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ARTICLE

Combined Injection of rAAV with Mannitol Enhances Gene Expression in the Rat Brain

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Recombinant adeno-associated viruses (rAAV) are highly efficient vectors for gene transfer into the central nervous system (CNS). However, a major hurdle for gene delivery to the mammalian brain is to achieve high-level transduction in target cells beyond the immediate injection site. Therefore, in addition to improvements in expression cassettes and viral titers, optimal injection parameters need to be defined. Here, we show that previous studies of somatic cell gene transfer to the mammalian brain have used suboptimal injection parameters, with even the lowest reported perfusion rates still excessively fast. Moreover, we evaluated the effect of local administration of mannitol to further enhance transgene expression and vector spread. Ultraslow microperfusion of rAAV, i.e., <33 nl/min, resulted in significantly higher gene expression and less injury of surrounding tissue than the previously reported rates of 100 nl/min or faster. Co-infusion of mannitol facilitated gene transfer to neurons, increasing both the total number and the distribution of transduced cells by 200–300%. Gene transfer studies in the CNS using rAAV should use very slow infusion rates and combined injection with mannitol to maximize transduction efficiency and spread.

Key Words: **rAAV; mannitol; injection parameters; CNS; gene therapy.**

INTRODUCTION

Efficient gene transfer into neurons is a major goal for molecular genetic studies of the nervous system and for the development of gene therapy for various human neurological disorders which currently have no effective treatment (reviewed in 1, 2). Various delivery systems and gene therapy strategies are under evaluation. Recombinant adeno-associated virus (rAAV) vectors offer many advantages for gene transfer into multiple tissues, including the central nervous system (CNS), such as their replication deficiency, ability to infect postmitotic neurons, low pathogenicity, lack of immunogenicity, high transduction efficiency, and long-term expression (3–10). However, further improvements in efficacy, stability, regulatability, and safety of rAAV as well as defined optimal injection parameters are necessary to fully realize the promise of this technology.

Direct *in vivo* gene transfer into the brain and spinal cord is usually limited to neurons within the immediate vicinity of the injection site, yet neuropathology associated with many disorders affects multiple brain regions or is widespread within a given brain structure. This suggests that improved vector diffusion and transduction may lead to increased therapeutic potential. It is now generally accepted that in contrast to manual "push" viral delivery, the use of a microprocessor-driven syringe for vector administration results in increased levels of gene expression (11). However, in contrast to the importance of continuous and nonpulsatile delivery of the vector, the volume of a viral suspension per se does not lead to an increase in transgene expression for a given vector particle dose (12). One approach to facilitating widespread distribution into the mammalian brain has been termed "convection-enhanced delivery," a method which uses large volumes of vector stocks delivered intraparenchymally at relatively fast rates over prolonged time intervals (13–15). An alternative to intraparenchymal injection is systemic intravascular delivery of viral vectors in combination with hyperosmotic mannitol for transient disruption of the blood–brain barrier. This strategy results in very limited brain parenchymal transduction in rodents (16–18). In one study, mannitol was added to an adenoviral vector stock with a nonquantitative description suggesting improved

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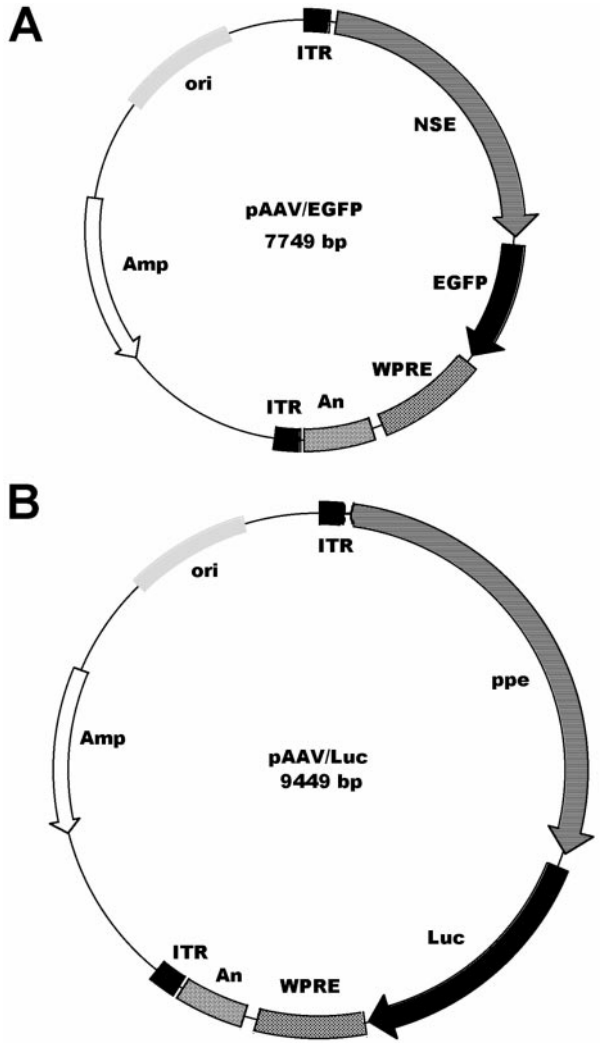

