doi:10.1006/mthe.2001.0520, available online at http://www.idealibrary.com on IDEAL

Targeted Retrograde Gene Delivery for Neuronal Protection

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The cellular heterogeneity and complex circuitry of the central nervous system make it difficult to achieve precise delivery of experimental and therapeutic agents. We report here an *in vivo* retrograde gene delivery strategy to target mature projection neurons using adeno-associated virus, a vector with low toxicity and the capacity for long-term gene expression. Viral delivery to axon terminal fields in the hippocampus and striatum resulted in viral internalization, retrograde transport, and transgene expression in specific projection neurons in entorhinal cortex and substantia nigra. Retrograde delivery of the anti-apoptotic gene *Bcl2l* (also known as Bcl-xL) protected entorhinal projection neurons from subsequent damage-induced cell death. Given the broad distribution of neurons affected by neurodegenerative diseases, gene delivery to both the terminal fields and the projection neurons through retrograde infection provides for strategic therapeutic intervention at both levels of the neural circuit. This approach may also facilitate experimental studies of defined neural circuits.

Key words: central nervous system, adeno-associated virus (AAV), gene transfer, gene therapy, retrograde transport, entorhinal cortex, substantia nigra, Alzheimer's disease, Parkinson's disease, virus labeling, *Bcl2l* (Bcl-xL)

INTRODUCTION

The loss or dysfunction of specific projection neurons leads to the manifestation of many neurological diseases. Examples include the loss of dopaminergic nigrostriatal projection neurons in Parkinson's disease [1], entorhinodentate projection neurons in Alzheimer's disease [2], and spinal motoneurons in amyotrophic lateral sclerosis [3]. Therapeutic protection of these projection neurons has been difficult because the broad anatomical distribution of these projection neurons and their relative inaccessibility have made direct local delivery of therapeutic agents problematic.

In vivo gene delivery to the central nervous system (CNS) has been facilitated by the development of new viral vectors, such as adeno-associated virus (AAV), that are capable of infecting post-mitotic neurons [4–8]. AAV is particularly attractive due to its low toxicity and immunogenicity and its long-term gene expression [5–9]. However, widespread, targeted *in vivo* gene delivery is hampered by the hetereogeneous cytoarchitecture of the CNS. Intracranial injection must deliver the vector to a specific location without damage to targeted cells or collateral infection of unintended cells. This precision of delivery is difficult to achieve because many target neuronal populations are physically intermixed with neurons with different functions and axonal projections. As these neuronal populations are defined by their terminal projection fields, it would be advantageous to deliver vectors to those projection fields for subsequent retrograde transport and infection of the vulnerable projection neurons.

To test the hypothesis that delivery of genes to projection neurons can be accomplished by retrograde transport of viral particles, we injected AAV containing the reporter green fluorescent protein (GFP) into the hippocampus and striatum. The resulting infection of projection neurons in the entorhinal cortex and the substantia nigra, respectively, presents a delivery strategy for using gene therapy for treating Alzheimer's and Parkinson's diseases. This gene delivery approach also has relevance to other neuronal populations whose extensive distribution, such as spinal motoneurons in amyotrophic lateral sclerosis, makes targeted therapeutic intervention difficult. Furthermore, we demonstrate that an anti-apoptotic gene, *Bcl2l* (also known as Bcl-xL), can be delivered *in vivo* to a

