functional Aβ protease activity, Vero cells were transduced with HSV-neprilysin at increasing MOI and, 2 days later, challenged with synthetic neprilysin. After 48 h, neprilysin expression was detected by Western blot analysis using an anti-APP primary antibody. Amyloid precursor protein expression was significantly reduced by HSV-APP/shRNA transduction; the magnitude of the effect was dependent on MOI. The fibroblast control confirms that equal amounts of protein were loaded in each lane. (c) Vero cells were transduced with either QOZHG or HSV-neprilysin. After 48 h, neprilysin expression was detected by Western blot analysis using anti-neprilysin antibody. A single band of expected size, whose intensity was MOI dependent, was detected after transduction with HSV-neprilysin, but not after transduction with QOZHG. (d) A murine neuronal cell line, N2A, was infected with HSV-neprilysin at MOI = 1 and examined microscopically 24 h later. The three panels show phase-contrast microscopy, fluorescence microscopy to detect GFP expression from the HSV-neprilysin vector and neprilysin expression, visualized by indirect immunofluorescence. To facilitate comparison between the images, the same transduced cell is marked with an arrowhead on each picture. Neprilysin expression appears localized at the cell membrane and around the nucleus, in contrast to GFP, which is located in the cytoplasm. (e) Vero cells were transduced with either QOZHG (MOI = 3) or HSV-neprilysin (MOI = 0.1, 1 or 3). After 48 h, 1000 pg/ml of synthetic Aβ1-40 peptide was added to the medium. After 5 h, residual Aβ40 was measured in the medium by enzyme-linked immunosorbent assay (ELISA). Cells transduced with HSV-neprilysin-degraded Aβ40 significantly more rapidly than cells transduced with QOZHG. The effect was MOI dependent and was abolished by addition of the specific neprilysin inhibitor thiorphan 150 μM.
Aβ_{40}. This was added to the culture medium for 5 h, following which the residual Aβ_{40} level in the medium was measured by ELISA. Synthetic Aβ_{40} was efficiently degraded by HSV vector-mediated neprilysin (Figure 2e). At an MOI = 3, the amount of Aβ_{40} remaining in the medium was 20% of the amount found after cells were transduced with an equivalent dose of control vector. As expected, Aβ-degrading activity was completely inhibited by the introduction of Thiorphan, formally proving that the biochemical protease activity observed was directly attributable to neprilysin (Figure 2e). Herpes simplex virus vector-expressed neprilysin also degraded synthetic Aβ_{42}, although with lower efficiency than observed for synthetic Aβ_{40} (data not shown).

**Generation of an amyloid-β peptide mouse model using a lentiviral vector**

To investigate the efficiency of both HSV-APP/shRNA and HSV-neprilysin in reducing Aβ accumulation in vivo,
we generated an Aβ mouse model using a lentivirus, LV-APP<sub>sw</sub>. The lentivirus was designed to express the APP<sub>751</sub> isoform containing the K670N/M671L Swedish double-mutation, under the control of HCMV IE promoter (Figure 3a). To facilitate the identification of cells transduced by LV-APP<sub>sw</sub>, a marker gene (DsRed; Clontech, Mountain View, CA, USA) was inserted downstream of an internal ribosomal entry site (IRES), so that both the APP<sub>sw</sub> gene and the DsRed gene are transcribed as a single bicistronic mRNA. The 293T cells transduced with LV-APP<sub>sw</sub> secreted high levels of Aβ<sub>40</sub> peptide compared with mock-transduced cells (Figure 3b). We stereotactically injected LV-APP<sub>sw</sub> into the hippocampus of 4-week-old C57BL/6J mice. At 10 days post-injection, robust Aβ<sub>40</sub> expression was detected at the injection site within the mouse hippocampus (Figure 3d), by indirect immunofluorescence using an Aβ<sub>40</sub>-specific antibody (Biosource, Camarillo, CA, USA) and a Cy5-conjugated secondary antibody. DsRed expression was also detected within the same cells, showing that the Aβ-expressing cells were transduced by the LV vector, and that Aβ<sub>40</sub> production was attributable to vector-mediated APP<sub>sw</sub> expression. In order to assess the type of cells that were transduced in these experiments, we carried out double detection studies, in which sections were labeled with an antibody to the neuron-specific isoform of the glycolytic enzyme enolase (NSE), and then the expression pattern of NSE compared with that of DsRed (Figure 3c). As previously reported with vesicular stomatitis virus-G-pseudotyped lentiviruses, transduced cells were almost exclusively neuronal.\textsuperscript{31} These data show that rapid neuronal overproduction of Aβ in vivo can be produced by inoculation of LV-APP<sub>sw</sub>, thereby generating a useful in vivo system to assay the effects of Aβ-targeting HSV vectors in the brain.