FIG. 1. Vector diagrams. (A) AAV/EGFP and (B) AAV/Luc plasmid constructs.

transgene expression in the rat brain (19). Mannitol has also been administered systemically in the setting of gene transfer experiments, with facilitation of the CNS distribution of the delivered gene protein product following intraventricular vector administration (20) and improved intraparenchymal spread of a nonviral vector (21). However, both ideal injection parameters and the local administration of mannitol remain poorly defined, with previous studies typically using flow rates ranging from 0.1 to 3 μl/min (12, 16, 19, 22, 23) and with no previous quantitative assessment on the use of mannitol delivered locally with the vector. Our goal was to extend these previous studies and specifically test the hypotheses that slower is better and that local mannitol significantly facilitates transduction efficiency.

MATERIAL AND METHODS

Generation of recombinant adeno-associated virus. The plasmids rAAV/PPE-Luc-WPRE (AAV/Luc) and rAAV/NSE-EGFP-WPRE (AAV/EGFP; Fig. 1) were created using standard molecular cloning procedures. Luciferase and EGFP (enhanced green fluorescent protein; Clontech) cDNAs were subcloned into an expression cassette consisting of a rat PPE (preproenkephalin) 2.7-kb promoter or a rat NSE (neuron-specific enolase) 1.8-kb promoter fragment, a woodchuck posttranscriptional regulatory element (WPRE (24)), and a SV40 poly(A) signal. Expression cassettes were subcloned into the pSUB201 rAAV backbone flanked by AAV inverted terminal repeats. The plasmids were subsequently packaged and purified according to (25) and (26) to yield high-titer recombinant AAV virus. Physical titer of virus stocks was measured using an AAV Titration ELISA Kit (Progen, Germany).

Animals and vector administration. Male Wistar rats (280–300 mg) were anesthetized with ketamine/xylazine (60 mg/6 mg per kg). An additional 1/3 of the initial dose of the anesthetic was administered during the surgical procedure. The experiments were carried out at the University of Auckland in accordance with Guidelines for Animal Care and protocols were approved by the University of Auckland Animal Ethics Committee.

AAV/Luc or AAV/EGFP (2 μl (7×10^9 particles/μl)) was injected stereotactically into the left and right hemispheres of the midstriatum with either 1 μl of 20% mannitol in PBS or 1 μl of 0.9% saline (3 μl total volume). The injection coordinates (AP +0.4 mm, ML ±3.0 mm, DV −6.6 mm) were measured from bregma, using Paxinos' and Watson's *Rat Brain Atlas.* An ultra-microsyringe pump controlled by a microprocessor (World Precision Instruments, Inc.) (11) was used to deliver the vector ± mannitol or PBS at a rate of 1 μl over defined time intervals ranging from 2 to 60 min with a 10-μl Hamilton syringe. Animals were left to recover for 3 weeks before testing.

Luciferase assay. After 3 weeks, animals were sacrificed under deep anesthesia, and their brains were removed, immediately frozen on dry ice, and stored at −80°C prior to sectioning. The brains were cut as a whole hemisphere or cryostat cut and were stored at −80°C. Luciferase activity was measured in luminescence per second (lum/s) using a luciferase assay kit (Promega) on a Wallac Jet luminescence counter. According to the standard measurements, 7 lum/s equaled 1 pg luciferase protein.

