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Recombinant adeno-associated virus vector: use for transgene expression and anterograde tract tracing in the CNS

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Abstract

We used a recombinant adeno-associated virus vector (AAV) to deliver a foreign gene, green fluorescent protein (GFP), into mature neurons in adult rat CNS in vivo. Microinjections of AAV as small as 50 nl transduced hundreds of neurons at the injection site. There was virtually no retrograde transport as fewer than one neuron per brain was found distant from the injection site that exhibited GFP immunoreactivity. The gene product, GFP, filled the entire neuronal cytoplasmic compartment; GFP immunoreactivity was robust in cell bodies, axons, and nerve terminals. There was no tissue damage at the injection sites or pathogenicity indicated by changes in astrocytic or microglial markers. There was no inflammatory response as judged by leukocytic invasion. Gene expression in transduced cells was robust and apparently permanent: there was no evidence of phenotypic reversion up to 12 weeks following infection. AAV is an excellent vector for introducing foreign genes into mature CNS neurons. Not only might it be an ideal vehicle for gene therapy, but also the GFP-containing AAV presents a new strategy for tracing long axonal pathways in the CNS, which is difficult with current tracers (PHAL, biotinylated dextrans).

Keywords

Parabrachial nucleus; Gene therapy; Green fluorescent protein; Rat

1. Introduction

The ability to induce the expression of identified genes by specific populations of neurons in the brain has long been a goal of molecular neurobiology. A variety of means has been used to accomplish this goal, including liposomes and recombinant retro- and adenoviral vectors [12]. Each of these methods has specific disadvantages, so that none has yet emerged which provides reliable and continuous gene expression in CNS neurons, in the absence of local inflammation or tissue damage.

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Recently, an adeno-associated virus (AAV) vector has been introduced which may circumvent many of these problems (see Ref. [14] for review). As the AAV vector DNA does not contain any viral coding sequences, there is no expression of viral proteins. As a result, the only exposure to viral proteins is the capsid, which is degraded soon after uptake. AAV then can potentially provide a means for introduction of foreign DNA to postmitotic cells, without inducing an immune reaction.

In tissue culture and in the CNS in vivo AAV has been capable of inducing long-lived, continuous expression of foreign genes by postmitotic neurons. If the injected AAV only transfects neurons at the injection site, and infection is without pathogenic consequences, the AAV vector might provide a long-sought method for CNS gene delivery. This vector could be used not only clinically for gene therapy, but also as a gene transfer tool for neuroscientists. However, the usefulness of AAV would be limited if the vector spreads throughout the brain in an unpredictable manner. In this study, we examined potential pathogenic responses to injection of a recombinant AAV containing the marker gene, green fluorescent protein (AAV-GFP). To assess transduction stability, we quantified the number of GFP immunoreactive neurons over time. Furthermore, we examined the spread of GFP through CNS pathways. Our observations suggest that AAV-GFP may prove to be an excellent anterograde tracer for long axonal pathways.

2. Materials and methods

Construction of rAAV vector plasmids

Recombinant AAV vector plasmids were constructed containing a synthetic form of the jellyfish green fluorescent protein gene, the so-called 'humanized' form, which is termed UF5 [15]. The recombinant pAcp-UF5 was derived from pSSV9 by excising all of the AAV coding sequences flanked by two Xba1 sites, leaving only the viral inverted terminal repeats (ITRs), and inserting an UF5 expression cassette in its place (Fig. 1). The UF5 expression cassette was constructed with a cytomegalovirus immediate-early (CMV-IE) promoter, the UF5 protein sequence, and an SV40 early region polyadenylation signal (SV40 pA).

Preparation of packaged Acp-UF5 vector

The AAV viral stock was raised as previously described [3], with the following exception. Briefly, semi-confluent 293 cells were infected with Adenovirus type5 at a multiplicity of infection of 10. One and a half hours later, the cells were co-transfected with 12 μ g of pAcp-UF5 plasmid and 4 μ g of pAd8 using a standard calcium phosphate precipitation method.