**Reduction of amyloid-β peptide expression by recombinant herpes simplex virus vectors in mouse central nervous system**

Using the lentiviral-based mouse model for Aβ overexpression, we tested HSV-APP-shRNA and HSV-neprilysin for their activity in reducing Aβ expression in vivo. The basic experimental paradigm was as follows: the hippocampus on one side of a mouse (the ‘test side’) was stereotactically co-injected with LV-APP<sub>sw</sub> and either HSV-APP/shRNA or HSV-neprilysin; the contralateral hippocampus (the ‘control side’) was co-injected with LV-APP<sub>sw</sub> and QOZHG, the parental HSV vector that is isogenic to HSV-APP/shRNA and HSV-neprilysin, except for the absence of Aβ-targeting genes. At 10 days or 4 weeks post-injection, brain sections were examined by indirect immunofluorescence for Aβ<sub>40</sub> and by fluorescence microscopy for expression of DsRed (expressed from LV-APP<sub>sw</sub>) and GFP (expressed from each of the HSV vectors). Figure 4a shows that the patterns of DsRed and GFP expression were essentially completely overlapping, suggesting that a population of neurons was co-transduced by the two different vectors. In each experiment, we first identified sections in which GFP expression was similar on each side by fluorescence microscopy, thus establishing that the model was comparing tissue with a similar level of transduction of test or control vector. We were also able to verify DsRed expression in test and control sides in the HSV-neprilysin experiments. We then compared sections from the test and control sides of each animal for expression and localization of Aβ<sub>40</sub>.

At 10 days post-injection, Aβ<sub>40</sub> immunoreactivity was greatly reduced in the hippocampus injected with HSV-APP/shRNA, compared with the QOZHG-injected control side (Figure 4b). Densities of Aβ<sub>40</sub> immunoreactivity were measured from images of at least three sections per brain using MetaMorph software and normalized to GFP expression, to control for any small degree of variability in transduction between sides. Figure 4c shows quantitative data derived from the densitometric analysis of sections exemplified by those shown in Figure 4a. The hippocampus on the test side, which was injected with HSV-APP/shRNA, displayed a significantly lower density of Aβ<sub>40</sub> expression compared with the control side, which was injected with QOZHG (P < 0.008). As the DsRed marker is expressed from the bicistronic APP<sub>sw</sub>-DsRed mRNA, shRNA targeting of APP would also be expected to prevent DsRed expression. As expected, the intensity of DsRed fluorescence was decreased in HSV-APP-shRNA-injected hippocampus, in comparison with the control side (P < 0.016).

A parallel set of experiments was carried out with a similar experimental design, but using HSV-neprilysin instead of HSV-APP-shRNA (Figure 5). At 4 weeks post-injection, brain sections were examined for neprilysin expression and Aβ<sub>40</sub> immunoreactivity, using the same procedures described above. Neprilysin expression was detected only on the side injected with the HSV-neprilysin vector (Figure 5a). This is in contrast to results reported elsewhere, where neprilysin expression was seen both ipsilateral and contralateral to vector transduction, and attributed to neuronal transport of the protein.\textsuperscript{32} Although the reasons for the difference between this study and the present report are unclear, the absence of detectable neprilysin expression on the control side means that Aβ<sub>40</sub> expression on the control side is both appropriate and legitimate as a control for comparison of Aβ<sub>40</sub> expression level on the neprilysin test side. Figure 5b and c show that HSV-neprilysin reduced Aβ<sub>40</sub> expression significantly in the test hippocampus, compared with the control hippocampus injected with QOZHG (P < 0.009). As expected, neprilysin expression did not affect DsRed expression, in contrast to the shRNA directed against the APP transcript.

