Systemic Central Nervous System (CNS)-targeted Delivery of Neuropeptide Y (NPY) Reduces Neurodegeneration and Increases Neural Precursor Cell Proliferation in a Mouse Model of Alzheimer Disease*

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Neuropeptide Y (NPY), one of the most abundant neurotransmitters in the central nervous system with roles in a variety of biological functions including: food intake, cardiovascular regulation, cognition, seizure activity, circadian rhythms, and neurogenesis. Reduced NPY and NPY receptor expression is associated with numerous neurodegenerative disorders including Alzheimer disease (AD). To determine whether replacement of NPY could ameliorate some of the neurodegenerative and behavioral pathology associated with AD, we generated a lentiviral vector expressing NPY fused to a brain transport peptide (apoB) for widespread CNS delivery in an APP-transgenic (tg) mouse model of AD. The recombinant NPY-apoB effectively reversed neurodegenerative pathology and behavioral deficits although it had no effect on accumulation of Aβ. The subgranular zone of the hippocampus showed a significant increase in proliferation of neural precursor cells without further differentiation into neurons. The neuroprotective and neurogenic effects of NPY-apoB appeared to involve signaling via ERK and Akt through the NPY R1 and NPY R2 receptors. Thus, widespread CNS-targeted delivery of NPY appears to be effective at reversing the neuronal and glial pathology associated with Aβ accumulation while also increasing NPC proliferation. Overall, increased delivery of NPY to the CNS for AD might be an effective therapy especially if combined with an anti-Aβ therapeutic.

Neuropeptide Y (NPY), one of the most abundant neuropeptides in the central and peripheral nervous system is synthesized in neurons and transported to pre- and post-synaptic vesicles where it is secreted (1). NPY is produced as preproenzyme that is processed first by removal of the secretory signal sequence and then by the peptidase convertase to generate NPY(1–39). Further editing by peptidase enzymes generates the mature NPY(1–36) (2).

This mature form binds primarily to three different receptors in the rodent brain, NPY R1, NPY R2, and NPY R5; which are all seven transmembrane receptors (3). Upon binding to the receptor, signaling pathways involve the activation of the G1/Go receptors and inhibition of cAMP synthesis followed by signaling through protein kinase C, mitogen-activated protein kinase (MAPK), inositol trisphosphate, and extracellular signal related kinase (ERK) (3).

NPY is associated with a variety of biologic functions including: food intake, cardiovascular regulation, cognition, seizure activity, circadian rhythms, and neurogenesis (4). Alterations in NPY have been associated with many neurodegenerative disorders including Downs syndrome (5), Huntington disease (6), and epilepsy (1), and reduced NPY levels have been reported in the CNS in Alzheimer disease (AD). Immunocytochemical studies have shown decreased NPY (7–12) and NPY receptors (13) immunoreactivity in the hippocampus of AD patients and NPY accumulation in dystrophic neurites around the amyloid plaques (14). In addition, reduced levels of NPY in the plasma (15) and cerebrospinal fluid (16–19) have been found correlating with the onset of AD. Thus reduction in NPY may play a role in pathology of AD or may be a secondary event associated with AD. In APP-tg models of AD the neuronal network dysfunction is associated with NPY alterations in the limbic system (20, 21) and intracerebral infusion of C terminus NPY fragments has been shown to be neuroprotective (22).

The pathogenesis of AD is associated with progressive accumulation of Aβ oligomers leading to synaptic loss, neuronal dysfunction, and death (23, 24). In addition, increased pro-inflammatory cytokine release that contributes to neuronal cell loss (25). The mThy1-APP-tg mouse model (line 41) that our group developed (26) recapitulates some of the aspects of this disease and has been useful for study of the pathogenesis of synaptic damage (26), neurogenesis defects (27), and behavioral deficits (28). For example, we have previously shown that intracerebral infusion of the NPY C terminus fragments into the APP-tg mouse model of AD ameliorates the behavioral and neurodegenerative pathology in this model (22).

In this study we wanted to develop a more long lasting, systemic delivery of NPY targeted to the brain to determine
whether this could ameliorate the neurodegenerative pathology and deficits triggered by Aβ accumulation. For this reason, we engineered a novel recombinant NPY with a blood-brain barrier transport tag. We have previously shown that fusion proteins containing a 38-aa peptide derived from apolipoprotein B (apoB) that recognizes the LDL-R in endothelial cells is capable of shuttling proteins into the CNS (29–32). This recombinant NPY was delivered by lentiviral vector via intraperitoneal injection to the liver for continuous expression and distribution in the blood. Using this method, we were able to show widespread distribution of NPY across the brain, reversal of neurodegeneration, and improved memory in the APP-tg mice. Furthermore, we showed increased proliferation of neural precursor cells in the subgranular zone of the hippocampus. NPY-apoB signaled via ERK and Akt through the NPY R1 and R2, respectively. Thus, widespread delivery of the brain-targeted recombinant NPY might represent a novel therapeutic for AD and other neurodegenerative diseases that show reduction in NPY.