Immunohistochemistry. Rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After being immersed in fixative overnight, brains were cryoprotected in 30% sucrose in PBS. Cryostat-cut sections 35 μm in thickness were rinsed in PBS containing 0.2% Triton X-100 (PBS–Triton) before being incubated in 1% H_2O_2 in 50% methanol for 1 h. After being rinsed again in PBS–Triton, sections were incubated overnight at room temperature with monoclonal antibodies against the neuron-specific nuclear antigen NeuN (27) (dilution 1:1000; Chemicon), GFAP (dilution 1:1000; Sigma), T cell helper/macrophages CD-4 (ox-35, dilution 1:100; Pharmingen), isolectin B4 peroxidase conjugated (dilution 1:50; Sigma), CD-8 suppressor/cytotoxic cells (dilution 1:50; Pharmingen), or polyclonal antibodies against EGFP (dilution 1:2000; Clontech) or luciferase (dilution 1:10,000; CorTex). Sections were processed using commercial antibodies and immunoreactivity was analyzed as previously described (28). Immunofluorescent labeling was detected using a Leica 4d TCS confocal microscope and DAB labeling was detected using a light microscope attached to a Leica DC camera supported by IM1000 Image Manager software (Leica Microsystem). The images were processed using Adobe PhotoShop 5.0 (Adobe Systems, Mountain View, CA).

Statistical analysis. Data are given as means ± SEM and were analyzed using Student's *t* test and ANOVA using the Systat (Evanston, IL) statistical software.

RESULTS

Relationship of Vector Infusion Rate and Transduction Efficiency

This study was undertaken to analyze different time periods of rAAV administration on transduction efficiency in rat brain using the luciferase-expressing vector. One microliter of AAV/Luc (7×10^9 particles/μl) was administered via stereotactic injection into a single midstriatum site over the time period of 2, 15, 30, 45, or 60 min into

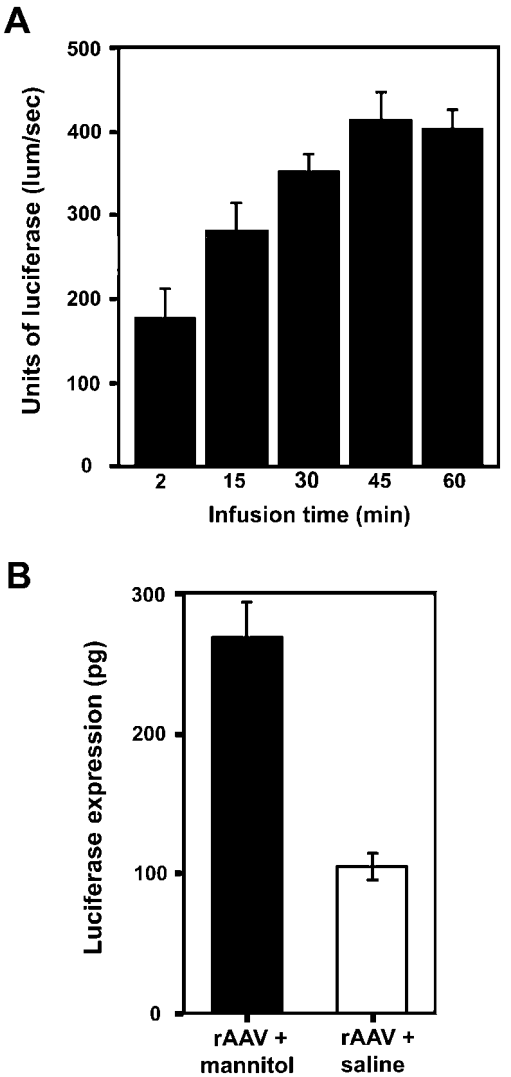

FIG. 2. Optimization of injection parameters. (A) Luciferase enzyme activity 3 weeks after administration of 1 μl of AAV/Luc delivered over 2, 15, 30, 45, or 60 min into the midstriatum. The level of luciferase expression was dependent on the vector infusion rate ($n = 30$, $F_{(4, 25)} = 7.97$, $P = 0.0003$). (B) Total luciferase expression in pg (means ± SEM) in the rAAV plus mannitol- and the rAAV plus saline-injected group after 3 weeks, showing that luciferase expression was 2.57 ± 0.74 times higher in the mannitol-treated group ($n = 10$, $P = 0.019$).

