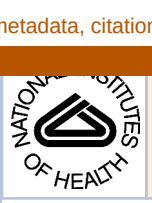

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Delivering multiple gene products in the brain from a single adeno-associated virus vector

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Abstract

For certain gene therapy applications, the simultaneous delivery of multiple genes would allow for novel therapies. In the case of adeno-associated virus (AAV) vectors, the limited packaging capacity greatly restricts current methods of carrying multiple transgene cassettes. To address this issue, a recombinant AAV (rAAV) vector was designed such that a furin proteolytic cleavage site (RKRRKR) was placed between the coding sequences of two genes (green fluorescent protein (GFP) and galanin), to allow cleavage of the chimeric protein into two fragments. In addition, these constructs contained the fibronectin secretory signal sequence that causes the gene products to be constitutively secreted from transduced cells. In vitro studies show that after transfection of HEK293 cells, the appropriate cleavage and constitutive secretion occurred regardless of the order of the genes in the transgene cassette. In vivo, infusion of rAAV vectors into the piriform cortex resulted in both GFP expression and significant galanin attenuation of kainic acid-induced seizure activity. Thus, the present results establish the utility of a proteolytic approach for the expression and secretion of multiple gene products from a single AAV vector transgene cassette.

Keywords

brain; AAV; multiple genes; furin; galanin

Introduction

The expression of multiple genes from a single virus vector might benefit any number of therapeutic gene therapies, from increasing the absolute number of gene products per virus vector to the delivery of distinct genes. However, to date, the delivery of multiple genes usually has required the use of additional vectors, particularly when adeno-associated virus (AAV) vectors are used. For example, Shen *et al*.[1] reported that delivery of a combination of genes including tyrosine hydroxylase, aromatic-L-amino acid decarboxylase and tetrahydrobiopterin increased the amount of dopamine production in a rat Parkinson's model. In this particular case,

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Conflict of interest

The authors declare no conflict of interest.

size constraints required these investigators to use three different recombinant AAV (rAAV) vectors to achieve expression of all three genes.

In the past 20 years, several different virus-based strategies have been explored to express two genes from a single vector, such as bicistronic vectors that use multiple promoters or internal ribosome entry sites. Unfortunately, several groups have reported a lack of efficacy with these vectors because of differential promoter silencing, transcriptional interference and unequal levels of gene expression.[2–5] Furthermore, the large size of multiple promoters or internal ribosome entry sites restricts the range of therapeutic genes that can be delivered, especially in the context of viral vectors with limited packaging capacity. Another approach for multiple gene delivery from a single vector relies on generating fusion proteins. As both proteins remain attached to each other, steric hindrance may alter the function of one or both proteins.[6,7] Many of these disadvantages might be circumvented by using a proteolytic processing strategy in which a chimeric protein is processed into separate bioactive peptides. First, unlike traditional fusion proteins, cleavage into separate peptides would increase the likelihood that each protein will retain its biological activity. Second, this method would ensure that each transduced cell expresses both gene products. Third, there would be only one promoter and one transcript, so differential promoter silencing, transcriptional interference and unequal levels of gene expression cannot occur. Fourth, proteolytic cleavage consensus sequences can be as small as two amino acids.[8] Finally, by using a single vector instead of a co-infection strategy, the total viral particle number can be reduced.

The following studies show that by using the furin cleavage consensus sequence arginine-lysine-arginine-arginine-lysine-arginine (RKRRKR) in rAAV vectors, one can obtain expression, appropriate cleavage and constitutive secretion of two separate proteins *in vitro*. Additionally, both genes retain biological function after vector delivery in the brain.

Results

In vitro characterization of multiple gene product delivery vectors

Plasmids were constructed where a single hybrid chicken beta (CB)-actin promoter drives expression of a single chimeric fusion protein containing the fibronectin secretory signal sequence (FIB)[9] and a furin consensus cleavage site (see Figure 1). Multiple constructs were made containing green fluorescent protein (GFP) and galanin in both possible orientations, to test whether GFP was correctly processed and cleaved from galanin whether it was 5′ or 3′ to the cleavage site. To detect GFP cleavage from galanin, GFP secretion was assayed by western blot. However, galanin cleavage could not be determined because of the extremely rapid degradation of galanin in serum.[10] Transfection of the various constructs into 293 cells produced fluorescence patterns similar to that seen by Haberman *et al*.[9] where cells transfected with unsecreted GFP exhibit a bright and uniform distribution of intracellular GFP (Figure 2a, panel 1) and a relatively dark background. In contrast, cells transfected with FIB-GFP show fluorescence concentrated around the perimeter of the cellular membrane and a high background, suggestive of GFP secretion into the media (Figure 2a, panel 2). Cells transfected with the various FIB-GAL-GFP chimeric proteins also show fluorescence concentrated around the perimeter, similar to those cells transfected with FIB-GFP (Figure 2a, panels 2–5). No fluorescence is detected when cells are transfected with the galanin plasmid alone (Figure 2a, panel 6).