Experimental Procedures

Construction of Lentivirus Vectors—NPY is produced as prepro-enzyme processed first by removal of the secretory signal in the endoplasmic reticulum to produce pro-NPY and then by the peptidase convertase to generate NPY(1–39) (2). These sequences are all located at the N terminus of the mature NPY protein, so to modify NPY additional sequences were added to the C terminus to prevent the interference with secretion of the protein. The human NPY cDNA (Open Biosystems) was PCR amplified with the addition of a C terminus V5 epitope tag and cloned into the third generation self-inactivating lentivirus vector (33) with the CMV promoter driving expression producing the vector LV-NPY. Addition of the 38-amino acid apoB LDL-R binding domain to the C terminus of wild-type NPY was performed by cloning the oligonucleotide coding for the apoB (29, 30, 32). Lentiviruses expressing the NPY (LV-NPY), NPY-apoB (LV-NPY-apoB), or empty vector (LV-control) were prepared by transient transfection in HEK293T cells (33).

Establishment of in Vitro Cultures—For these experiments we used the rat neuroblastoma cell line B103 (34) or adult rat hippocampal neural progenitor cells (NPCs) (Chemicon) (35). NPCs were grown in DMEM/Ham’s F-12 medium containing B27 supplements without vitamin A (Gibco). NPCs were plated onto polylysine-coated glass coverslips and differentiated in DMEM/F-12 medium containing N2 supplements (Gibco) for 4 days. All cells were then infected with LV-control, LV-NPY, or LV-NPY-apoB at a multiplicity of infection of 40. For NPC experiments with NPY R1/2 inhibitors, neurons were pretreated with (S)-N2-[[1−2−[4−[4−[(R),5−11 dihydro−6(6h)− oxodi benz[e]azepin−11–yl]−1−piperazinyl]−2−oxoethyl]−cyclopentyl]−acetyl]−N−[2,1,2−dihydro−3,5(4H)−dioxo−1,2−diphenyl−3H−1,2,4−triazol−4−yl]−ethyll−argininamide (BIIE0246, Y2 receptor inhibitor, Tocris Biosciences, Ellisville, MO), R−N2−(diphenylacetyl)−N−(4−hydroxyphenyl)−methylargininamide (BIBP3226, Y1 receptor inhibitor, Sigma), or vehicle control (PBS) at 1 μM for 24 h as previously described (36) followed by incubation with CHO cell-derived Aβ oligomers for 24 h.

Natural Aβ was prepared according to Walsh et al. (37) (kindly provided by Dr. Eddie Koo) by incubating control CHO cells or CHO cells expressing the APP V717F mutation (also referred as 7PA2 cells) with B27-conditioned media for 16 h. Total Aβ concentration was determined as previously described (38). Neurons were treated with 100 pm natural Aβ for 24 h. One set of cells were grown in plates for immunoblot analysis or the lactate dehydrogenase cell death assay (Promega) and the other set grown on coverslips and fixed in 4% paraformaldehyde for immunohistochemistry. For this purpose, coverslips were immunolabeled with antibodies against recombinant NPY epitope tag, V5 (Life Technologies), NPY R1 (GeneTex), NPY R2 (GeneTex), or MAP2 (Life Technologies) followed by secondary antibodies tagged with tyramide red or FITC and imaged with the laser scanning confocal microscope. Images were analyzed with the NIH ImageJ program to assess levels of pixel intensity.

For co-culture analysis, 5 × 10⁴ B103 cells were plated onto poly-L-lysine-coated glass coverslips and 1 × 10⁵ 293T cells previously infected with LV-control, LV-NPY, or LV-NPY-apoB vectors were plated onto 12-well cell culture inserts containing a 0.4-μm PET membrane (Fisher Scientific). Cultures were incubated separately for 6 h to allow cells to attach and then co-cultured 24 h (39). Cultures were then treated with recombinant oligomerized Aβ (5 nM) or vehicle (DMSO) for 24 h (40). Cultures were fixed in 4% paraformaldehyde for immunohistochemistry.