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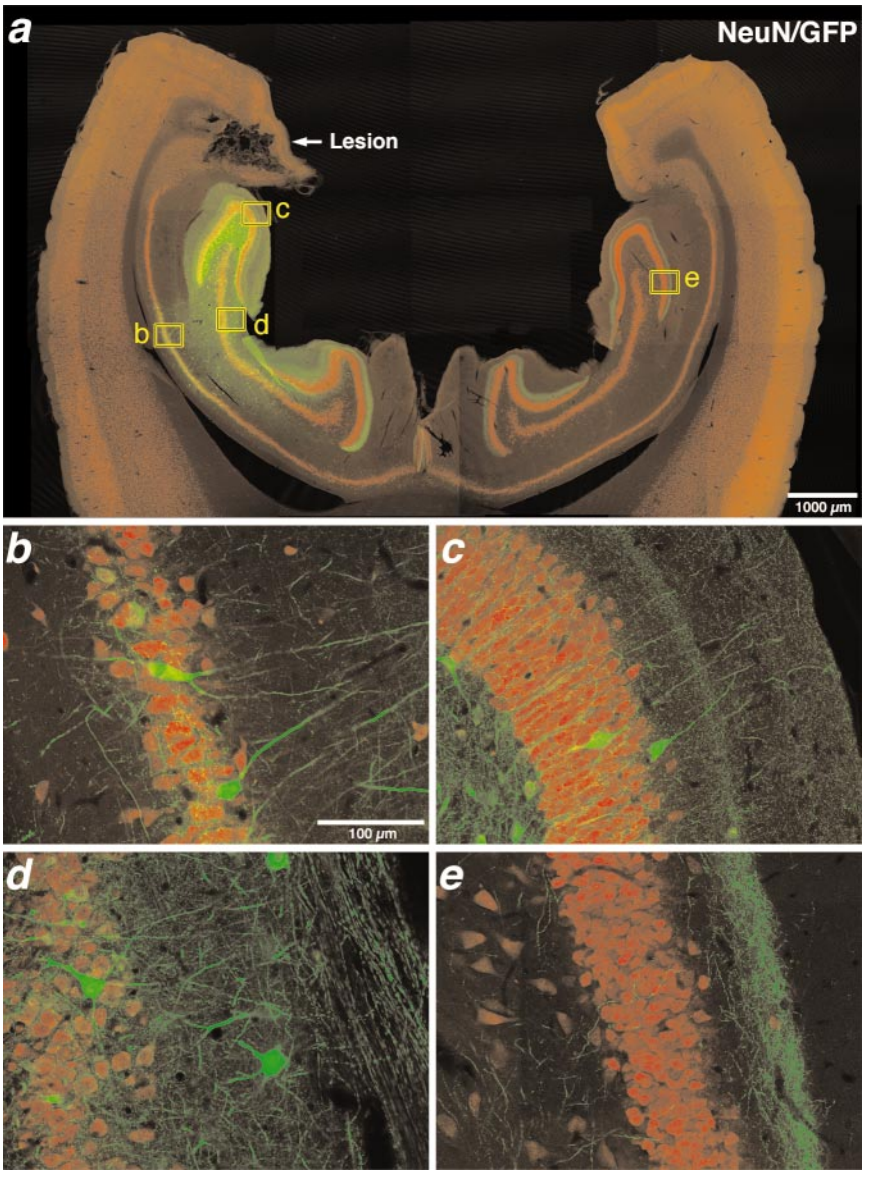

FIG. 1. Anterograde GFP filling of hippocampal neurons demonstrates neurotropic infection by AAV. (A) Unilateral injection of AAV-GFP into the right hippocampus infects neurons, detected by antibodies against the neuronal marker NeuN (red), in all hippocampal subfields, as shown in subsequent high-power views of the indicated boxed regions on this horizontal section through the dorsal hippocampus. The GFP gene product (green) fills neuronal cell bodies and anterogradely fills axonal processes including commissural projections. The location of the perforant pathway lesion is also indicated. Infection is shown of Area CA1 neurons (B), dentate granule neurons (C), and Area CA3 neurons (D). (E) Anterograde GFP filling of hippocampal commissural projections to the non-injected hemisphere.

pathway-specific projection neuron population and that the retrograde transport, infection, and expression of this gene product can protect these neurons from subsequent injury.

RESULTS

Retrograde Infection of Projection Neurons

To determine whether genes can be delivered to projection neurons by retrograde transport of viral particles, we injected AAV containing the reporter gene GFP into the hippocampus and striatum. Within 2 weeks following intrahippocampal viral injection, there was robust expression of gene product in neurons of all hippocampal subfields, with the greatest concentration of GFP-positive neurons in the dentate hilus (Fig. 1). Consistent with an earlier report [10], there was little infection of dentate granule neurons (Fig. 1C). Infection of neurons was also demonstrated by the anterograde filling of processes with diffused GFP [8]. Hilar mossy cells of the hippocampus project to the molecular layer of both the ipsilateral and contralateral granule cell layer [11] and, accordingly, GFP filled both ipsilateral and contralateral projections (Figs. 1C and 1E). There was little or no host immune response against the virus or GFP transgene, based on the absence of GFAP hypertrophy or CD4/CD8-positive cells; neither was vascular cuffing observed (data not shown).