**Discussion**

The purpose of these studies was to generate and characterize gene transfer reagents that decreased Aβ expression in vitro and in vivo. The data shown here establish three important new points: HSV vectors can be used to express RNAi reagents; lentiviral gene transfer can be used to construct a convenient in vivo model of Aβ accumulation; and the HSV vectors generated in this study are effective at suppressing Aβ accumulation in vivo, with potential consequences for the development of gene therapy approaches for AD.

**Short hairpin RNA expression using a herpes simplex virus vector**

We provide the first demonstration that shRNA may be expressed at appropriate levels from the HSV vector...
backbone in order to target a cellular gene; the anti-APP shRNA efficiently blocked expression of the APP gene and reduced \( \text{A}_\beta \) peptide production in vitro and in vivo. Although the vector-expressed shRNA has not been mutated to formally demonstrate that this molecule is a functional siRNA, the possibility that the observed reduction in APP expression is a nonspecific consequence of dsRNA hairpin expression is very unlikely. The expression was driven by the RNA pol-III H1 promoter. As the behavior of a heterologous cis-acting
regulatory element inserted within a viral vector backbone can be unpredictable, demonstration of shRNA expression at sufficient levels to produce a measurable change in gene expression is a key finding that allows us to further develop the vector platform for targeting dominant alleles in vivo through RNAi. In this study, vector-mediated downregulation of Aβ expression in vivo diminished greatly by 4 weeks post-injection, and we presume that the H1 pol-III promoter was inactivated over this time period. This is possibly a reflection of the generalized repression of gene expression that affects the HSV genome, with the notable exception of the viral latency genes. Identification of regulatory elements that allow long-term expression of shRNA from the HSV vector genome will be important before anti-APP RNAi can be applied in the study of transgenic animal models or developed as a therapeutic reagent. Recent literature implies that RNA pol-II promoters can express shRNA efficiently. Consequently, we are investigating whether the pol-II-dependent latency active promoter

![Image](https://example.com/image.png)

**Figure 4** Herpes simplex virus (HSV) vector-mediated amyloid precursor protein-short hairpin RNA (APP-shRNA) expression reduces amyloid-β peptide (Aβ) accumulation in vivo. (a) LV-APPshRNA (1 x 10^7 transducing units (TU)) and HSV-APP/shRNA (1 x 10^7 pore-forming unit (PFU)) were co-injected into the right hippocampus (‘test side’), whereas LV-APPshRNA (1 x 10^7 TU) and QOZH (1 x 10^7 PFU) were co-injected into the left hippocampus (‘control side’) of C57BL/6J mice. At 10 days post-injection, brain sections were examined for Aβ40 expression, by indirect immunofluorescence, and expression of the DsRed and green fluorescent protein (GFP) reporters by fluorescence microscopy. Amyloid-β40 immunoreactivity, DsRed fluorescence and GFP fluorescence were of similar in intensity on the control side. However, the fluorescent signal attributable to both Aβ40 and DsRed was much weaker on the test side compared with GFP fluorescence. (b) The fluorescent signal from three sections similar to those shown in panel a was analyzed quantitatively. The signal intensities (pixels/image normalized to GFP pixels/image) of both Aβ40 (**P < 0.008) and DsRed (*P < 0.016) were significantly lower on the test side, injected with HSV-APP/shRNA, than the control side, injected with QOZH.