Treatment of Animals—Mice expressing human APP751 (Line 41) under control of the mThy-1 promoter (APP-tg) were generated as previously described (26). Non-tg and APP-tg mice received a single intraperitoneal injection of LV-control, LV-NPY, or LV-NPY-apoB (n = 10/group) of 1 × 10⁴ transducing units in a volume of 300 μl. We, and others have previously shown that this method of vector delivery results in lentiviral transduction of the liver (sinusoidal cells and to a lesser extent hepatocytes) and the spleen (splenocytes) where the secreted protein is expressed and transported by the blood to the blood-brain barrier (32, 41). Treatment started when mice were 9 months of age when Aβ accumulation has lead to neurodegeneration, gliosis, and plaque generation. The treatment was well tolerated, and no weight loss or other complications were noted. Treatment lasted 3 months with all mice 12 months of age by the end of the study. We have previously shown that lentivirus delivery by intraperitoneal injection can sustain transgene expression up to 3 months (29, 39). To label dividing cells including those involved in neurogenesis, 24 h prior to sacrifice, mice received a single intraperitoneal injection of BrdU (150 mg/kg) (42). All experiments were carried out in accordance with guidelines from the NIH regarding the care and use of animals for experimental procedures and approved by the University Institutional Animal Care and Use Committee at the University of California at San Diego under protocol number S0221.

Water Maze Testing—As previously described (43), to evaluate the functional effects of treatment in mice, groups of APP-tg and non-tg animals were tested in the water maze. For this purpose, a pool (diameter 180 cm) was filled with opaque water (24°C) and mice were first trained to locate a visible platform
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(34, 45). For immunoblot analysis, 20 μg of total protein per lane was loaded on 4–12% BisTris SDS-PAGE gels and blotted onto polyvinylidene fluoride membranes. Membranes were probed with antibodies against full-length NPY (Bachem), C-terminal NPY (Life Technologies), epitope tag V5 (Life Technologies), or NPY R2 (GeneTex). Incubation with primary antibody was followed by species-appropriate incubation with secondary antibody tagged with horseradish peroxidase (Santa Cruz Biotechnology). Blots were probed with antibodies against full-length NPY (Bachem), C-terminal NPY (Life Technologies), epitope tag V5 (Life Technologies), or NPY R2 (GeneTex). Incubation with primary antibody was followed by species-appropriate incubation with secondary antibody tagged with horseradish peroxidase (Santa Cruz Biotechnology) and visualization with enhanced chemiluminescence. Analysis of all immunoblots was performed with a Intas Instruments EthoVision video tracking system (San Diego Instruments) set to analyze two samples per second.

Locomotor activity was measured as previously described (44). Briefly, mice were placed in polycarbonate cages (42 × 22 × 20 cm) placed into frames (25.5 × 47 cm) mounted with two levels of photocell beams at 2 and 7 cm above the bottom of the cage (San Diego Instruments). These two sets of beams were used for the recording of both horizontal (locomotion) and vertical (rearing) behavior. A thin layer of bedding material was applied to the bottom of the cage. Mice were tested for 20 min.

Immunoblot Analysis—Immunohistochemistry—At the end of the experiment, animals were transcardially perfused with physiological saline and brains were collected. Brains were divided sagittally into right and left hemispheres. The left hemispheres were fixed in 4% paraformaldehyde in phosphate-buffered saline and sequentially sectioned on the vibratome at 40 μm and stored at −20 °C in cryoprotective medium. The right hemispheres were snap-frozen and stored at −80 °C for subsequent protein extraction. Sections were immunostained with antibodies against Aβ (clone 4G8, Senetek), NeuN (neuronal marker, Millipore), GFAP (astroglial marker, Millipore), Map2 (Life Technologies), synaptophysin (Millipore), BrdU (Accurate Chemical and Scientific Corp), Iba1 (Wako), doublecortin (DCX) (Santa Cruz Biotechnology), or proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology) and imaged with an Olympus BX51 bright field digital microscope or a laser scanning confocal microscope. Stereological analysis of NeuN, BrdU, DCX, and PCNA immunoreactivity was conducted by the dissector method using the Stereo-Investigator System (MBF Bioscience). A minimum of 100 cells was counted per animal with 3 fields per animal. The results were averaged and expressed as cell counts per 0.1 mm³. Digital images were analyzed with the ImageQuant 1.43 program (NIH) to determine synaptic density (MAP2 and SY38) (46, 47). Optical density measurements were obtained using the ImageQuant software, and quantification was performed by correcting against background signal levels.

Double Immunolabeling—To determine co-localization between BrdU and neuronal markers, double labeling experiments were performed as previously described (48). Vibratome sections were immunolabeled with an antibody against NeuN (neurons) with the Tyramide Signal AmplificationTM Direct system (NEN Life Sciences) and an antibody against BrdU with a FITC-tagged secondary antibody (Vector Laboratories, 1:75). Additional double labeling experiments were performed with antibodies against V5 (tyramide red) NeuN or GFAP (FITC); activated caspase-3 (tyramide red) and MAP2 (FITC), and DCX (tyramide red) and BrdU (FITC). Cell nuclei were stained using ProLong® Gold Antifade (Molecular Probes). Sections were imaged with a Zeiss ×63 objective on an Axiosvert 35 microscope (Zeiss) with an attached MRC1024 laser scanning confocal microscope (Bio-Rad) (49).