Western blots of these samples from Figure 2a were performed following immunoprecipitation with a poyclonal GFP antibody, to further characterize gene products in the conditioned media and cell lysates. As seen in Figures 2b–c, lane 1 shows that in the absence of the FIB, the GFP is expressed but not secreted into the medium. Conversely, for CB-FIB-GFP, the GFP is expressed and secreted into the medium (Figures 2b–c, lane 2). For the CB-FIB-EGFP-

RKRRKR-GAL transfection, GFP is cleaved from galanin and secreted into the medium, whereas some unprocessed protein is detected in the cell lysate (Figures 2b–c, lane 3). Similarly, after CB-FIB-GAL-RKRRKR-EGFP transfection, GFP is cleaved from galanin and secreted into the medium whereas some unprocessed protein is detected in the cell lysate (Figures 2b–c, lane 4). Transfection of CB-FIB-GAL-EGFP, the construct that lacks a protease cleavage site, results in the secretion of an uncleaved chimeric fusion protein into the medium (Figures 2b–c, lane 5). A small amount of degraded protein is present in the cell lysate, but does not get secreted (Figures 2b–c, lane 5). Finally, transfection of CB-FIB-GAL shows that the GFP antibodies do not crossreact with galanin (Figures 2b–c, lane 6). Taken together, GFP is cleaved from the chimeric protein and secreted into the medium regardless of the relative position to the furin cleavage site.

In vivo functional test of multiple gene product delivery vectors

To test the *in vivo* function of these proteins, we packaged our multiple gene product delivery constructs into rAAV2 vectors where the CB promoter drives expression of the FIB-GAL-RKRRKR-EGFP or FIB-EGFP-RKRRKR-GAL coding sequence. Then, 3 μl of virus (1.0×10^{12} viral particles per ml) was infused bilaterally into the piriform cortex of rats, as described earlier.[11] Control rats received no infusion, as we have previously shown that vectors expressing and secreting GFP (AAV-FIB-EGFP), as well as vectors expressing peptides that lack secretion sequences (AAV-GAL) have no effect on seizure sensitivity, seizure behaviors or seizure-induced cell death.[9,11] One week later, all rats received a 10mg kg^{-1}, intra-peritoneal (ip) dose of kainic acid, and subsequently, the time to limbic seizure behaviors was recorded.

Presence of GFP protein *in vivo* shows the transduction patterns after injection of AAV-CB-FIB-GAL-RKRRKR-EGFP or AAV-CB-FIB-EGFP-RKRRKR-GAL, and confirms that the viruses are functional in both orientations (Figure 3). Again, the localization of GFP to the periphery of the transduced neurons is supportive of secretion. As seen in Figure 4, all groups exhibited wet dog shakes with similar latency, validating the efficacy and uniformity of the absorbed dose of kainic acid. With respect to galanin expression, all vector-treated animals showed a significant delay in the onset of class IV seizures (**t*-test; $P \leq 0.01$). This significant increase in the latency to seizures shows that the galanin gene product is in fact expressed, secreted and functional regardless of whether it is 5′ or 3′ of the cleavage site in our multiple gene product delivery vectors.

To eliminate the possibility that galanin would still be functional as an uncleaved chimeric protein *in vivo*, we also made virus from the noncleavable construct FIB-GAL-EGFP and tested it in the kainic acid paradigm. We found no significant difference between the FIB-GAL-EGFP-treated rats ($N = 6$) and controls ($N = 5$) for the latency to wet dog shake behaviors (FIB-GAL-EGFP—54±3 min; untreated controls—58±7 min) or class VI seizure behaviors (FIB-GAL-EGFP—78±6 min; untreated controls—80±9 min; data not shown).

Discussion

The present results establish the utility of a proteolytic approach for the expression and secretion of multiple gene products from a single AAV vector. When a furin cleavage consensus site was inserted between the coding sequences for two separate proteins, *in vitro* findings show first that a chimeric fusion protein is produced within the cell. Subsequently, intracellular processing cleaves the chimeric protein and constitutively secretes the cleaved proteins. Our results are supported by previous experiments in which the delivery of two protein subunits was obtained with a single vector cassette.[12,13]

The data also show that a single FIB sequence is sufficient for secretion of both gene products, and that the FIB-dependent trafficking of the chimeric protein into the constitutive secretory

pathway must occur before furin cleavage. If furin cleavage occurred before the entry into the FIB pathway, then constitutive secretion of both proteins would require two FIB sequences. Additionally, the western blot data suggest that a few amino acids from the FIB sequence may be left on the 5′ end of the gene immediately following it (Figure 2b, compare lanes 2–3 to lane 4). In fact, we have confirmed this finding by mass spectrometry (data not shown). Although function is not compromised for FIB-mediated secretion of galanin, GFP or NPY,[9,11,14] these uncleaved residues may be a problem for genes whose functional domains are very close to the n-terminus of the protein.