The hippocampal formation receives input from various cortical, subcortical, and commissural projections. Preliminary observations of labeled projection neurons from these regions prompted us to systematically evaluate retrograde infection following AAV delivery. Cortical input to the hippocampus arises from primarily glutamatergic projection neurons in layer II of the entorhinal cortex traveling via the perforant pathway to form the entorhinodentate projection [12,13]. Within 2 weeks of AAV delivery to the hippocampus, GFP was expressed in entorhinal layer II neurons (Figs. 2A–2C). Expression levels varied between individual layer II neurons, but three-dimensional sampling using confocal microscopy revealed that, within the portion of the entorhinal cortex projecting to the site of injection, more than 80% of layer II neurons expressed some GFP. We detected a few GFP-expressing neurons in other brain regions projecting to the viral injection sites. We have not explored this observation, but likely factors contributing to the extent of retrograde transport include projection fiber and viral receptor densities at the site of injection [14,15].

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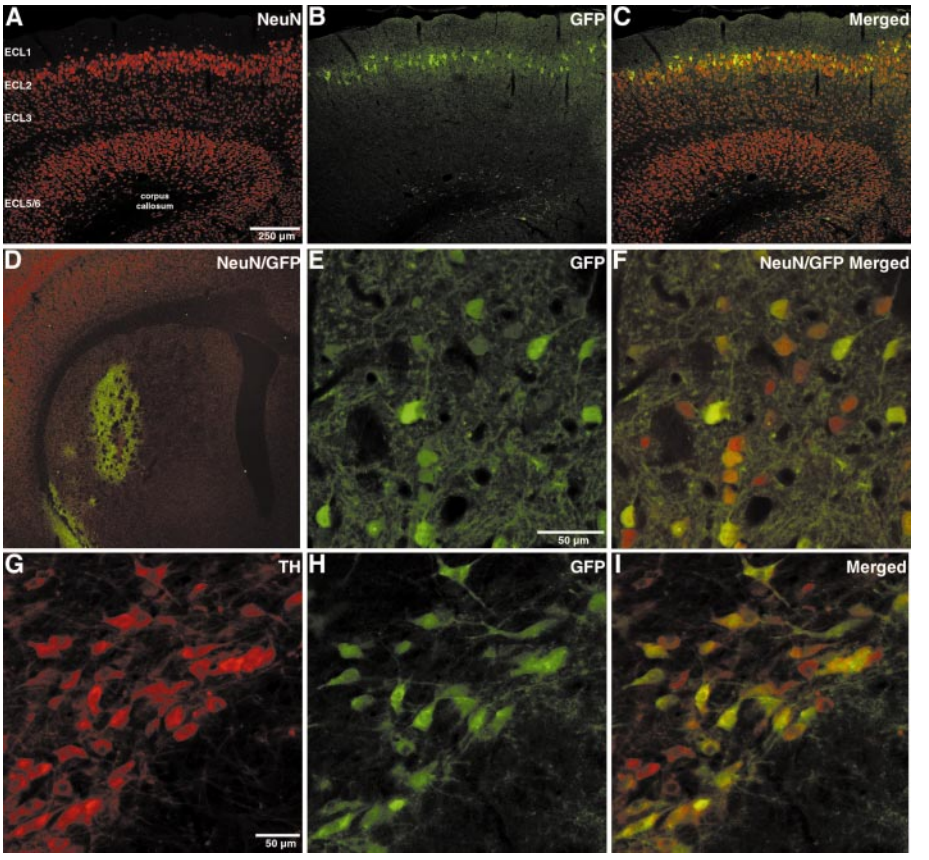

FIG. 2. AAV retrogradely infects specific projection neurons. (A–C) Intrahippocampal injection of AAV-GFP specifically infects entorhinal cortex projection neurons to the dentate gyrus in layer II (ECL2). See also Fig. 4. (D) Intrastriatal injection of AAV-GFP produces local infection of neurons. (E) GFP expression in center of the striatal injection site. (F) Merge of neuron-specific staining (red, NeuN) to demonstrate neurotropic infection by AAV. (G–I) Intrastriatal AAV-GFP injection produces specific retrograde infection of tyrosine-hydroxylase-positive (TH- red) projection neurons in the substantia nigra.