Statistical Analysis—Values are expressed as average mean ± S.E. To determine the statistical significance we used one-way analysis of variance with Dunnett’s post hoc test when comparing to the control condition. Additional comparisons were done using Tukey–Kramer or Fisher post hoc tests. The differences were considered to be significant if p values were less than 0.05.

Results

Generation of the LV-NPY-ApoB and Neuroprotective Effects against Aβ—We, and others have shown that delivery of recombinant NPY promotes the survival of neurons challenged with Aβ (22, 50, 51). Given this proof of principle data, we wanted to develop a novel NPY construct that can be delivered systemically and will target the CNS. For this purpose, we developed a lentiviral vector expressing NPY with a blood-brain barrier targeting sequence, ApoB (Fig. 1A). Delivery of this vector by intra-peritoneal injection would result in transduction of the liver and spleen, thus expressing the recombinant NPY for distribution through the bloodstream. This would allow widespread distribution in the whole brain rather than single concentrated bolus injections that would have resulted from stereotoxic delivery of the recombinant protein alone or by viral vector delivery to a brain region. The vectors were designated LV-NPY (wild-type NPY) and LV-NPY-apoB (wild-type NPY with the addition of the LDL-R binding domain of ApoB). The new vector was used to infect B103 neuronal cells in vitro where similar levels of expression and secretion to wild-type LV-NPY were detected by immunoblot (Fig. 1B) and immunohistochemistry (Fig. 1C).

We first determined if the fusion NPY-apoB retained the neuroprotective effects from Aβ oligomers as the wild-type NPY. Neuronal B103 cells were infected with LV-NPY, LV-NPY-apoB, or LV-control and then treated with recombinant Aβ oligomers for 16 h (Fig. 1 D–F). Treatment with Aβ induced activated caspase-3 indicating induction of apoptosis in the neuronal cells treated with the LV-control vector. Similarly, treatment with recombinant Aβ reduced the neurofilament MAP2 indicating reduced neuronal viability. In contrast, those
neuronal cells treated with LV-NPY or LV-NPY-apoB are resistant to the Aβ-induced apoptosis and showed no change in MAP2 expression (Fig. 1, D–F). Thus, addition of the 38-amino acid apoB LDL-R to the NPY neuropeptide does not appear to affect expression, secretion, or activity of the NPY protein in the context of Aβ neuronal toxicity.

To determine whether the NPY-apoB retained autocrine effects, we established an in vitro co-culture system (39) consisting of human fibroblasts (293T cells) expressing the NPY or NPY-apoB protein separated from neuronal cells (B103 cells) by a 0.4-μm membrane that allows only the diffusion of soluble proteins (Fig. 2A). To these cultures, we added oligomerized Aβ42 as described above and then evaluated the effects on neuronal cell death by assessing the levels of activated caspase-3 immunoreactivity and neuronal fitness by immunolabeling with an antibody against MAP2 to determine neurite length. Similar to results with NPY or NPY-apoB expressing neuronal cells, neuronal B103 cells that were exposed to fibroblast expressed NPY or NPY-apoB in a paracrine manner were protected from oligomeric Aβ-induced apoptosis and showed increased MAP2 expression (Fig. 2B–D). Therefore, the NPY-apoB acts in a paracrine manner to protect neurons from Aβ-induced neurotoxicity in vitro.

The NPY-ApoB Fusion Protein Traffics into the CNS and Concentrates in the Cortex and Hippocampus of Non-tg and APP-tg Mice—To determine whether the NPY-apoB fusion peptide penetrates into the brain, non-tg and APP-tg mice received a single intraperitoneal injection of LV-control, LV-NPY, or LV-NPY-apoB and analyzed by immunoblot for expression of NPY in cell lysates or cell culture supernatant. Transduced B103 neuronal cells were immunostained for recombinant protein expression by NPY and the epitope tag V5.

H9262 transduced B103 neuronal cells were immunostained for recombinant protein expression by NPY and the epitope tag V5. A, diagrammatic representation of the lentiviral vectors (LV). The NPY (LV-NPY) and NPY-apoB (LV-NPY-apoB) were cloned into the 3rd generation lentivirus vector. B, B103 neuronal cells were transduced with LV-control, LV-NPY, or LV-NPY-apoB and analyzed by immunoblot for expression of NPY in cell lysates or cell culture supernatant. C, transduced B103 neuronal cells were immunostained for recombinant protein expression by NPY and the epitope tag V5, D, double immunolabeling for MAP2 (green) and activated caspase-3 (red) in B103 neuronal cells treated with CHO cell-derived oligomeric Aβ for 24 h following transduction with LV-control, LV-NPY, or LV-NPY-apoB. E, image analysis of active caspase-3 and MAP2 staining, results are expressed as pixel intensity. * indicates statistical significance (p < 0.05) compared with LV-control treated with Aβ oligomers by Student’s t test, unpaired, two-tailed. Arrows indicate neurite extensions of B103 cells. Scale bar = 10 μm.