The *in vitro* findings also indicate that the proteins are correctly processed intracellularly regardless of position relative to the cleavage sequence before secretion from the transfected cells. Similarly, the absence of GFP–galanin fusion protein in the medium suggests a 100% efficient cleavage for those proteins that are constitutively secreted. These data are somewhat contradictory to the data obtained in previous experiments.[15] Gaken *et al*.[15] found that furin-mediated cleavage of two cytokines from cells transduced with retroviral vectors results in only around 50% cleavage efficiency. This difference in cleavage efficiency may be explained by variations in the endogenous level of furin between cell lines. Alternatively, the change in cleavage efficiency may be because of the use of a different furin cleavage consensus sequence.

In addition to being correctly processed and secreted *in vitro*, both proteins retain their function after direct brain injection of cleavable vector cassettes. However, galanin loses its therapeutic efficacy as an uncleaved chimeric fusion with GFP. Thus, we have established that a proteolytic strategy can be effective in delivering at least two functional proteins from a single AAV vector. Even though these findings provide a ‘proof of principle’, the antiseizure effect was not as robust as was found in earlier studies in which galanin alone was delivered.[11] Certainly, the level of GFP expression seemed somewhat less than the level found with an AAV-CB-FIB-GFP vector (data not shown). The explanation for this reduction in efficacy will require further detailed investigation, and most likely will improve as vector development progresses.

These findings clearly show that it is possible to deliver multiple proteins from a single AAV vector *in vivo*, thus increasing the potential to treat polygenic diseases. Although two or more distinct proteins can be generated using this technique, it may also be used to manipulate the ratios of these proteins by inserting multiple copies of the same protein in tandem. Ultimately, as most neurological diseases such as epilepsy involve numerous genes, second messenger pathways and downstream targets, coordinating the delivery of multiple therapeutic genes will facilitate the development of effective treatments.

Materials and methods

Cloning plasmids for multiple gene product delivery vectors

The plasmids pTR-CB-EGFP, pTR-CB-FIB-EGFP and pTR-CB-FIB-GAL were generated as described earlier.[9] Briefly, gene expression is driven by the hybrid CB-actin promoter, the mature coding sequence of the transgene is followed by the SV-40 polyA, and the cassette is flanked by AAV2 TRs. The plasmid EGFP-N1 was purchased from BD-Biosciences-Clontech (San Jose, CA, USA), and the GFP was digested out using AgeI–NotI restriction sites. The fragment was gel purified and ligated into the AgeI–NotI sites of the AAV2 plasmid backbone, resulting in the plasmid pTR-CB-EGFP. Then the annealed FIB oligonucleotides were ligated into the AgeI site resulting in the plasmid pTR-CB-FIB-EGFP. The galanin sequence was amplified by RT–PCR from rat brain RNA using primers directed to the mature peptide sequence, such that melting and reannealing of two separate PCR products resulted in 5′ AgeI and 3′ NotI overhangs.[16] The 3′ primer included a stop codon to properly terminate translation. The sequence of the primers used is as follows: (Gal 5′ long) 5′-CCG GTA ATG GGC TGG ACC CTG AAC-3′, (Gal 5′ short) 5′-TA ATG GGC TGG ACC CTG AAC-3′, (Gal 3′ short)

5′-GC TCA TGT GAG GCC ATG CTT G-3′, (Gal 3′ long) 5′-GGC CGC TCA TGT GAG GCC ATG CTT G-3′. The PCR product was ligated into the AgeI–NotI sites of the AAV2 plasmid backbone, resulting in the plasmid pTR-CB-GAL. Then the annealed FIB oligonucleotides were ligated into the AgeI site resulting in the plasmid pTR-CB-FIB-GAL. Using pTR-CB-FIB-GAL as a backbone, the other plasmids were generated by PCR so the furin cleavage consensus sequence arginine-lysine-arginine-arginine-lysine-arginine (RKRRKR) was cloned in frame between two genes. Briefly, two pairs of oligonucleotides were designed for each construct, such that a forward and reverse primer corresponded to each gene plus 3 of the six amino acids that make up the furin consensus sequence (see Table 1). The oligonucleotides included the restriction sites NheI and NotI that would be used for cloning. Two fragments each containing the gene of interest plus half of the furin consensus sequence were generated by PCR, then the products were digested with NheI and NotI, and ligated into the plasmid backbone using a sticky-blunt-sticky ligation. All plasmids were sequenced by the UNC Lineberger Cancer Center DNA Sequencing Facility to verify accuracy.