Functional titer assay

Serial dilutions of AAV-GFP vector were added to a 24 well plate which had been seeded with 0.5×10^5 293 cells per well. After one and a half hours transduction at 37°C, the cells were fed with 1 ml DMEM containing 10% fetal calf serum and incubated for 2 days. Then the green cells were counted under the fluorescence microscope to determine the vector titer per ml, which in this case was 6 million functional transducing units per ml.

CNS injection

AAV-GFP was injected into the brains of 12 adult, male Sprague–Dawley rats. Briefly, rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and injections were placed stereotactically under aseptic conditions through a burr hole into the parabrachial nucleus. A glass micropipette air pressure injection system was used, as previously described. Injections consisting of either 50 nl ($n=6$) or 500 nl ($n=6$) of DMEM containing roughly six functional AAV units/nl (see above for titering method), were placed via a micropipette with a 20 μ m tip diameter. To avoid tissue damage injections were made slowly over a 10 min (50 nl) or 30 min (500 nl) interval. Wounds were closed with wound clips and animals were allowed to recover and survive for either 2, 4, 6, 8, 10, or 12 weeks. One rat with each size injection was killed at each survival point, by deep chloral hydrate anesthesia followed by perfusion through the heart with 10% phosphate buffered formalin.

Histology

Brains and spinal cords were equilibrated with 20% sucrose overnight, then cut on a freezing microtome into four series of 40 μ m coronal sections. Sections were stored in phosphate buffered saline (PBS) containing 0.2% sodium azide until use. In some cases, sections were viewed directly for GFP fluorescence. One series of sections from each brain and all sections from each spinal cord was stained with an antiserum against GFP (Molecular Probes) using an immunoperoxidase method with biotinylated goat anti-rabbit antiserum and an ABC kit (Vector Labs), followed by staining with diaminobenzidine hydrochloride (DAB) 0.5% and H₂O₂ 0.2%, as previously described, e.g., Ref. [2]. Additional series of sections through the injection site were stained with antisera against glial fibrillary acidic protein (GFAP, rabbit polyclonal; Sigma) or monoclonal antibodies against leukocyte common antigen (CD45, mouse OX1 clone; Pharmingen) or microglial cells (CD11b/c, mouse IgG OX42; Pharmingen). Tissue was mounted on glass microscope slides and the sections stained for GFP were subjected to a silver–gold DAB enhancement procedure [9].

Digital photomicrographs were taken with a Kodak 460 DCS camera mounted on a Zeiss microscope. Digital images were prepared using ‘Adobe Photoshop’ on an Apple computer. Images were sharpened, brightness and contrast were adjusted, and then images were printed with a Kodak 8600 dye sublimation printer.

3. Results

3.1. Stable, efficient neuronal transduction

At each injection site, clusters of neurons were found that expressed GFP (Fig. 2A, Fig. 3). In the first four cases, sections were initially viewed under fluorescence optics, and green fluorescent neuronal profiles were seen at each injection site. Subsequently, immunocytochemical staining for GFP was found to identify larger numbers of neurons, and did not fade, and so this method was used to quantify gene expression in all cases.

Because the GFP filled out the cell bodies and proximal dendrites, we could visually identify the cell type that was transduced (Fig. 3). In all cases, cells were medium to large sized (12–30 μ m diameter in the long axis) and had clearly identifiable long dendrites. In some cases,

initial axon segments could also be identified. In no case were cells labeled that resembled astrocytes, oligodendrocytes, or microglia. Hence, the GFP expression appeared to be restricted to neurons.

Quantification of the numbers of GFP-immunoreactive neurons at each time point demonstrated that expression was stable across time. The number of GFP-immunoreactive neurons in rats injected with 50 nl AAV at 12 weeks was essentially identical to that at 2 weeks. Statistical comparison of the numbers of neurons in the 2, 4, and 6 week animals (336 ± 130 ; $n=3$) compared to the 8, 10, and 12 week animals (340 ± 98 ; $n=3$), showed no significant difference ($p > 0.5$; Student's *t*-test). The average cell size (as measured by the widest region perpendicular to the long axis of the cell) in each group was not significantly different: $7.5 \pm 1.3 \mu\text{m}$ for the 2, 4, 6 week group vs. $7.6 \pm 1.6 \mu\text{m}$ for the 8–12 week group. Therefore the number of cells in each group could be compared directly; no correction factor was used.