To determine specifically which cells within the CNS take up the recombinant NPY or NPY-apoB, we performed double immunofluorescence for the virus-expressed NPY (V5) and the neuronal marker (NeuN) or astrocyte marker (GFAP) in the hippocampus (Fig. 4). Virus vector-expressed NPY and NPY-apoB co-localized with the neuronal marker NeuN (Fig. 4, A and B) with a significant increase in co-localization observed with the NPY-apoB (4×) over the NPY protein. In contrast, no
V5 epitope-tagged NPY (virus vector expressed) was observed co-localizing with astrocytes (GFAP) (Fig. 4, C and D).

Systemically Delivered Brain-targeted NPY-ApoB Is Neuroprotective and Promotes Proliferation of NPCs in the Hippocampus of APP-tg Mice—To determine the effects of systemically delivered NPY or NPY-apoB on AD-like neuropathology including accumulation of Aβ/H9252 in the APP-tg mouse, we performed immunohistochemical analysis with an antibody (monoclonal, 4G8) against Aβ/H9252. At this age, all APP-tg mice displayed considerable amyloid deposition in the neocortex and hippocampus (Fig. 5A). There was little to no difference in the accumulation of Aβ following treatment with either LV-NPY or LV-NPY-apoB compared with LV-control (Fig. 5, A and B). APP-tg mice, similar to Alzheimer patients, show increased astrogliosis that accompanies the Aβ/H9252 accumulation (31) (Fig. 5, C and D). Treatment of APP-tg mice with LV-NPY-apoB vector significantly reduced the astrogliosis in this model compared with non-tg mice (Fig. 5, C and D). The APP-tg mice with LV-NPY-apoB vector significantly reduced the astrogliosis in this model compared with non-tg mice that received either LV-NPY or LV-control (Fig. 5, C and D). The APP-tg mice show an increase in numbers of cells positive for Iba1 staining the hippocampus compared with non-tg mice (Fig. 5, E and F) indicating microgliosis. These appear to be activated microglia based on morphology. Treatment with LV-NPY or LV-NPY-apoB does not affect the number of microglia in the hippocampus in these mice (Fig. 5, E and F) nor does it affect the activation state of the cells.

Compared with non-tg mice, the LV-control-treated APP-tg mice showed reduced numbers of NeuN positive neurons in the CA3 regions of the hippocampus (Fig. 6, A and B). Treatment with LV-NPY-apoB ameliorated the loss of NeuN positive cells in the hippocampus to near non-tg levels compared with APP-tg mice that received either LV-NPY or LV-control (Fig. 6, A and B).

It has been proposed that Aβ neurotoxicity targets the synapses (52–54). Indeed, compared with non-tg mice, the LV-control-treated APP-tg mice showed reduced levels of the post-synaptic marker (MAP2) (Fig. 6, C and D) and the pre-synaptic marker (synaptophysin, SY38) (Fig. 6, E and F). Compared with the LV-control and LV-NPY-treated APP-tg mice, delivery of the LV-NPY-apoB vector ameliorated the loss of both MAP2 and SY38 in the APP-tg mice to levels comparable with the non-tg mice (Fig. 6, C–F). Similarly, examination of apoptotic neurons by co-staining for MAP2 and activated caspase 3 showed that the APP-tg mice have increased numbers of apoptotic neurons in the hippocampus (Fig. 6, G and H). This was significantly reduced only by treatment with LV-NPY-apoB (Fig. 6, G and H). Thus, although LV-NPY-apoB had no effect on the levels of Aβ in the APP-tg mouse, it was able to ameliorate the neurodegenerative pathology including a reduction in astrogliosis, neuronal apoptosis, and maintenance of the normal levels of neuronal markers such as NeuN, MAP2, and SY38.

We recently showed that our APP-tg mouse displays neurogenesis deficits in the hippocampus (27, 31, 55). To determine whether delivery of LV-NPY or LV-NPY-apoB to the brain altered neurogenesis in the subgranular (SG) zone of the dentate gyrus of the hippocampus, APP-tg and non-tg mice were examined by immunohistochemistry for incorporation of BrdU, indicating proliferating cells. Consistent with previous
studies, compared with the LV-control non-tg mice, the LV-control or LV-NPY-treated APP-tg mice displayed a 30 and 40% reduction in BrdU-positive NPCs in the dentate gyrus (Fig. 7, A and B). In contrast non-tg and APP-tg mice that received LV-NPY-apoB showed a 20–30% increase in the basal levels of neurogenesis as reflected by the numbers of DCX positive cells (Fig. 7, C and D). This was further verified by double labeling for BrdU and DCX to show that these immature neural precursor cells were dividing (Fig. 8, A and B). To investigate if increased neurogenesis was associated with proliferation of NPCs, immunostaining with an antibody against PCNA was performed. Compared with the LV-control-treated non-tg mice, the LV-control or LV-NPY-treated APP-tg mice showed similar levels of PCNA positive cells in the dentate gyrus (Fig. 7, E and F). In contrast non-tg and APP-tg mice that received the LV-NPY-apoB presented a 20–30% increase in the prolifera-
tion of NPCs as reflected by the numbers of PCNA positive cells (Fig. 7, E and F).