Cell culture and transfection

293 cells were maintained at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and penicillin–streptomycin. Cells were plated at a density of 5.5×10^5 cells per ml medium in 60 mm dishes the day before transfection. Cells were transfected using the polyethyleneimine technique.[17] Forty-eight hours after transfection, cells were imaged then medium and lysates were collected. Briefly, medium was cleared by centrifugation at 2500 r.p.m. at 4 °C for 5 min to remove any cellular debris. Cells were scraped and rinsed with 2 ml Dulbecco phosphate-buffered saline (DPBS) then lysed using 1 ml lysis buffer (50 mM Tris pH8.0, 150 mM NaCl, 50 mM NaF, 1% NP-40) per 60 mm plate. Lysates were cleared by spinning in centrifuge at maximum speed at 4 °C for 5 min, supernatant was transferred to a clean tube.

Immunoprecipitation and western blotting

Immunoprecipitation of GFP was performed on cleared medium and lysates. Briefly, medium and lysates were preincubated with 20 μl Protein A Sepharose beads (GE Healthcare Life Sciences, Piscataway, NJ, USA) and rocked for 1 h at 4 °C to remove nonspecific binding. Supernatant was transferred to a clean tube. For medium 50 μl Protein A beads and 10 μl Living Colors Full-Length A.V. Polyclonal antibody (BD-Biosciences) was added. For lysates 25 μl Protein A beads and 5 μl Living Colors Full-Length A.V. Polyclonal antibody (BD-Biosciences) was added. Samples were incubated overnight on a rocker at 4 °C. Supernatant was removed by vacuum and beads were washed three times using 1 ml DPBS. Load dye was added to beads along with water and betamercaptoethanol, then samples were boiled 5 min and cooled to room temperature before loading on 10% Bis–Tris gel (Invitrogen, Carlsbad, CA, USA). Samples were transferred by western blot to a nitrocellulose membrane and blocked using 10% nonfat milk in Tris buffered saline+0.1% Tween (TBST). Blot was incubated at 4 °C overnight in 1:1000 dilution of primary antibody Living Colors A.V. Monoclonal antibody JL-8 (BD-Biosciences). Blot was rinsed three times for 5 min in TBST then incubated in a 1:3000 dilution of goat-anti-mouse HRP (Pierce, Rockford, IL, USA). Signal was detected using the West femto-chemiluminescence kit (Pierce) according to the manufacturer's instructions.

rAAV production, purification and characterization

Recombinant AAV2 was produced and purified as described earlier[18] with the following modifications: 293 cells (10 cm × 15 cm plates per prep) were transfected with 60 μg transgene plasmid, 120 μg XX-680 and 100 μg PXR2 through the polyethyleneimine technique. Nuclei were isolated and lysed using sonication. AAV particles were purified by cesium chloride

density gradient. Peak fractions were determined through dot blot hybridization, and extensively dialyzed against DPBS+10% (wt/vol) D-sorbitol. Final titer was determined by dot blot hybridization (modified Southern blot) using a probe against the CB promoter sequence. To determine the infectious quality of the virus preps, infectious center assays were also performed using C12 cells (293 cells with Rep stably integrated) as described earlier.[17]

Experimental animals

The animals were pathogen-free male Sprague–Dawley rats obtained from Charles Rivers. They were maintained in a 12 h light–dark cycle and had free access to food and water. All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85–23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Usage Committee.

rAAV vector microinjection

For AAV infusions, rats were first were anesthetized with 50mg kg^{-1}, ip pentobarbital and placed into a stereotaxic frame. Using a 32 gauge stainless steel injector and a Sage infusion pump, the rats received 3 μl virus (1.0×10^{12} viral particles per ml) over 20 min into the piriform cortex (interaural line 6.7 mm, lateral 6.0 mm, vertical 8.4 mm), according to the atlas of Paxinos and Watson.[19] The injector was left in place for 3 min post infusion to allow diffusion of virus away from the injector. In all cases, the incision was sutured, and the animals were allowed to recover for 7 days.

In vivo *detection of AAV-derived GFP*

Although the GFP is likely being secreted in these animals, it is very stable with a fairly long half-life (around 80 h).[20] This allows us to visualize the pattern of AAV transduction through GFP immunohistochemistry. Ten days after piriform cortical infusion of AAV vectors containing GFP, the animals received an overdose of pentobarbital (100 mg kg^{-1}, ip) and subsequently were perfused transcardially with ice-cold 100 mM sodium PBS (pH 7.4), followed by 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). After overnight fixation in the paraformaldehyde–phosphate buffer, tissue sections (40 μm thick) were taken using a Leica vibrating blade microtome and rinsed in PBS. The sections were mounted and GFP fluorescence was visualized on an Olympus IX-70 fluorescent microscope (Center Valley, PA, USA).

Kainic acid treatment

Seven days after piriform cortical infusion of the AAV vectors, the animals received a 10 mg kg^{-1}, ip dose of kainic acid (Cayman Chemical Co., Ann Arbor, MI, USA). Using the limbic seizure scale of Racine,[21] the latency was recorded to classes III, IV and V limbic seizure behavior for up to 180 min post kainic acid. Four hours after kainic acid treatment, the animals received an overdose of pentobarbital (100 $mgkg^{-1}$, ip) and subsequently were perfused transcardially with ice-cold 100 mM sodium PBS (pH 7.4), followed by 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). After overnight fixation in the paraformaldehyde–phosphate buffer, 40 μm thick tissue sections were taken and rinsed in PBS. The sections were mounted and the infusion placement was determined.