six rats per group. After 3 weeks, luciferase protein expression was detected in the whole hemisphere, measuring enzyme activity with a luciferase assay. There was a steady increase in luciferase enzyme activity over administration time of rAAV (Fig. 2A) starting from 177.9 ± 34.2 lum/s, measured in the group which received the virus over a period of 2 min, increasing over 282.1 ± 33.3 lum/s, after rAAV administration over 15 min, to 413.9 ± 36.5 lum/s in the group to which the virus was administered over 45 min. Following a delivery period of 60 min, an enzyme activity of 401.9 ± 21.1 lum/s was measured, showing that there was no improvement of the transduction efficiency using a period of injection longer than 45 min. There was no spread to contralateral hemispheres. Thus, these data indicate that slower speed of virus administration leads to higher transduction efficiency ($n = 30$, $F_{(4, 25)} = 7.97$, $P = 0.0003$). Moreover, according to light microscopy analysis, the slower infusion rates resulted in less injury of brain tissue than faster infusion rates (data not shown). Together, the luminescence and histological results show that there is an optimum time speed of vector administration of 1 μl (7×10^9 particles/μl) over 30–45 min to achieve optimum levels of luciferase expression, giving a flow rate of 22–33 nl/min.

Enhancement of rAAV Transduction and Spread in Vivo by Hypertonic Mannitol

Using the optimum infusion rate (33 nl/min) for the given volume of vector (3 μl), a separate group of 16 animals was analyzed to determine the effect of mannitol on gene expression and vector distribution. These rats were injected with AAV/Luc (7×10^9 particles/μl) with or without mannitol into the midstriatum. As a control, the contralateral side of each rat was injected with rAAV and saline instead of rAAV and mannitol. Three weeks after the injection, the expression of the luciferase gene was determined using a luciferase assay both in sequential sections and in the whole hemisphere. The total enzyme activity in the mannitol-treated group (M group) reached 1614.76 ± 339.18 lum/s (258.36 ± 54.26 pg) compared to 627.66 ± 127.67 lum/s (100.42 ± 20.42 pg) in the saline-treated group (S group) and thus was 2.57 ± 0.74 times higher in the M group than in the S group (Fig. 2B; $n = 10$, $P = 0.019$).

In order to analyze virus spread in more detail, AAV/EGFP (7×10^9 particles/μl) was injected into the striatum with or without mannitol (Fig. 3). Sequential sections taken every 35 μm were immunohistochemically stained using a EGFP-specific antibody and DAB detection showing that the rAAV administered together with mannitol was much more widely distributed than in the control hemisphere without mannitol. Higher magnification of brain tissue at the injection site showed that more transduced cells were present and distributed over 4 mm in the mannitol hemisphere (Fig. 4A) vs fewer transduced cells with distribution limited to 2 mm in the control sides (Fig. 4B). Double labeling using the neuronal marker NeuN showed that more than 95% of cells transduced with AAV/EGFP were neurons as expected from use of the NSE promoter and AAV tropism for neurons (Fig. 4C). These data clearly illustrated that virus spread was strongly enhanced in brain when it was injected together with mannitol.

This was confirmed by quantitative data analyzing sequential brain sections after delivery of AAV/Luc (Fig. 5A). These sections, taken every 400 μm and analyzed in the luciferase assay, revealed that the spread of the virus was 3 ± 0.6 times greater with mannitol administration. For example, luciferase expression >13 pg was detectable over a distance of −0.8 to 1.6 mm from the needle track in the M group. However, in the S group, it was detectable over