To determine if retrograde infection is unique to hippocampal projections, we injected AAV-GFP into the striatum and examined projection neurons within the substantia nigra pars compacta for expression of GFP. The dopaminergic nigrostriatal projection provides important modulatory input to the striatum and the progressive degeneration of this pathway produces the clinical manifestations of Parkinson's disease [1]. As observed in the hippocampus, delivery of AAV in the striatum produced substantial infection of local neurons (Figs. 2D–2F). Within 2 weeks of injection to the striatum, there was robust expression of GFP in tyrosine-hydroxylase-positive neurons of the substantia nigra pars compacta (Figs. 2G–2I).

Projection Neurons Retrogradely Transport Viral Particles

To discriminate between retrograde transport of GFP protein and true infection of projecting neurons by retrogradely transported AAV viral particles, we injected AAV particles conjugated to the fluorescent dye, Cy3, into either the hippocampus or striatum. Previous work has shown that careful conjugation of this probe does not alter the infectivity of the virus [10,16]. To demonstrate that labeled virus was fully infectious, HEK 293 cells were infected with Cy3-AAV-GFP. Confocal microscopic analysis showed that virus attached to the cell membrane within minutes; by 30 minutes, virus had localized within the nucleus, and GFP expression was observed by 24 hours (data not shown).

Examination of entorhinodentate and nigrostriatal projection neurons 24 hours following *in vivo* delivery of Cy3-conjugated AAV to the hippocampus and striatum, respectively, revealed the presence of Cy3 particles within the cytoplasm and nucleus of these projection neurons (Figs. 3A–3D). Systematic sampling of the labeled region revealed that 65% of substantia nigra pars compacta neurons and 90% of entorhinal layer II neurons contained Cy3-conjugated viral particles. Intraventricular delivery of the microtubule depolymerizing agent colchicine completely blocked the retrograde transport of Cy3-conjugated AAV particles at 24 hours after injection, demonstrating that viral particles were moved by specific retrograde axonal transport (data not shown). Furthermore, adjacent, non-projecting glial cells did not contain Cy3-conjugated AAV particles, suggesting that retrograde transport of viral particles had occurred by such an intracellular mechanism.

To determine if GFP was actively transcribed in the projection neurons, we carried out RT-PCR analysis 2 weeks following delivery of AAV to the hippocampus or striatum (Fig. 3E). We detected viral message at high levels at the hippocampal and striatal injection sites of both animals, as well as at lower levels in the entorhinal cortex and substantia nigra (Fig. 3E, arrow). Viral genomic DNA was detected in both injection areas and faintly in one substantia nigra region (Fig. 3E, arrowhead). The viral transport studies and the RT-PCR data indicate that the virus was retrogradely transported from the injection sites, the recombinant viral genome was converted to

double-stranded DNA, mRNA was transcribed and spliced, and GFP was translated.

Neuroprotection by Anti-apoptotic Gene Expression

We next asked whether targeted retrograde delivery of a therapeutic gene could protect or modulate the function of a population of projection neurons. Entorhinal layer II neurons suffer severe degeneration early in the progression of Alzheimer's disease [2,17,18]. By transecting the perforant pathway in a rodent model, we have been able to produce selective degeneration of layer II entorhinal neurons [13,19]. Previously, we found that local delivery of the anti-apoptotic gene *Bcl2l* protected septal neurons from injury [20]. Here, we injected AAV containing *Bcl2l* into the hippocampus to determine whether retrograde delivery and expression of *Bcl2l* would protect entorhinal layer II projection neurons from subsequent injury [21]. Virally delivered BCL2L was produced as a fusion protein with GFP to permit localization of infected cells, because it is not possible to discriminate by immunocytochemical detection between endogenous rodent BCL2L and the gene product alone. To demonstrate that this BCL2L/GFP fusion protein was functional, we infected HEK293 cells with either AAV-GFP or AAV-BCL2L/GFP and found that only the BCL2L/GFP gene product successfully protected cells from staurosporine- or tyrphostin-induced apoptotic cell death (data not shown).