Statistics

Treatment effects were evaluated using a two-tailed *t*-test where a $P<0.05$ was required for significance. Mean scores were calculated for each vector-treated group then used for comparative analysis against untreated controls. If class IV seizure behavior was not observed the animal was given a maximal score of 180 min.

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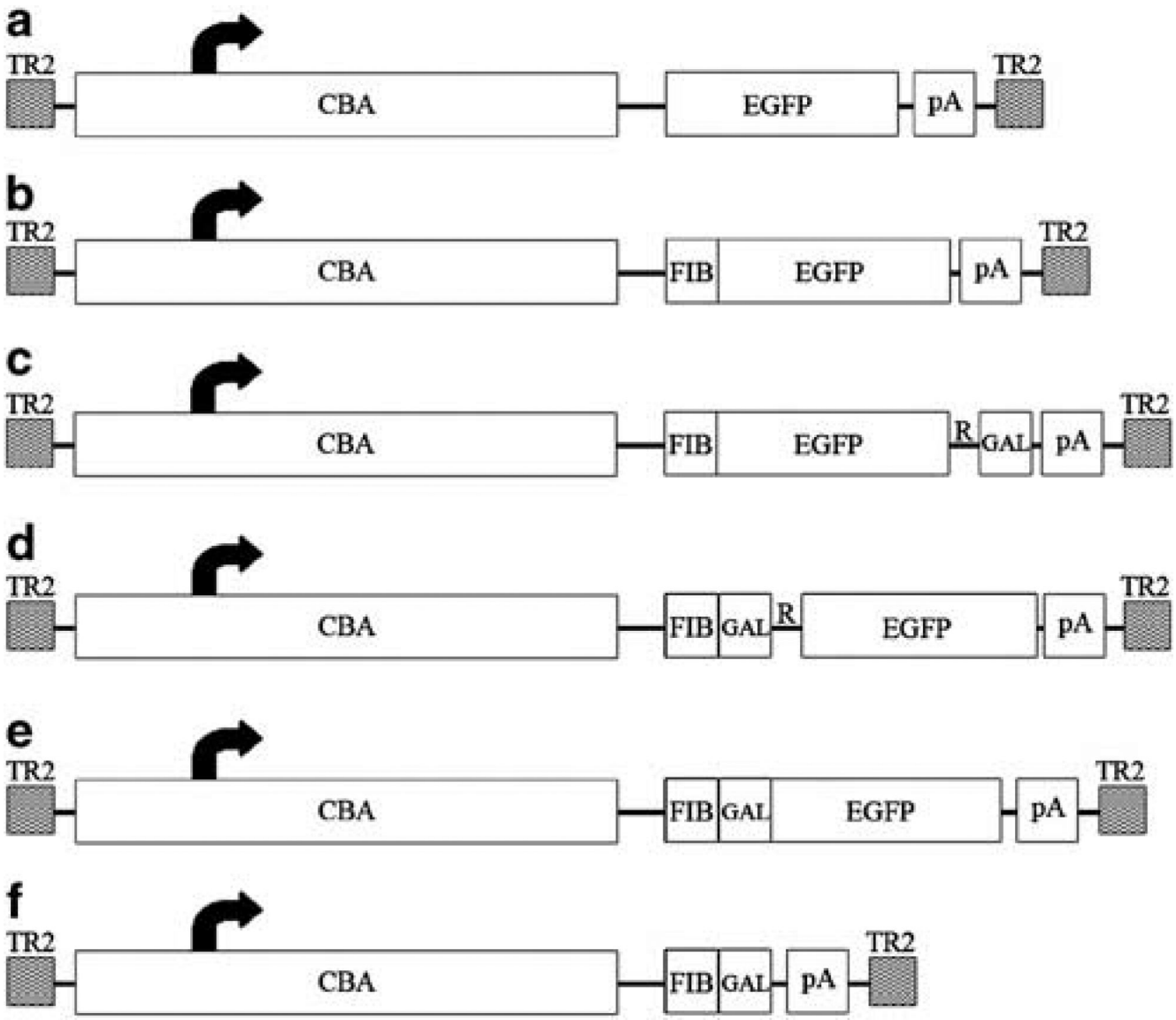

Figure 1.