![Image](https://example.com/image.png)

**Figure 5** Herpes simplex virus (HSV) vector-mediated neprilysin expression reduces amyloid-β peptide (Aβ) accumulation in vivo. LV-APPshRNA (1 x 10^7 transducing units (TU)) and HSV-neprilysin (1 x 10^7 pore-forming unit (PFU)) were co-injected into the right hippocampus (‘test side’), whereas LV-APPshRNA (1 x 10^7 TU) and QOZH (1 x 10^7 PFU) were co-injected into the left hippocampus (‘control side’) of C57BL/6J mice. At 4 weeks post-injection, brain sections were examined for (a) neprilysin and (b) Aβ40 expression, by indirect immunofluorescence, and expression of the DsRed and green fluorescent protein (GFP) reporters by fluorescence microscopy. (a) Neprilysin expression (blue) was only detected on the test side, which was injected with HSV-neprilysin; as expected, expression was co-localized with expression of GFP from the HSV vector. Neprilysin expression was also co-localized with DsRed expression, indicating that LV-mediated amyloid precursor protein (APP) expression occurred in the same cells as HSV-mediated neprilysin expression. Scale bar, 60 μm. (b) Aβ40 immunofluorescence (blue) was greatly reduced on the test side, injected with HSV-neprilysin, compared with the control side, injected with QOZH. DsRed and GFP expression were similar on the two sides. Scale bar, 60 μm. (c) The fluorescent signal from three sections similar to those shown in panel b was analyzed quantitatively. The signal intensity (pixels/image normalized to GFP pixels/image) of Aβ40 was significantly lower on the test side, injected with HSV-neprilysin, than the control side, injected with QOZH (**P < 0.009). DsRed expression was unaffected by HSV-neprilysin.
LAP2, which has been shown to direct long-term expression of therapeutic transgenes in the central nervous system,\textsuperscript{25} can be used either to express shRNA directly \textit{in vivo} or to prevent inactivation of a chimeric LAP2/pol-III promoter.

\textbf{An in vivo model of amyloid-β peptide overproduction}

We have developed a rapid \textit{in vivo} model of Aβ production by using a recombinant LV to overexpress a mutant form of APP. A similar approach has been reported in animal models of Parkinson’s disease, using...
LV\textsuperscript{35} or AAV\textsuperscript{36} to overexpress \(\alpha\)-synuclein, and Huntington's disease, using AAV-mediated expression of expanded polyglutamine tracts\textsuperscript{37} or LV-mediated expression of Huntingtin.\textsuperscript{38} Aside from its intrinsic flexibility and convenience,\textsuperscript{39} the major attraction of this type of model system is that the vector can be delivered to species that are not amenable to transgenic manipulation, such as rats and monkeys, but which have special relevance to the study of human brain function and pathology. The main advantage of using the LV system from the standpoint of the present studies is that the rapid elevation in tissue \(A\beta\) levels produced by vector transduction allows us to test different vectors for their desired effects in targeting \(A\beta\) expression over a short time course \textit{in vivo}, without the need to breed special transgenic lines of rodents. We did not examine the animals at later time points in these studies. Consequently, we do not know whether the elevation of \(A\beta\) seen in these experiments was an irreversible accumulation, or simply a dynamic increase in steady-state \(A\beta\) level, secondary to the elevation in production resulting from vector transduction and \(A\beta\) protein expression. Amyloid plaque formation was not observed in the experiments reported here, possibly as a result of the early time points post-injection at which the observations were made. Most of the \(A\beta\)-specific signal observed on immunohistochemical analysis carried out in these studies appeared intracellular; it is likely that this represents a processed, pre-secretory form of \(A\beta\), located in the endoplasmic reticulum, the site of APP post-translational processing. From the standpoint of gene therapeutic development, we were encouraged to find that this population of \(A\beta\) peptides was reduced by both APP RNAi and neprilysin, because soluble intracellular \(A\beta\) peptides have been implicated in triggering neuronal apoptosis.\textsuperscript{40} We are currently exploring this model further, both biochemically and pathologically, by evaluating whether long-term expression of LV-\(A\beta\)\textsuperscript{35} or co-expression of the microtubule-associated protein Tau, which is deposited in the intracellular neurofibrillary tangles of AD, will reproduce more extensive AD-like pathological changes in this model. Regarding the validity of using the LV model to test the effect of HSV vectors, it should be stressed that the well-known trans-activation of the lentiviral LTR region by HSV ICP\textsuperscript{0} could not have confounded the results reported here, because the test vectors were compared with an isogenic control HSV vector that expresses ICP\textsuperscript{0} similarly.