Within 2 weeks following injection of AAV-BCL2L/GFP into the hippocampus, entorhinal layer II neurons showed expression of GFP in a distribution and intensity equivalent to those of AAV-GFP (Fig. 4A). Layer II neurons appeared healthy after infection with either construct, and quantitation revealed no cell loss following expression of the functional *Bcl2l* transgene (Fig. 5). Transection of the perforant pathway produced significant death of entorhinal layer II neurons after 2 weeks in animals expressing only GFP, with over 60% loss of layer II neurons (Fig. 5). Surviving neurons still expressed GFP, but were shrunken and surrounded by apoptotic bodies and an increased population of glial cells (Fig. 4B). In contrast, expression of BCL2L protected entorhinal layer II neurons from the significant injury-induced death seen with expression of GFP alone (Fig. 5). Entorhinal neurons appeared healthy and continued to express the BCL2L/GFP transgene (Fig. 4C). In agreement with the protective effect reported with locally delivered anti-apoptotic genes [22–24], these data suggest that expression of *Bcl2l* in targeted projection neurons is sufficient to protect neurons against substantial injury.

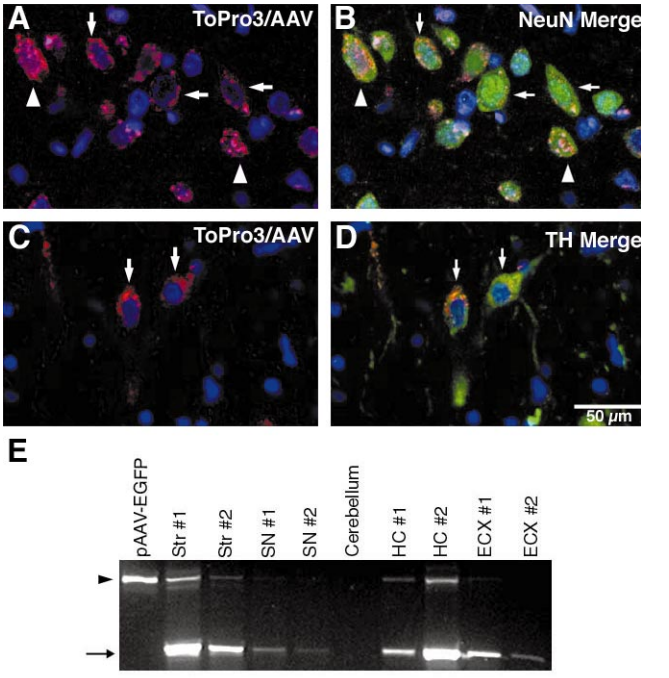

FIG. 3. AAV viral particles are retrogradely transported to entorhinal and nigral projection neurons and the transgene is actively transcribed in these cells. (A) AAV viral particles conjugated to the fluorophore Cy3 (red) are present in a population of entorhinal layer II cells (arrows, intracytoplasmic; arrowheads, intranuclear) detected by the DNA stain ToPro3 (blue) following injection to the ipsilateral dentate gyrus. (B) Merging the image in (A) with the neuronal marker, NeuN (green), shows that the viral particles are within entorhinal neurons. (C) AAV-Cy3 tagged virus injected into the striatum detected in cells (arrows) of the ipsilateral substantia nigra. (D) Detection with tyrosine hydroxylase (TH, green) reveals the presence of viral particles within TH-positive nigral neurons. (E) RT-PCR analysis of viral expression revealed appropriate 300-bp transcripts (arrow) in the substantia nigra (SN) of animals receiving striatal injections (Str) and in the entorhinal cortex (ECX) of animals receiving hippocampal (HC) injections of AAV-GFP. At the site of injection, viral genomic DNA generated a 900-bp product (arrowhead) in a control reaction using a pAAV-GFP vector plasmid.