Diagram of vector constructs. (**a**) CB-EGFP is a negative control used in immunoprecipitation in western blot (IP/WB) experiments to show that GFP is expressed but not secreted in the absence of an FIB sequence. (**b**) CB-FIB-EGFP is a positive control used in IP/WB experiments for secretion of GFP into the medium. (**c**–**d**) CB-FIB-EGFP-RKRRKR-GAL and CB-FIB-GAL-RKRRKRE-GFP are used both for *in vitro* cleavage and secretion assay, and used for *in vivo* studies to test the function of both gene products. Note that the R designates the position of the cleavage sequence 'RKRRKR' in these constructs. (**e**) CB-FIB-GAL-EGFP is a noncleavable control for *in vitro* and *in vivo* experiments. (**f**) CB-FIB-GAL is a negative control used in IP/WB experiments to show that the GFP antibody does not crossreact to galanin or other nonspecific proteins.

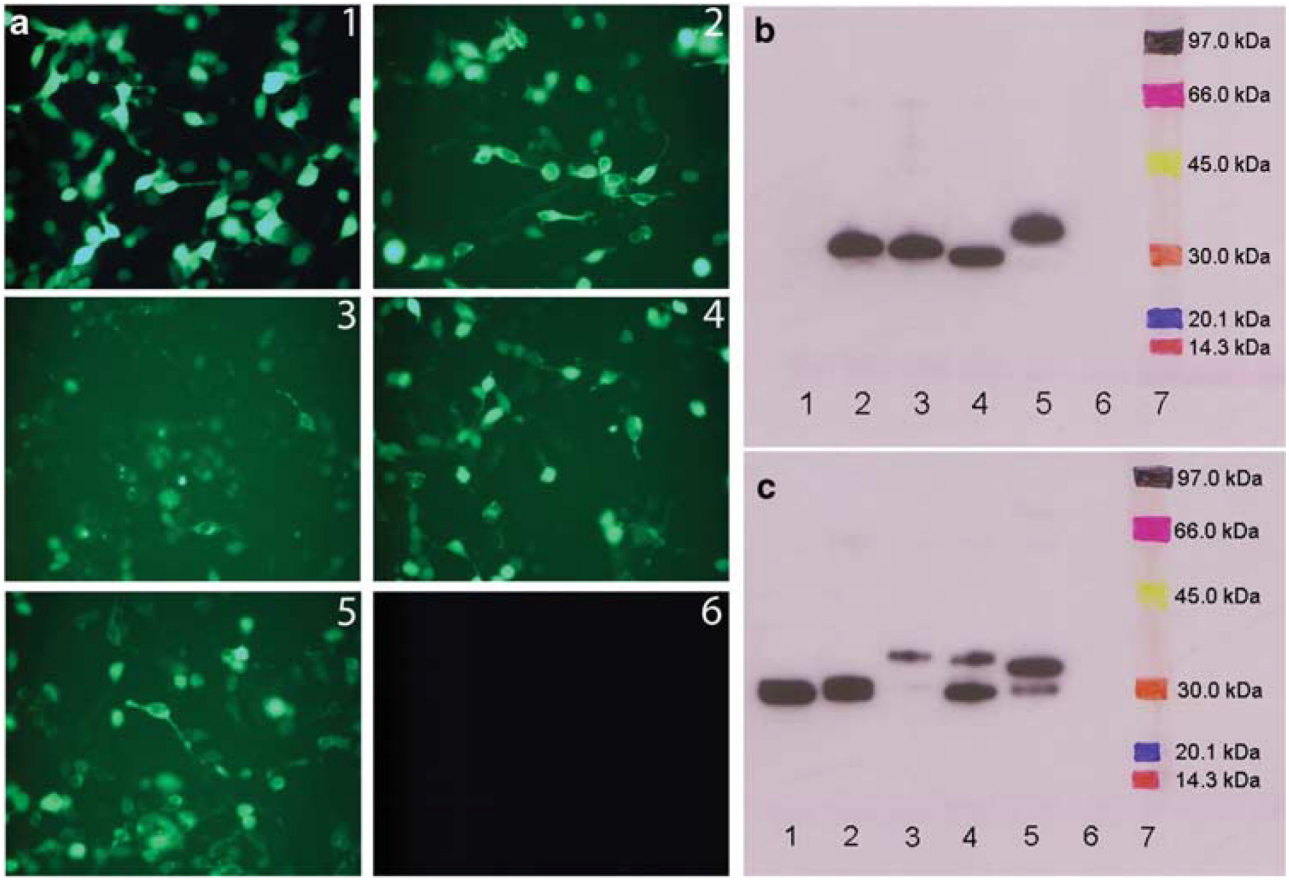

Figure 2.

In Vitro characterization of fluorescence patterns, cleavage, and secretion of proteins derived from multiple gene product delivery vectors. (**a**) GFP expression patterns in transfected 293 cells. Panel 1 is CB-EGFP, panel 2 is CB-FIB-EGFP, panel 3 is CB-FIB-EGFP-RKRRKR-GAL, panel 4 is CB-FIB-GAL-RKRRKR-EGFP, panel 5 is CB-FIB-GAL-EGFP and panel 6 is CB-FIB-GAL. After the samples were imaged, they were collected as conditioned media (**b**) and cell lysates (**c**). Samples were immunoprecipitated with a polyclonal GFP antibody, then loaded on gels for Western blotting. Lane 1 is CB-EGFP, lane 2 is CB-FIB-EGFP, lane 3 is CB-FIB-EGFP-RKRRKR-GAL, lane 4 is CB-FIB-GAL-RKRRKR-EGFP, lane 5 is CB-FIB-GAL-EGFP, lane 6 is CB-FIB-GAL and lane 7 is a Rainbow marker of known proteins from Amersham, size indicated to the right.