Gene therapy for Alzheimer's disease?

We have shown that \textit{in vivo} viral delivery of anti-\(A\beta\) siRNA or neprilysin using HSV vectors can efficiently reduce \(A\beta\) peptide production. This significant finding has potential consequences for the design and deployment of anti-\(A\beta\) reagents as possible anti-AD therapeutics. Given the central role of \(A\beta\) in neuritic plaques, a pathological hallmark of AD, it is legitimate to hypothesize that reduction of APP levels \textit{in vivo}, with a commensurate reduction in \(A\beta\) production, will slow or prevent progression of AD. However, this approach raises concerns that physiological perturbation may occur in the brain, secondary to loss of APP function. This concern is partially allayed by the finding that 'knockout' mice that do not express APP do not display overt abnormalities during embryonic or post-natal development.\textsuperscript{42} However, the contribution of APP to adult brain function has yet to be fully delineated; the intracellular domain of APP, released by \(\gamma\)-secretase cleavage, appears to have important signaling and transcriptional regulatory functions that might be perturbed by APP downregulation.\textsuperscript{43,44} Furthermore, adult APP null mice showed hypersensitivity to evoked seizures, minor motor defects and variable gliosis.\textsuperscript{45,46} In rare familial AD cases, allele-specific RNAi\textsuperscript{47} may present an alternative therapeutic strategy that would not be subject to similar concerns about loss of APP function, although this option is not applicable in the majority of AD cases, in whom no pathogenic APP mutations are present. Further studies are clearly indicated to determine the effects of APP knockdown in the brain \textit{in vivo}, and in this regard our HSV-APP/shRNA vector may prove an invaluable tool, because it could be deployed to assess the neurobiological and behavioral effects of hippocampal APP knockdown in primates.

We delivered neprilysin using a replication-defective recombinant HSV vector, and showed that it degraded \(A\beta\)\textsubscript{40} \textit{in vivo}. The reduction in \(A\beta\) level was comparable to that observed using RNAi, but lasted longer; the difference is most likely attributable to the choice of \(cis\)-acting regulatory elements, in that the neprilysin expression cassette was driven by a chimeric promoter composed of the HSV latency promoters fused to the IE promoter of cytomegalovirus. Our data showing that neprilysin reduces \(A\beta\) levels \textit{in vivo} are in agreement with previous studies in which LV\textsuperscript{48} and AAV\textsuperscript{52} vectors were employed to deliver the neprilysin gene in transgenic animal models. However, before considering \textit{in vivo} delivery of neprilysin further as a candidate therapeutic approach to AD, it will be necessary to determine the effects of its long-term overexpression. Neprilysin degrades many substrates with important physiological functions in the brain, such as enkephalins, endorphins and substance P, raising the possibility that its dysregulation could cause significant disruption of normal brain function. This issue could be studied in biologically relevant systems \textit{in vivo} using the viral vector described here. Should long-term neprilysin expression prove deleterious, it may be possible to restrict its substrate range to selectively target \(A\beta\) through protein engineering.

The next phase of these studies is to effect long-term expression of neprilysin or APP-shRNA \textit{in vivo}, in transgenic murine models of AD. These studies will be crucial in determining whether the short-term \textit{in vivo} biochemical actions of the vectors (i.e. reducing \(A\beta\) levels \textit{in vivo}) that we show here will translate into prevention of histological end points such as plaque formation or cell loss. To enable this crucial question to be adequately addressed, it will be necessary to characterize further the best way of using available \(cis\)-acting elements to drive expression of shRNA and neprilysin from the HSV backbone, in order to ensure that long-term expression at relevant levels can be achieved. These promoter studies are ongoing.