DISCUSSION

The targeted infection of projection neurons in the entorhinal cortex and the substantia nigra and the delivery of a therapeutic transgene demonstrate a delivery strategy of potential use for gene therapy of CNS disorders, particularly as shown in this work for Alzheimer's and Parkinson's diseases. Targeted retrograde gene delivery using AAV offers several advantages for therapeutic intervention in CNS disease and injury. Genes are delivered to both the target of the neural pathway and the projecting neurons, providing for gene expression at both levels of the neural circuit. Retrograde targeting can facilitate gene delivery to projection neurons that present difficult surgical access for direct viral injection. Finally, targeted retrograde gene delivery overcomes the problem of achieving adequate and specific distribution of virus to projection neurons with wide anatomical distribution. For example, the targeted delivery to entorhinal projection neurons of layer II (Figs. 2A–2C) would not be possible with direct injection of virus into the entorhinal cortex. Similar challenges face delivery to other projection neuron populations, such as spinal motoneurons in ALS.

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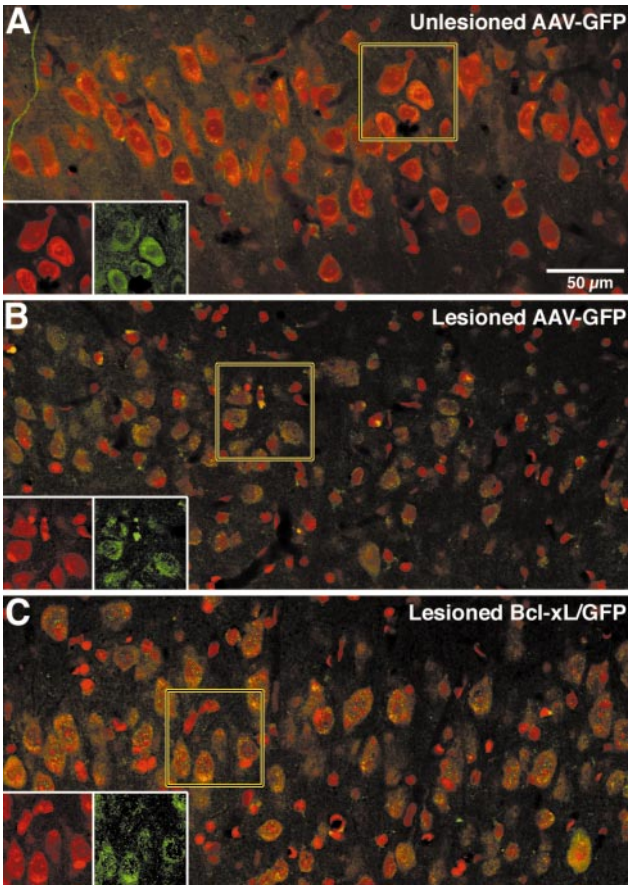

FIG. 4. Retrograde *in vivo* gene delivery of the anti-apoptotic gene *Bcl2l* (BclxL) protects entorhinal neurons against subsequent injury. (A) Entorhinal layer II projection neurons to the dentate gyrus form a distinct band of cells in the uninjured entorhinal cortex. Projection neurons are large cells with an RNA-rich cytoplasm (propidium iodide; red). Merging the propidium iodide image with colocalized GFP expression (green) results in cells with an orange hue. The insets show individual staining of the region boxed in yellow discriminating between the nucleic acid staining and the GFP expression. Entorhinal neurons infected with the BCL2L/GFP construct appear indistinguishable from infection by AAV-GFP alone. (B) Prior retrograde *in vivo* AAV gene delivery of the reporter gene GFP fails to protect vulnerable layer II neurons from subsequent lesion of the perforant pathway, resulting in neuronal loss or atrophy of remaining neurons. Glial cell numbers are increased in response to injury and apoptotic bodies are seen (insets). GFP is still expressed within surviving neurons (insets). (C) *In vivo*, retrograde delivery of AAV-BCL2L/GFP protects entorhinal neurons from perforant pathway lesion. Individual entorhinal neurons appear healthy as in (A) and continue to express BCL2L/GFP.