Individual neurons were filled in a Golgi-like manner (Fig. 3), so that local dendrites, axons, and even spines were clearly identifiable. Smooth black reaction product filled the cytoplasm, and could be traced into the axon. The injection site, the parabrachial nucleus, was chosen because it has well known connections, which allowed us to assess anterograde and retrograde transport. In the entire series, only two retrogradely labeled neurons were encountered. In one animal a single cell was seen in the central nucleus of the amygdala and in another, one cell in the spinal cord; both regions are known to provide inputs to the PB. Some cell bodies as much as $500 \mu\text{m}$ from the pipette tip were labeled, but only if they had a dendrite extending into the injection site.

3.2. Absence of tissue damage or pathogenicity

Selected tissue sections were stained from each case using an immunoperoxidase method for GFAP, CD11b/c and CD45. In no case, did we find greater GFAP-IR at the injection sites compared to the contralateral brain site (Fig. 2B). In only one case (AAV8), in which 500 nl was injected, there was a small collection of CD11b/c-IR microglia at the injection site (not shown). In the other 11 cases the injection sites had no change in CD11b/c staining (Fig. 2C). In several cases GFAP and CD11b/c-IR elements were visible along the pipette track through the inferior colliculus where the shank of the pipette passed through the brain. We found no evidence for leukocytic infiltration of the injection sites in the CD45 stained sections (Fig. 2D).

3.3. Anterograde transport of GFP

In every case GFP-IR anterogradely labeled axons were followed from the site of AAV-GFP injection to their terminal sites in the forebrain, brainstem and spinal cord (Figs. 4 and 5). The filling of axons and terminals was comparable to other intracellular transport markers, such as *Phaseolus vulgaris*-leucoagglutinin (PHA-L) or biotinylated dextrans injected at these sites (e.g., Refs. [5,13]. In each case the expected pattern of terminal fields was found based on the known projections of the PB [6]; there were no known projections that were not labeled, suggesting that the AAV-GFP transduction did not exclude any particular subset of neurons. Furthermore, the characteristic appearance of PB terminal fields was comparable to

that seen following injection of PHA-L in previous studies [1]. For example, fibers from the external lateral subnucleus formed characteristic ramifications that surrounded individual neurons in the central nucleus of the amygdala (Fig. 5). A striking characteristic of AAV-GFP tracing was that very long projection pathways were labeled. Individual labeled axons could be followed for several millimeters in horizontal sections of the spinal cord. In some cases labeled axons and terminals could be seen as far from the injection site as the sacral spinal cord.

4. Discussion

Our results indicate that AAV can be used as a vector to safely transduce cells in the CNS without causing gliosis or cellular inflammatory reactions. The expression seems to be limited to neurons at the site of injection, and is both stable and reliable. The expressed GFP filled the cytoplasm and all of the neuronal processes, down to the most distal axon terminals. Thus, this method is potentially suitable as a very powerful anterograde tracer of long pathways in the CNS.

We have confirmed and extended the findings of other reports of transduction of CNS neurons in vivo by AAV [8,10,11]. These groups also found that foreign gene expression persisted for months following transfection, although no quantitative assessment of the duration of expression was made. One group found that, in addition to neurons, GFAP-IR cells were transduced with an AAV vector containing a CMV promoter [8]. In contrast, Peel et al. [11] found that only cells of neuronal morphology expressed GFP when AAVs with neuron-specific enolase (NSE) or platelet-derived growth factor (PDGF) promoters were used. Neither group reported tissue damage beyond that of the injection process itself (however, injection volumes exceeded those used in our study by 40–100 fold). Although difficulties determining the AAV vector titer make relative efficiency difficult to compare (see below), transduction efficiencies between 2–10% have been reported [8,11]. Markers for inflammatory processes were not examined in previous studies.