In conclusion, we have shown that recombinant replication-defective genomic HSV vectors effectively delivered reagents that reduced the levels of \(A\beta\) peptide \textit{in vitro} and \textit{in vivo}. Herpes simplex virus vectors expressing neprilysin, or RNAi against APP, will form the basis for further \textit{in vivo} studies on the neurobiology
of APP and neprilysin. Furthermore, in our view, these vectors seem worthy of further exploration as possible therapeutic reagents for AD.

Materials and methods

Constructs

Short hairpin RNA (shRNA) to the APP transcript was constructed by annealing two oligonucleotides, 5'-gacctcctgatcaatgtaagctgaagagctcatgtgcatgtttggaaa-3' and 5'-agatttctaaacactgatagttgctgcctgtaacgacgtacttacgt-3', and cloning the resulting dsDNA into the BglII–HindIII sites of the pH1 pol-III promoter vector (a gift from Dr Paul Robbins, University of Pittsburgh, Pittsburgh, PA, USA). Human neprilysin cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from human liver first-strand cDNA using primers 5'-tgaggtgatgggcaagtc-3' and 5'-cctctacctgtaacccccagt-3', and cloning the resulting constructs into the HindIII–Apal sites of the pCDNA3 (Invitrogen-Gibco, Carlsbad, CA, USA) vector, and the insert was sequenced to ensure that no point mutations were introduced during the PCR amplification. pIRES-Bleo-APP was made by inserting a HindIII–NotI restriction fragment of the cDNA encoding human APP751 (a gift from Dr Ruth Perez, University of California, San Diego, CA, USA) into the EcoRV–NotI sites of pIRES-BleO (BD Bioscience-Clontech, Palo Alto, CA, USA).

Cell lines, cell culture, transfection and infection

All cells were cultured at 37°C in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Carlsbad, CA, USA) containing 5% fetal bovine serum (Invitrogen-Gibco), 1 mM L-glutamine (Invitrogen-Gibco), penicillin (100 IU/ml; Invitrogen-Gibco) and streptomycin (100 µg/ml; Invitrogen-Gibco). 7Wiso cells were a gift from Dr Paul Robbins, University of Pittsburgh, Pittsburgh, PA, USA) and were used for selection of stably transfected cell lines. Cells in suspension were infected with Lipfectamine Plus (Invitrogen-Gibco). 7W cells were a gift from Dr Edward Koo (University of California, San Diego, CA, USA) and were used for selection of stably transfected cell lines. Cells in suspension were infected with recombinant HSV-1 or lentivirus for 1 h at 37°C.

Herpes simplex virus vector construction and production

The APP-shRNA and neprilysin cDNA expression constructs were inserted into a HSV-1 U1 targeting plasmid and recombined into U141 locus of QOZHG backbone vector by homologous recombination (protocol and detailed explanation provided in Burton et al.49). QOZHG (genotype ICP4-, ICP27::HCWVp-GFP, β-ICP22, β-ICP47, U141::ICP0pLacZ)29 is based on the d106 backbone,29 a gift from Dr Neal DeLuca (University of Pittsburgh). The genomes of QOZHG, HSV-neprilysin and HSV-APP/shRNA are depicted schematically in Figure 2a. The vectors were propagated in complementation 7B Vero cells, which stably express ICP4 and ICP27. Recombinants were screened by clear/blue plaque assays using X-Gal staining, purified by three rounds of limiting dilution and verified by Southern blot analysis. High titer and high-purity vector stocks were prepared as described.49

LV vector construction and production

cDNA encoding human APP751, carrying the Swedish mutation (a gift from Dr Edward Koo, University of California, San Diego, CA, USA) was cloned into the pIRES-DsRed-polyA+ plasmid (Clontech). The Mulu/DraI fragment, containing the APPshRNA-IRES-DsRed cassette, was inserted into the ClaI/Xhol sites of the pHR lentiviral transfer plasmid (a gift from Dr Didier Trono, University of Geneva, Geneva, Switzerland; Naldini et al.31). LV-APPshRNA viral vectors were produced in 293T cells by co-transfection of packaging plasmids and transfer plasmid.31 The supernatant was collected and concentrated by ultracentrifugation at 50,000 g for 90 min. The titer of transducing units (TU) was estimated by quantification of DsRed fluorescent 293T cells, 5 days after transduction with serial dilutions of the viral preparation.