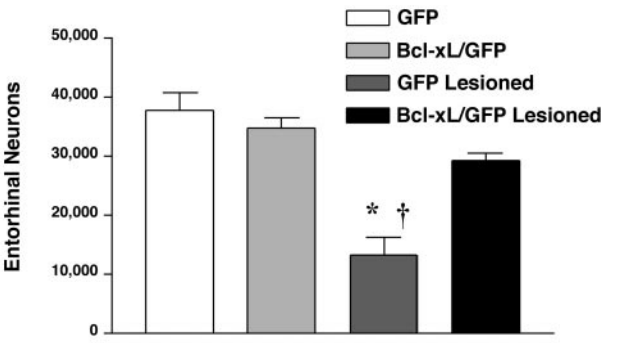

FIG. 5. Quantitation of entorhinal layer II neurons shows no toxic effect of BCL2L (BclxL) expression in unlesioned animals. A perforant pathway lesion produced a significant 60% loss of GFP-expressing entorhinal neurons relative to unlesioned animals ($P < 0.001$; asterisk). In contrast, there were more than twice as many surviving neurons in BCL2L-expressing animals, demonstrating significant protection by this transgene relative to GFP expression alone ($P < 0.01$; dagger).

While there have been numerous reports that AAV vectors of any serotype are not retrogradely transported or are transported in only a limited fashion [7,8,25,26], we have reported that AAV undergoes a specific transport sequence by internalization and rapid, microtubule-mediated transport to the nucleus of projection neurons in an efficient manner (Fig. 3). The differences between our findings and others could be explained by vector titer, purity, or injection into axonal pathways that have increased viral receptors for AAV entry, as well as sensitivity of detecting the transgene. So far, gene therapy studies have only used adenovirus, herpes simplex virus, and pseudo-rabies virus for delivering transgenes in a targeted retrograde delivery strategy [27–31]. While these studies have shown very encouraging results targeting large numbers of cells by using the transport properties of the virus, toxicity from these vector systems can limit expression duration and yield variable results. The ability to use AAV in a retrograde delivery strategy may advance study of the functional modulation of CNS circuitry by delivering experimental transgenes to effect a gain or loss of function in identified neural circuits.

Recently, a link between neurodegeneration of layer II neurons of the entorhinal cortex and cognitive impairment has been established. Significant losses of layer II neurons were found in patients experiencing mild cognitive impairment and mild to moderate Alzheimer's disease, suggesting that the onset of cognitive decline correlates with the onset of neuronal loss that occurs in the hippocampus and the entorhinal cortex [2,17,18,32]. Therefore, targeted delivery of anti-apoptotic or other therapeutic genes to vulnerable projection neurons may be a useful neuroprotective strategy for early stages of neurodegenerative disease. These results demonstrate an ability to efficiently target areas within the nervous system by a safe, long-term expressing AAV vector that may permit the development of more efficient delivery strategies to target diseased areas within the brain.

MATERIALS AND METHODS

Virus production. Recombinant AAV-2 carrying eGFP (Clontech) or a GFP-BCL2L fusion (human BCL2L cDNA; John Reed, Burnham Inst.) driven from the human CMV promoter was produced in HEK293 cells (ATCC) by calcium phosphate transient transfection of vector plasmid and pAAV/Ad8 helper plasmid, followed by infection with adenovirus dl312 (MOI 2.0;

[33]). Virus was purified by two CsCl density gradients, dialysis, and heating to 56°C for 1 hour. Recombinant virus titers were 5×10^{10} infectious particles per milliliter. All viral stocks were tested and found to be free from contaminating adenovirus [33].

Surgery and histology. All surgical procedures were performed in accordance with NIH guidelines and with institutional approval. F344 female rats (140–160 g; Harlan Sprague-Dawley) were deeply anesthetized and positioned in a stereotaxic frame for right-unilateral injection into the hippocampus (AP–4.0, ML–3.0, and DV–2.5 from dura) or striatum (AP+0.2, ML–3.0, and DV–4.0 from dura). Viral suspension (3 μl per site at 5×10^{10} infectious particles/ml) was injected at a rate of 0.3–1 μl/min. Animals received either rAAV-GFP (hippocampus, $n = 12$; striatum, $n = 6$) or rAAV-Bcl2l/GFP (hippocampus, $n = 12$). Animals were transcardially perfused 4 weeks after viral injection with 4% paraformaldehyde and serial 50-μm horizontal sections (hippocampal-injected animals) or coronal sections (striatal-injected animals) were produced by freezing sliding microtomy. Multiple immunofluorescent labeling [6,19] used antibodies against NeuN (mouse, 1:50, R. Mullen, Univ. Utah), tyrosine hydroxylase (TH, rabbit, 1:1000, Chemicon), anti-GFAP (guinea pig, 1:1000, Advanced Immunochemical), anti-CD4 and CD8 (both mouse, 1:1000, Pharmingen), and anti-GFP (rabbit, 1:500, Clontech) to enhance detection of the reporter gene. Specificity of anti-GFP detection was verified by staining control, uninfected tissue. Donkey anti-species antibodies conjugated to biotin, FITC, Cy3, or Cy5 and Streptavidin-FITC (1:250; all from Jackson Immunoresearch) were used for detecting primary antibodies. Fluorescent DNA stains used were DAPI (30 ng/ml), propidium iodide (PI, 1:1000), or ToPro3 (1:5000, all from Molecular Probes). Microscopy was performed using confocal microscopes (BioRad MRC1024UV or Olympus FluoView 200).