To determine whether these newly generated neuronal precursor cells were migrating and differentiating into new neurons, we performed double-immunohistochemistry for BrdU, the marker of cell proliferation, and NeuN, the marker of neurons. Although the LV-NPY-apoB vector significantly increased NPC proliferation in the subgranular zone, there was no concomitant increase in new neurons in the hippocampus in mice that received this vector compared with mice that received LV-control (Fig. 8, C and D).

The alterations in synaptic markers in APP-tg mice have been associated with memory and learning deficits beginning at 3–6 months of age as measured in the Morris water maze (56). To determine whether the neuronal and synaptic improvements observed following treatment with the LV-NPY-apoB vector could ameliorate these deficits, APP-tg mice that received LV-NPY-apoB were able to learn the location of the hidden platform significantly faster and at a rate similar to non-tg mice (Fig. 9A). In contrast, APP-tg mice that received the LV-NPY-apoB vector spent significantly more time in the target quadrant than APP-tg that received either LV-NPY or LV-control vectors. Next, mice were analyzed for locomotor activity in the open field. Compared with non-tg mice that received LV-control and LV-NPY-treated APP-tg mice displayed increased activity; in contrast APP-tg mice that received the LV-NPY-apoB injections displayed levels of activity comparable with non-tg controls. No effects were observed in rearing. Taken together these studies showed that LV-NPY-apoB ameliorates the neurodegeneration, increases neural precursor cell proliferation, and reverses learning and memory alterations in the APP-tg mice.

The Neuroprotective Effects of NPY-ApoB against Aβ Oligomers Involves Signaling via ERK and Akt through the NPY R1 and -R2—NPY acts primarily through 3 receptors in the rodent brain: NPY R1, NPY R2, and NPY R5 of which R1 and R2 are located in the subventricular zone and the subgranular zone of the hippocampus and are known to be involved in neurogenesis (57). To determine which receptor the NPY-apoB is acting through, we utilized an in vitro rat NPCs model. LV-NPY-and LV-NPY-apoB-infected rat NPCs expressed increased levels of full-length NPY and the C-terminal NPY (Fig. 10A) as well as
showed abundant expression of both NPY R1 and NPY R2 (Fig. 10, A–C). Thus the NPCs used in this study express both the NPY R1 and NPY R2 receptors.

NPCs infected with LV-control, LV-NPY, or LV-NPY-apob were challenged with Aβ oligomers in the presence of an NPY R1 inhibitor (BIBP3226) or NPY R2 inhibitor (BIIE0246). Aβ oligomers significantly reduced the viability of the rat NPCs after 16 h treatment; however, prior infection with LV-NPY or LV-NPY-apob was able to prevent the Aβ oligomer-associated cell death as measured by MAP2 staining or lactate dehydrogenase assay (Fig. 10, D–F). Addition of either the NPY R1 or NPY R2 inhibitor reduced the survival affected by LV-NPY or LV-NPY-apob in the Aβ challenged NPCs. The NPY R1 inhibitor reduced the NPY effect by 25%, whereas the NPY R2 reduced the NPY effect by 40% (Fig. 10, E and F). Thus, NPY-apob produced from the lentivector is active at protecting the NPCs from toxic effects of Aβ acting through both NPY R1 and NPY R2.

FIGURE 5. Effects of systemically delivered LV-NPY-apob on Aβ plaques and gliosis in the APP-tg mouse. A, hemibrains from non-tg and APP-tg mice that had received a single intra-peritoneal injection of LV-control, LV-NPY, or LV-NPY-apob were immunostained for Aβ and analyzed with the laser scanning confocal microscope (green). B, computer aided image analysis for % Aβ staining in the neurophil of the hippocampus. C, bright field microscopy analysis of serial longitudinal vibratome sections from the non-tg and APP-tg immunostained with an antibody against GFAP following treatment with LV-control, LV-NPY, or LV-NPY-apob vector. D, computer aided image analysis for numbers of GFAP positive cells in the molecular layer of the CA1 of the hippocampus. E, bright field microscopy analysis of serial longitudinal vibratome sections from non-tg and APP-tg immunostained with an antibody against Iba-1. Stereological estimates (dissector method) of total Iba-1 positive microglia counts were measured in the hippocampus. Top row represents a low power overview (×40, scale bar = 250 µm) and panels in the bottom rows display higher magnification images (×400, scale bar = 50 µm) of the corresponding sections. Scale bar for E = 50 µm. *, indicates one-way analysis of variance with Dunnett’s post hoc test, p < 0.05 compared with non-tg mice that received LV-control. #, indicates one-way analysis of variance with Dunnett’s post hoc test, p < 0.05 compared with APP-tg mice that received LV-control.
the NPY R1 and NPY R2 receptors and signaling through ERK and AKT, respectively.