4.1. Technical considerations

Intracerebral brain injections of nanoliter volumes made with glass micropipettes causes little or no tissue damage at the injection site, although pipette tracks are occasionally observable by small amounts of gliosis or the presence of a few erythrocytes. On the other hand, microliter (as opposed to nanoliter) injections of fluid into the brain causes significant damage at the injection site, even when the solution is delivered very slowly. This is not unexpected as the volume of 1 μ l of fluid is 1 mm³, about the size of the entire parabrachial nucleus. Because previous studies employing AAV vectors for CNS gene delivery have used volumes of 1–5 μ l [8,10,11], damage due to the injection itself could have obscured small injurious effects of the AAV vector. In contrast, we have shown that injections of 50–500 nl AAV produced no damage, gliosis, or leukocytic infiltration.

Successful transduction of small populations of neurons without significant damage requires a vector preparation of high enough titer and transduction efficiency such that nanoliter volumes may be sufficient for transduction in the intact CNS. In order to calculate transduction efficiency, the number of transducing particles injected must be known. Several

means have been used to estimate the titer of infectious particles in a given AAV preparation (see Refs. [3,8,11]). However, to date there is no standardized method that permits comparison between different preparations from different laboratories. Investigators typically test batches of AAV vectors with tissue culture assays that measure the number of transduced cells based on their expression of the foreign gene product. However, because transduction efficiency may depend upon cell type, in vitro assays are not necessarily predictive of efficacy in vivo. Indeed, earlier studies have reported that the transduction efficacy may vary depending upon the brain region into which the AAV vector is introduced [8,10]. In our study, the in vitro titer assay predicted 6 million functional units per ml. Therefore, our 50 nl injection should have contained 300 infectious particles. Our counts revealed more than 300 labeled cells in a single series of every fourth section. It is not a simple matter to estimate the total number of transduced cells per brain, because we did not use stereological methods, but if more than 900 cells were labeled per brain then the transduction efficiency must be at least three times that measured in vitro. This is a striking improvement in efficiency compared to earlier studies and critical for achieving effective transduction with injections small enough to avoid tissue damage.

Another important issue is identifying the best promoter for expressing foreign genes in neurons. The CMV promoter is believed to be universally active in neurons, but not in glial cells. Hence, we chose this as the promoter in our AAV. Our results are consistent with this specificity, at least in the parabrachial region. However, AAV vectors with CMV promoters have been reported to transduce glial cells, although with a neuronal preference, in the striatum [8]. AAVs with NSE or PDGF promoters have been reported to infect only neurons in the spinal cord [11]. Therefore, the usefulness of a given promoter will need to be determined for the brain region under investigation. The molecular fate of AAV DNA (chromosomal integration vs. episomal) in vivo is unknown, although both localizations have been reported in different tissue culture preparations (see Xiao et al. [14] for review). Therefore, cell type may also influence the success of long-term transduction in this regard.

4.2. Use of AAV vectors for gene transfer

The availability of an agent for delivering foreign genes into neurons would allow a variety of manipulations of the CNS, for neurobiological experimentation as well as for gene therapy to treat many conditions. Earlier studies by Kaplitt et al. [8] demonstrated that AAV vectors can be used to transfer genes such as tyrosine hydroxylase into CNS neurons and to ameliorate symptoms in conditions such as experimental parkinsonism. However, the clinical use of the AAV vector for treatment of neurological disease would depend upon its achieving long-term stable expression of the foreign gene in the absence of any local inflammation. Our results confirm earlier studies claiming these properties, and in addition, provide a quantitative demonstration that AAV vector-derived foreign gene expression does not change over at least a 3-month period. In addition, this has been the first study using sufficiently non-traumatic injection methods to verify that there is essentially no activation of microglial, astroglial, or leukocytic inflammatory mediator cells at any time point beyond 1 week after the AAV injection. Usefulness of the AAV for gene therapy or neurobiological studies would also depend upon the ability to control the population of neurons that are transduced. In contrast to other viral vectors that may be retrogradely transported by neurons

in remote regions of the brain, the AAV appears to transduce only local neurons. This characteristic will allow crucial control over the population of neurons that is affected.