Western blot analysis

Protein samples were harvested from six-well plates using radioimmunoprecipitation assay lysis buffer with added protease inhibitors (Sigma). The lysates were analyzed on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Western blots were performed using the following primary antibodies and dilutions: APP and Aβ-clone 6E10, 1:400 dilution (Signet, Dedham, MA, USA); neprilysin-anti-CD10, clone SN5c/L4-1A1, 1:1000 dilution (Sigma). Anti-rabbit or anti-mouse horse-radish peroxidase-conjugated secondary antibodies (Sigma) were used at a dilution of 1:5000. Signal was visualized by chemiluminescence (ECL; Amersham, Piscataway, NJ, USA).

Measurement of amyloid-β peptide and assay of amyloid-β peptide degradation in vitro

Culture media were centrifuged at 500 g for 5 min to remove cell debris, before assay for Aβ40 or Aβ42 using isoform specific ELISA kits (Biosource, Camarillo, CA, USA). Standard curves were derived using synthetic Aβ, allowing confirmation that the assays were linear and quantitative, and determination of sample concentrations. For the Aβ degradation assay, 48 h after infection with QOZHG or HSV-neprilysin virus, Vero cells were washed with 1 x phosphate-buffered saline and incubated with medium containing Aβ40 or Aβ42 (Biosource) at an initial concentration of 1000 pg/ml, supplemented with 1 µM ZnCl2, and in the absence of serum. Media were collected after 5 h of incubation and assayed for remaining Aβ using the ELISA described above. The neprilysin inhibitor, Thiopran (Sigma), was used at a concentration of 150 µM. Each experiment was performed at least three times for statistical analysis.

Animals and surgical procedure

All animal experiments were performed in accordance with institutional guidelines and with Institutional Animal Use and Care Committee (IACUC) approval. C57BL/6 male mice (Jackson Laboratory, Bar Harbor,
ME, USA) were injected with LV-APP<sup>env</sup> (1 × 10<sup>5</sup> TU) and either HSV-neprilysin or HSV-APP/RNAi (1 × 10<sup>7</sup> pore-forming unit), in a total volume of 1 µl at a rate of 0.2 µl/min, into the hippocampus of the right hemisphere using a stereotaxic frame (Stoelting, Wood Dale, IL, USA). As a control, LV-APP<sup>env</sup> plus QOZHG was injected into the contralateral hippocampus. Five mice were injected with each vector (n = 5). At 10 days to 45 weeks after injection, animals were transcardially perfused under deep anesthesia with phosphate-buffered 10% formalin and their brains removed for immunohistochemical analysis.

**Immunohistochemistry and imaging analysis for quantification**

Cryosections (14 µm) were made from fixed brains embedded in Cryo-Gel (Instrumedics, Hackensack, NJ, USA). Primary antibodies and dilutions were: Aβ – rabbit anti-β-amyloid 40 antibody (1:150; Biosource); neprilysin – monoclonal anti-CD10 antibody (clone SN5c/L4-1A1, 1:50; Ancell); neuron-specific enolase – rabbit anti NSE (1:300; Spring Bioscience, Freemont, CA, USA). Fluorescent images were captured using an Olympus Provis fluorescence microscope (Olympus Optical, Melville, NY, USA) and MagnaFire software (v2.0; Karl Storz Imaging Inc., Tuttlingen, Germany). The density of fluorescent signal in each captured image was measured using MetaMorph software (v6.2r5; Universal Imaging Corporation, Downingtown, PA, USA). Data were collected from three sections per mouse for quantitative analysis and normalized to GFP expression. Statistical comparison between the two sides of each mouse brain was carried out using a two-tailed pairwise t-test, assuming equal variance.

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