The hippocampus of non-tg and APP-tg mice that received LV-control, LV-NPY, or LV-NPY-apoB were analyzed by immunoblot for NPY R1 and NPY R2 receptors. Similar expression levels of both receptors were observed in both mice (Fig. 12A). Similar to results observed in vitro in the rat NPCs, treatment of non-tg or APP-tg mice with the LV-NPY-apoB resulted in phosphorylation of ERK and AKT (Fig. 12, A–C). Therefore, the NPY-apoB was able to penetrate the brains of non-tg and APP-tg mice to a significantly greater degree than NPY alone and thus was able to bind to and activate NPY R1/NPY R2 receptors in the hippocampus inducing neurogenesis in the subgranular zone.

FIGURE 6. Delivery of systemic LV-NPY-apoB ameliorates neurodegeneration in the hippocampus of APP-tg mice. Non-tg and APP-tg mice were injected with LV-control, LV-NPY, or LV-NPY-apoB and after 3 months, mice were immunostained for the (A) neuronal marker, NeuN, (C) post-synaptic marker, MAP2, or the (E) pre-synaptic marker, synaptophysin (SY38), and analyzed with a digital bright field or laser scanning confocal microscope. B, stereological estimates (dissector method) of total NeuN positive neuronal counts was measured in the hippocampus. Percent area of MAP2 (D) and SY38 (F) immunostaining in the hippocampus. Scale bars = overview, 200 μm; hippocampus, 40 μm. G, double immunostaining for the neuronal post-synaptic marker MAP2 (green) and the apoptotic marker activated caspase 3 (red). H, computer added image analysis of the % cells displaying co-localization between MAP2 and activated caspase 3. * indicates statistical significance (p < 0.05, one-way analysis of variance, post hoc Fisher) compared with non-tg LV-control treated mice. # indicates statistical significance (p < 0.05, one-way analysis of variance, Dunnett’s post hoc test) compared with APP-tg treated LV-control or LV, LV-NPY-treated mice.
Discussion

We report here the construction and characterization of a lentivirus expressing a novel brain targeting and penetrating the NPY protein for widespread delivery to the CNS following systemic administration. NPY-apoB was taken up by neurons across the hippocampus ameliorating neurodegeneration and increasing proliferation of neural precursor cells in the subgranular zone of the dentate gyrus. NPY-apoB signaling occurred through both NPY R1 and NPY R2 receptors. Although increased NPC proliferation was observed, there was little to no increase in basal levels of neuronal differentiation in the hippocampus. Although increased NPY-apoB trafficking to the CNS did not reduce Aβ accumulation, there was an increase in neurogenesis and synaptic density that was accompanied by behavioral improvements. Systemic delivery of the fusion NPY-apoB to the CNS may prove to be a practical approach for replacing the loss of this neuropeptide often observed in AD and other neurodegenerative diseases.

We observed increased proliferation of NPCs in the subgranular zone but failed to observe an increase in differentiation of these new cells into the hippocampus. It has been previously reported that NPY promotes self-renewal of neural progenitor cells in the subgranular zone of the dentate gyrus (61); however, it only induces neuronal differentiation in the subventricular zone where it fails to promote self-renewal (62, 63). In fact, previous reports have shown that the increase in proliferation in the subgranular zone is through the NPY R1 receptor via the ERK pathway (58, 64, 65); a receptor that we show in this report is activated by our recombinant NPY-apoB consistent with these previous findings. Although we did not investigate the subventricular zone in this study due its relevance to AD, it would be interesting to examine it in the future.

NPY R1 is expressed on astrocytes in the hippocampus (66) and NPY R1 and NPY R2 receptors are both expressed on microglia in the hippocampus. Interestingly, although NPY R2 activation has been shown to play a role in microglia survival in methamphetamine-induced microglia cell death (67), NPY R1 receptor activation has been shown to inhibit microglia cell activation (68) and the production of the pro-inflammatory cytokine IL-1β as well as downstream components such as iNOS (68). Inflammation and increased gliosis is a hallmark of AD and is observed in the APP-tg model used in this study (31). Delivery of the NPY-
FIGURE 8. Double immunolabeling analysis of the effects of NPY-apoB in the differentiation of neuroblasts into neurons in the hippocampus. APP-tg and non-tg mice were injected with LV-control, LV-NPY, or LV-NPY-apoB and 3 months later treated with BrdU. Brains were fixed in paraformaldehyde and (A) representative sections were double-immunostained for BrdU (green) and (A) DCX (red) or (C) NeuN (red) and analyzed with the laser scanning confocal microscope. Top row shows co-localization of signal. Computer added image analysis of the % cells displaying co-localization between BrdU and DCX (B) or NeuN (D). Scale bar 40 μm. * indicates statistical significance (p < 0.05, one-way analysis of variance, Dunnett’s post hoc test) compared with non-tg, LV-control treated mice. # indicates statistical significance (p < 0.05, one-way analysis of variance, post hoc Tukey-Kramer) compared with APP-tg-, LV-control-, or LV-PY-treated mice.