Viral transport detection. For viral transport studies, rAAV-GFP was produced with pXX6, a helper plasmid for use in adenovirus-free AAV packaging [34], purified by four CsCl gradients to ensure high purity, and labeled with *N*-hydroxysuccinimidyl ester Cy3 reagent (Amersham), as described [16,35]. Animals received intracranial injection of Cy3-conjugated AAV (hippocampus, $n = 3$; striatum, $n = 3$) and were perfused 24 hours later. As a control to demonstrate that viral infection was specific, infection of 293 cells was prevented by competition with monoclonal antibody A20 (1:5, American Research Products) that binds intact virion particles. For transport blocking studies, 3 μl of colchicine (10 mg/ml, Sigma) was injected into the right lateral ventricle ($n = 8$, saline control $n = 2$). After 12 hours, Cy-3 conjugated AAV was injected into hippocampus ($n = 4$) or striatum ($n = 4$) as above and the animals were perfused 24 hours later. One animal from each colchicine group died before perfusion.

Expression analysis. Tissue from hippocampal ($n = 2$) or striatal ($n = 2$) rAAV-GFP injected animals was collected after 2 weeks using RNase-free materials and reagents. RNA was isolated from both the injection sites and projection neuron populations, in addition to a control region (cerebellum). Total RNA was isolated from the tissue using the RNAzol B reagent (Tel-Test, Inc.). Reverse transcription was performed with the Superscript kit (Life Technologies) using oligo dT primer. For amplification, the 5′ primer, 5′-GTGGATCCTGAGAACTTCAG-3′, was homologous to the 5′ untranslated region of the rAAV-GFP transcript, whereas the 3′ primer, 5′-AAGTCGTGCTGCTTCATGTGG-3′, was homologous to GFP. Thirty cycles of PCR were performed (1 minute each at 94°C, 60°C, and 72°C) using *Taq* DNA polymerase (Promega). PCR products were analyzed by electrophoresis on a 3% agarose gel. We used amplification primers flanking an intervening sequence from the human β-globin intron at the 5′ end of the transcript to distinguish single-stranded viral DNA from mRNA. Amplification from single- or double-stranded viral genomic DNA generated a 900-bp product in a control reaction using pAAV-GFP vector plasmid as the template, whereas mRNA yielded a 300-bp product.

Neuroprotection studies. Two weeks following viral injection, half of the hippocampus-injected animals (AAV-GFP, $n = 6$; AAV-Bcl2l/GFP, $n = 6$) received right-unilateral perforant path lesions as described [13,19]. Quantification of entorhinal layer II neuron number was performed on a one in six series of propidium-iodide-stained sections for each animal using the optical fractionator procedure (MicroBrightField, Inc.) as described [19,36,37]. Statistical analysis was performed by multi-way ANOVA followed by a Bonferroni post-hoc analysis of means differences between groups (GraphPad Software).

ACKNOWLEDGMENTS

We thank Cynthia Pervan (Chicago Medical School), Lynne Randolph-Moore, Marie-Claude Senut, and Steve Suhr (Salk Institute) for advice and assistance, and Mary Lynn Gage (Salk Institute) for critical reading of the manuscript. This work was supported by a T.L.L. Temple Foundation Award from the Alzheimer's Association, NIH/NIA, Project ALS, and The Pasarow Foundation.

RECEIVED OCTOBER 3; ACCEPTED DECEMBER 3, 2001.

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