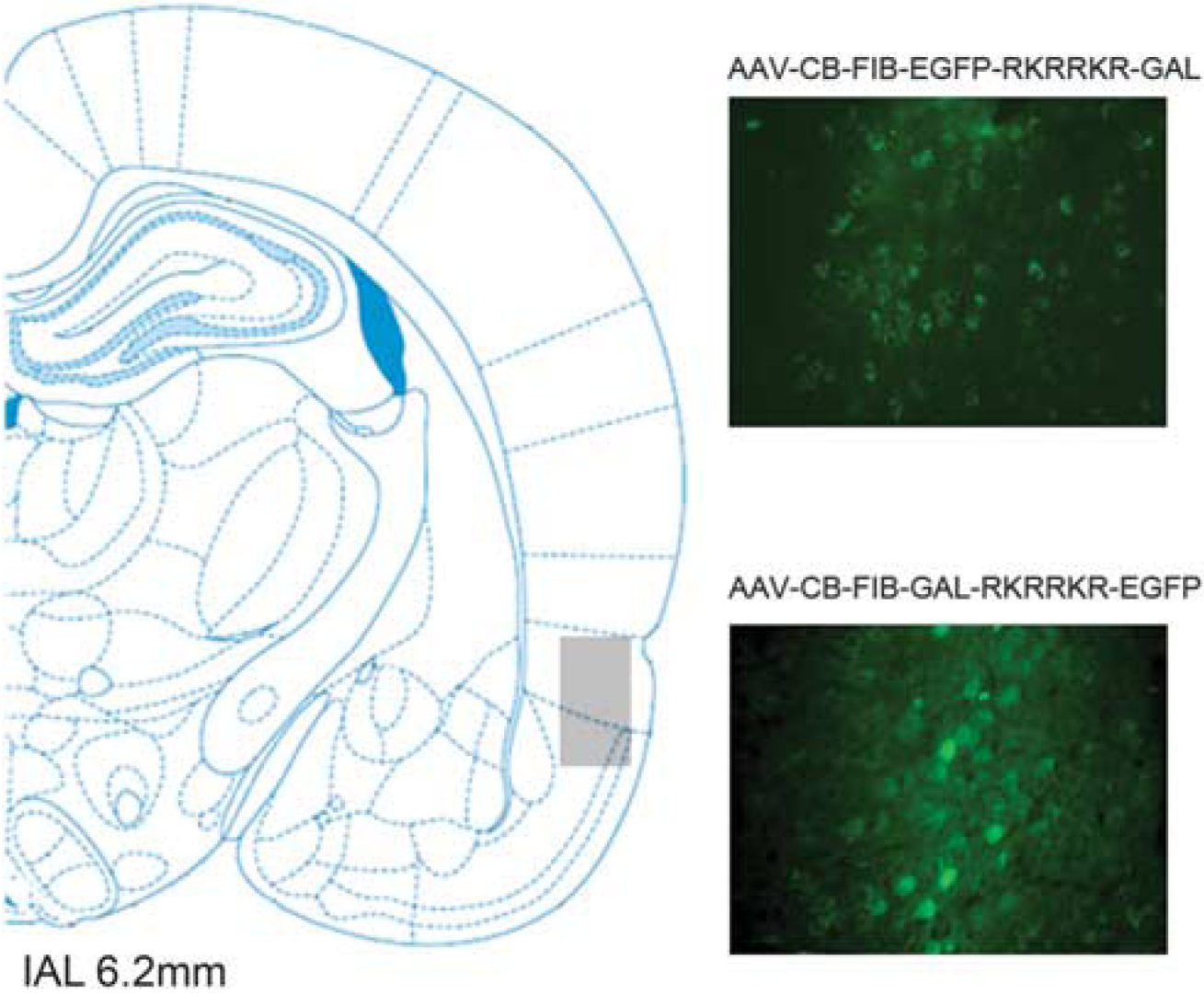

Figure 3.

Immunofluorescence of neurons in the piriform cortex that have been transduced with GFP-containing vectors. The shaded area in the coronal section shows the range of placement within the piriform cortex of the AAV vector microinjections. Sections were taken at the level of the needle track and processed for GFP fluorescence. GFP marks the virus transduction pattern, and confirms that *in vivo* our constructs are functional in both orientations.