4.3. Use of the AAV-GFP vector as an agent for anterograde tracing of axons

The current generation of anterograde tracers, such as PHA-L and biotinylated dextran amines (BD), provides an excellent method for tracing most axonal projections in the brain. However, these tracers have two major deficiencies that AAV-GFP does not appear to have. First, both PHA-L and BD injection sites show not only labeled neurons that have taken up and transported the tracer, but also neurons and in the case of BD, tissue macrophages [4], that contain tracer and may not have transported it. Furthermore, the injection sites have a 'smudgy' appearance due to the tracer present in the extracellular space. For these reasons it is not possible to define unequivocally the exact region of uptake with respect to the staining of the diffusion zone of the tracer (see Ref. [5] for discussion). In contrast, AAV-GFP injection sites stained with antibodies against GFP only showed Golgi-like stained cells that had been transduced and synthesized GFP; GFP does not appear to be released into the extracellular space. Therefore, one can see the exact population of neurons that express GFP and can transport it anterogradely.

A second benefit of using AAV-GFP for anterograde tracing is the complete absence of labeling due to tracer uptake by fibers of passage. Uptake and anterograde transport would not cause labeling because gene expression in nerve terminals is very unlikely. Uptake and retrograde transport of the AAV vector in our studies simply did not occur; in only two cases did we find a single GFP-immunoreactive neuron outside of the injection site. In both of these cases the neuron was located in a region known to project to the PB. Therefore, uptake by nerve terminals and not fibers of passage was likely in these cases.

A third benefit of using AAV-GFP as an anterograde tracer is that one can stain the brain months after the injection. This is not possible with PHA-L or BD, both of which are degraded and begin to fade from the injection site by about 4–5 weeks and from labeled axons thereafter.

On the other hand, AAV-GFP transduced cells continue to express and show GFP immunoreactivity for months and possibly longer. By waiting several weeks following surgery, it should therefore be possible to trace axonal pathways in the mammalian brain that are too long to follow with PHA-L or BD. For example, it has not yet been possible to trace certain long descending corticospinal pathways with PHA-L (see Ref. [7]). Finally, in addition to tracing very long axonal projections, long term expression of AAV-GFP may be useful in experiments in which the surgery must be done long before the brain is examined. For example, if it proves to be entirely non-toxic, AAV-GFP may be injected into the brains of non-human primates such as chimpanzees, or even human volunteers, at the time of neurosurgery for other reasons, allowing tracing of pathways months or years later, when the subjects die of natural causes. In this capacity, AAV-GFP may hold the potential for unlocking some of the more recalcitrant mysteries of the human brain.