FIGURE 9. Treatment with systemic brain targeted LV-NPY-apoB ameliorates behavioral deficits in APP-tg mice. Three months after intraperitoneal injections with LV-control, LV-NPY, or LV-NPY-apoB, memory and learning were assessed by the Morris water maze. A, mice trained on the cued platform on days 1–3 and then tested for spatial learning on days 4–7 were analyzed for total swim distance. B, probe test was performed at day 8, number of entrances of the mouse in the target quadrant containing the hidden platform were quantified. Open field was used to assess locomotor activity. C, total activity was measured by beam breaks 2 cm above the cage floor and D, rearing was measured by beam breaks 7 cm above the cage floor. * indicates statistical significance p < 0.05 when compared with non-tg controls. One-way analysis of variance with Dunnett’s post hoc test. n = 10 mice per group.
Neuronal progenitor cells are protected from Aβ challenge by NPY and NPY-apoB through NPY R1 and NPY R2 receptors. Rat hippocampal progenitor cells were infected with LV-control, LV-NPY, or LV-NPY-apoB. A, representative immunoblot, and B and C, immunofluorescence for the expression of NPY, NPY R1, and NPY R2. D, infected neuronal progenitor cells were then challenged with CHO cell-derived oligomeric Aβ for 24 h in the presence of NPY R1 inhibitor (BIBP3226), NPY R2 inhibitor (BIIE0246), or vehicle (PBS) and examined by immunofluorescence by staining for MAP2. E, MAP2 staining was quantified. F, cell cultures were assayed for cell death via the lactate dehydrogenase assay. * indicates statistical significance, p < 0.05 when compared with cultures treated with Aβ oligomers. Two tailed, unpaired, Student’s t test. n = 3 culture replicates per group.
apoB to the brain resulted in widespread reduction in astrogliosis consistent with the finding that NPY is able to repress neuroinflammatory responses and neurodegeneration, thus downregulating a key component of the pathology associated with AD. Interestingly, we did not see a change in the numbers of microglial cells in APP-tg mice treated with NPY-apoB. Thus, activation of both NPY R1 and NPY R2 signaling pathways in microglia cells in the hippocampus could reduce inflammation and also does not affect microglia proliferation.

In addition to regulating neurogenesis and astrogliosis in the CNS, NPY regulates circadian rhythms and food intake. Many neurodegenerative diseases, including AD, manifest with alterations in circadian rhythms (4, 69, 70) and one prominent clinical feature of AD is weight loss correlating with disease severity (71). In fact, several studies have reported that one of the earliest symptoms of AD, manifesting in pre-clinical mild cognitive impairment, may be early weight loss (72–75). Although neither weight nor circadian rhythms were monitored in this short-term study, the delivery of systemic NPY-apoB may be beneficial to these other symptoms of neurodegenerative diseases.

We previously reported that the extracellular endopeptidase nephrilysin cleaves the full-length NPY generating a prominent C-terminal fragment composing amino acids 21–36 and 31–36 (22, 31). This C-terminal fragment of NPY increases neuroviability and neuroproliferation in the APP-tg mice (22, 31). In this report, we showed that systemic delivery of the NPY-apoB increased CNS concentrations of this C-terminal NPY fragment.

The recombinant NPY-apoB protein produced from the lentiviral platform contains a V5 epitope tag and the 38-amino acid ApoB LDL-receptor binding domain slightly increasing the size of the recombinant vector-produced NPY-V5 protein (Fig. 1B). Because this size difference was so small, this was observed only on gels where sufficient protein separation was allowed to occur. The in vivo immunoblot analysis of recombinant NPY-V5 and NPY-V5-apoB shows the size separation on the blot probed with the
anti-V5 antibody; however, on a separately run gel that was probed for full-length NPY and the CT-NPY, insufficient size separation generates the appearance of similar protein size (Fig. 3D).

Although NPY-apoB was effective at reversing the neuronal pathology and behavioral deficits as well as increasing proliferation of neural precursor cells, there was no effect on the accumulation of Aβ in the CNS of APP-tg mice. Thus, NPY-apoB might be an effective therapeutic; however, one that would be most effective with an anti-Aβ therapy in combination. Passive immunization (76) and CNS delivery of Aβ proteases (30, 31) have been effective at reducing the accumulation of Aβ in the brain and may be effective in combination with CNS-targeted NPY-apoB at treating the many symptoms associated with AD.

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