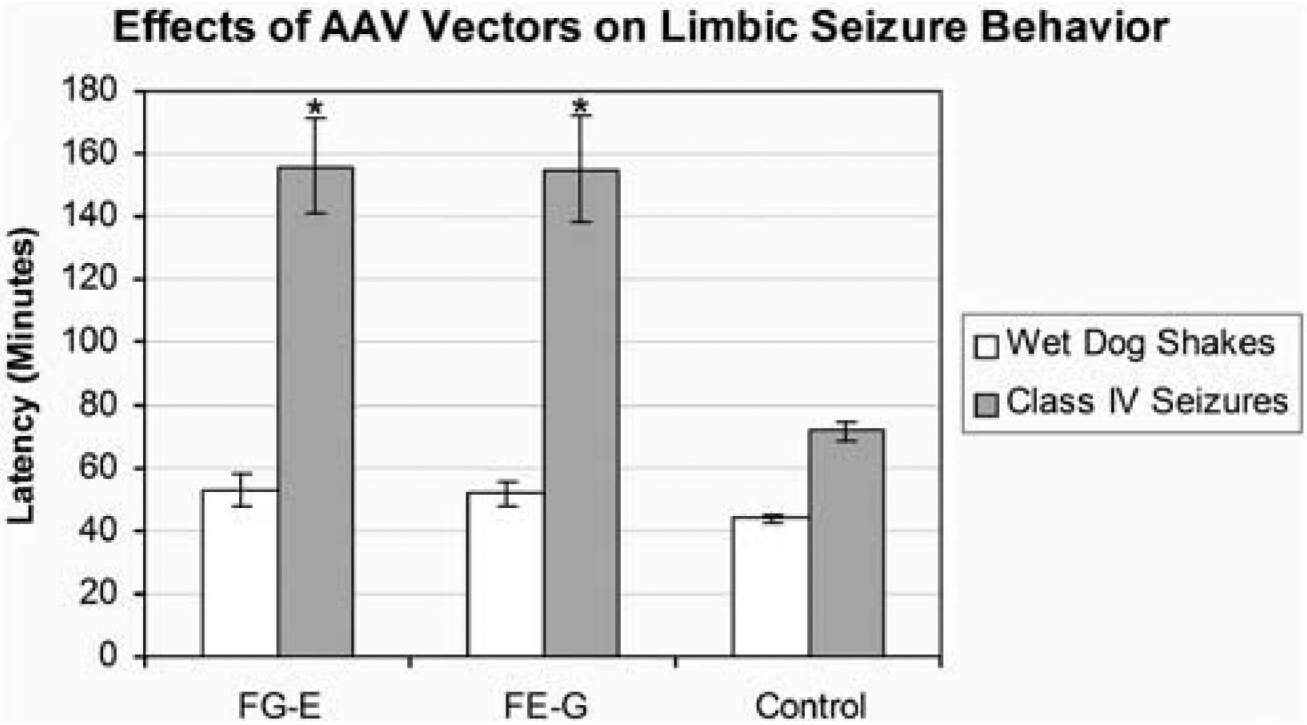

Figure 4.

The effects of multiple gene product delivery vectors on limbic seizure behavior. The latencies to wet dog shakes and limbic seizure behaviors were determined for 180 min post kainic acid. FGE is AAV2-CB-FIB-GAL-RKRRKR-EGFP ($N = 5$, three out of five rats had no class IV seizures), FE-G is AAV2-CB-FIB-EGFP-RKRRKR-GAL ($N = 6$, four out of six rats had no class IV seizures), and Control is kainic acid only with no vector ($N = 5$, all five rats had class IV seizures). * Signifies significance of $P \leq 0.01$ compared to Control using two-tailed *t*-test. Error bars indicate standard error of the mean.

Table 1

PCR Primers for multiple gene product delivery vectors

Construct name	Primers used	Melting temperature (°C)
TR-CB-FIB-GAL-RKRRKR-EGFP	F1 = GTACGGAAGTGTTACTTCTGCTC	55.0
	R1 = 5′PTCTCTTTCTTGTGAGGCCATGCTT	56.0
	F2 = 5′PAGAAAGAGAATGGTGAGCAAGGGCGAGGAGC	66.0
	R2 = CTTATCATGTCTGGATCCCCGCGGCC	64.0
TR-CB-FIB-EGFP-RKRRKR-GAL	F1 = GTACGGAAGTGTTACTTCTGCTC	55.0
	R1 = 5′PTCTCTTTCTCTTGTACAGCTCGTC	56.0
	F2 = 5′PAGAAAGAGAGGCTGGACCTGAACAGCG	64.0
	R2 = CTTATCATGTCTGGATCCCCGCGGCC	64.0
TR-CB-FIB-GAL-EGFP	F1 = GTACGGAAGTGTTACTTCTGCTC	55.0
	R1 = 5′PTGTGAGGCCATGCTTGTCGCT	56.0
	F2 = 5′PATGGTGAGCAAGGGCGAGGAGCTG	63.0
	R2 = CTTATCATGTCTGGATCCCCGCGGCC	64.0