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References

1. Bernard JF, Alden M, Besson JM. The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: a phaseolus vulgaris leucoagglutinin (PHA-L) study in the rat. *J Comp Neurol.* 1993; 329:201–229. [PubMed: 8454730]
2. Chamberlin NL, Saper CB. Differential distribution of AMPA-selective glutamate receptor subunits in the parabrachial nucleus of the rat. *Neuroscience.* 1995; 68:435–443. [PubMed: 7477954]
3. Du B, Wu P, Boldt-Houle DM, Terwilliger EF. Efficient transduction of human neurons with an adeno-associated virus vector. *Gene Ther.* 1996; 3:254–261. [PubMed: 8646557]
4. Earle KL, Mitrofanis J. Identification of transient microglial cell colonies in the forebrain white matter of developing rats. *J Comp Neurol.* 1997; 387:371–384. [PubMed: 9335421]
5. Feil K, Herbert H. Topographic organization of spinal and trigeminal somatosensory pathways to the rat parabrachial and Kolliker–Fuse nuclei. *J Comp Neurol.* 1995; 353:506–528. [PubMed: 7759613]
6. Fulwiler CE, Saper CB. Subnuclear organization of the efferent connections of the parabrachial nucleus in the rat. *Brain Res Rev.* 1984; 7:229–259.
7. Hurley KM, Herbert H, Moga MM, Saper CB. Efferent projections of the infralimbic cortex of the rat. *J Comp Neurol.* 1991; 308:249–276. [PubMed: 1716270]
8. Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat Genet.* 1994; 8:148–154. [PubMed: 7842013]
9. Kitt C, Levey A, Friedman L, Walker V, Koliatsos V, Raskin L, Price DL. Immunocytochemical visualization of cholinergic fibers in the monkey neocortex: enhanced visualization using silver nitrate. *Soc Neurosci Abstr.* 1988; 14:631.
10. McCown TJ, Xiao X, Li J, Breese GR, Samulski RJ. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res.* 1996; 713:99–107. [PubMed: 8724980]
11. Peel AL, Zolotukhin S, Schrimsher GW, Muzyczka N, Reier PJ. Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type-specific promoters. *Gene Ther.* 1997; 4:16–24. [PubMed: 9068791]
12. Verma IM, Somia N. Gene therapy—promises, problems and prospects [news]. *Nature.* 1997; 389:239–242. [PubMed: 9305836]
13. Wylie DR, Linkenhoker B, Lau KL. Projections of the nucleus of the basal optic root in pigeons (*Columba livia*) revealed with biotinylated dextran amine. *J Comp Neurol.* 1997; 384:517–536. [PubMed: 9259487]
14. Xiao X, Li J, McCown TJ, Samulski RJ. Gene transfer by adeno-associated virus vectors into the central nervous system. *Exp Neurol.* 1997; 144:113–124. [PubMed: 9126160]
15. Zolotukhin S, Potter M, Hauswirth WW, Guy J, Muzyczka N. A humanized green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J Virol.* 1996; 70:4646–4654. [PubMed: 8676491]

Fig. 1. Schematic of the Acp-UF5 vector. The following abbreviations are used: ITR—inverted terminal repeats, CMV—cytomegalovirus promoter, *Xba*I and *Sal*I—restriction sites, SV40 pA—the simian virus 40 polyadenylation sequence.

Fig. 2.

Digital photomicrographs of AAV injection site. Shown are ipsi- (A) and contralateral (A') fields of an injection site stained with anti-GFP antiserum in case AAV10. An adjacent series of sections was stained for GFAP. B and B' show the section adjacent to that shown in A and A'. Note that the GFAP staining in the external lateral subnucleus is always slightly greater than the other PB subnuclei. Staining was no different on the ipsi- (B) compared to contralateral (B') to the AAV injection. Panel C shows microglial staining in another section through the injection site of the same brain. D shows staining for leukocyte common antigen (CD45). The insets in B, C, and D are higher magnification images of stained cells. The scale bar shown in the inset in D denotes 10 μm . A higher magnification of the boxed area in panel A is shown in Fig. 3.

Fig. 3. High magnification photomicrograph of AAV-GFP transduced neurons. The area shown is a higher magnification of the boxed area at the ventrolateral tip of the superior cerebellar peduncle in Fig. 2A. Note the Golgi-like filling of GFP immunoreactive cells of neuronal morphology (compare with insets in Fig. 2).

Fig. 4. High magnification photomicrographs of terminal fields of GFP-immunoreactive neurons. Terminal fields were located in the rostral ventrolateral medulla (A), spinal trigeminal nucleus (B), lateral hypothalamus (C), and spinal cord (D). Scale bar in A applies to A–C. Scale bar in D denotes 50 microns.

Fig. 5. Photomicrograph depicting GFP-IR terminal fields in the central nucleus of the amygdala. Note that nerve terminals form dense clusters surrounding target neurons (arrowheads and inset). The scale bar in the inset denotes 20 μm .