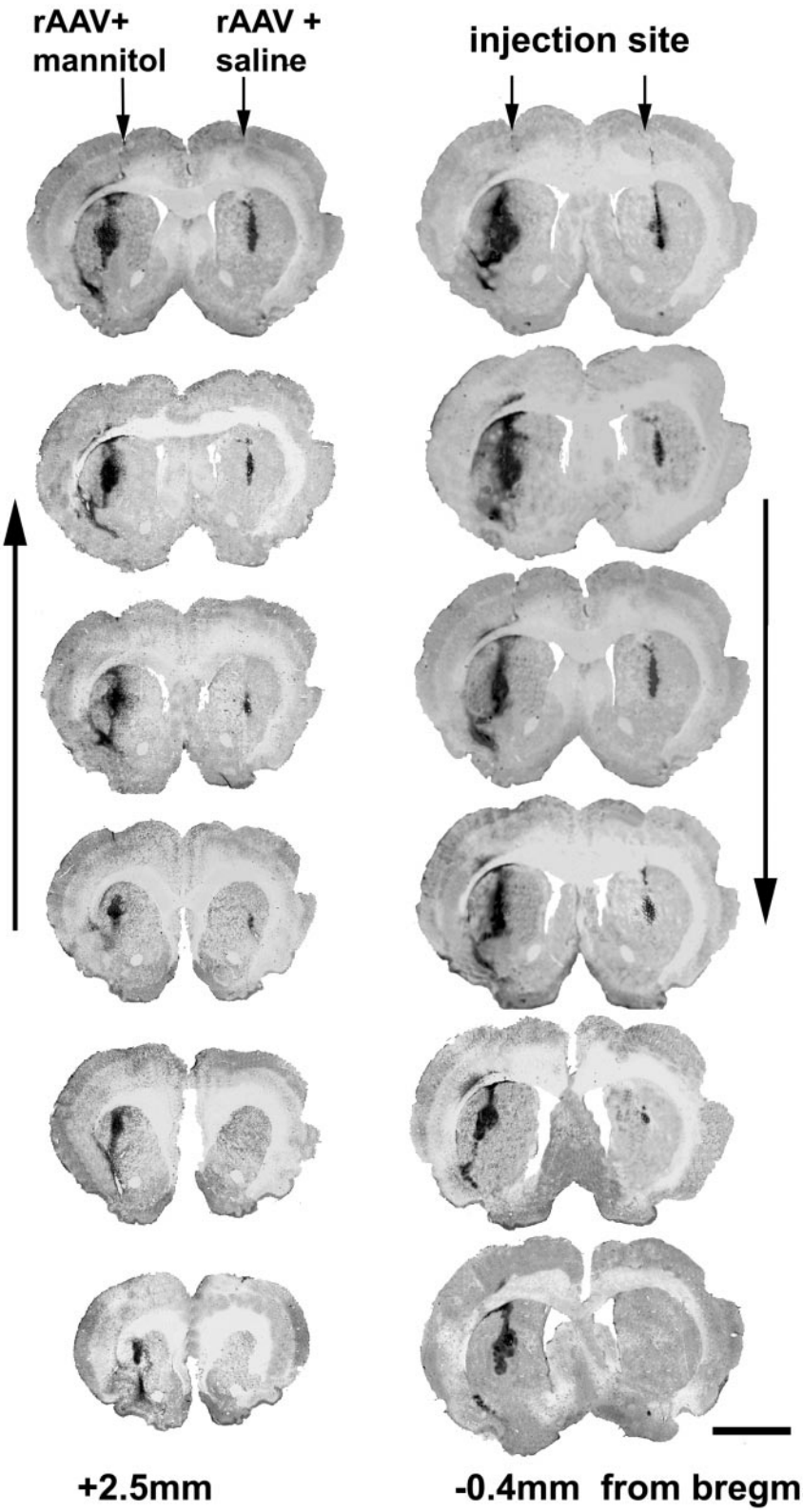

FIG. 3. Effect of rAAV delivered with or without mannitol on vector distribution. Representative sections between +2.5 and −0.4 mm from bregma indicating the injection site (arrows) of 3 μl of AAV/EGFP (7×10^9 particles/μl) with mannitol (left hemisphere) and with saline (right hemisphere) are shown using DAB detection of EGFP expression. Visual inspection showed that rAAV spread was about three times higher after administration with mannitol. Scale bar, 4 mm.

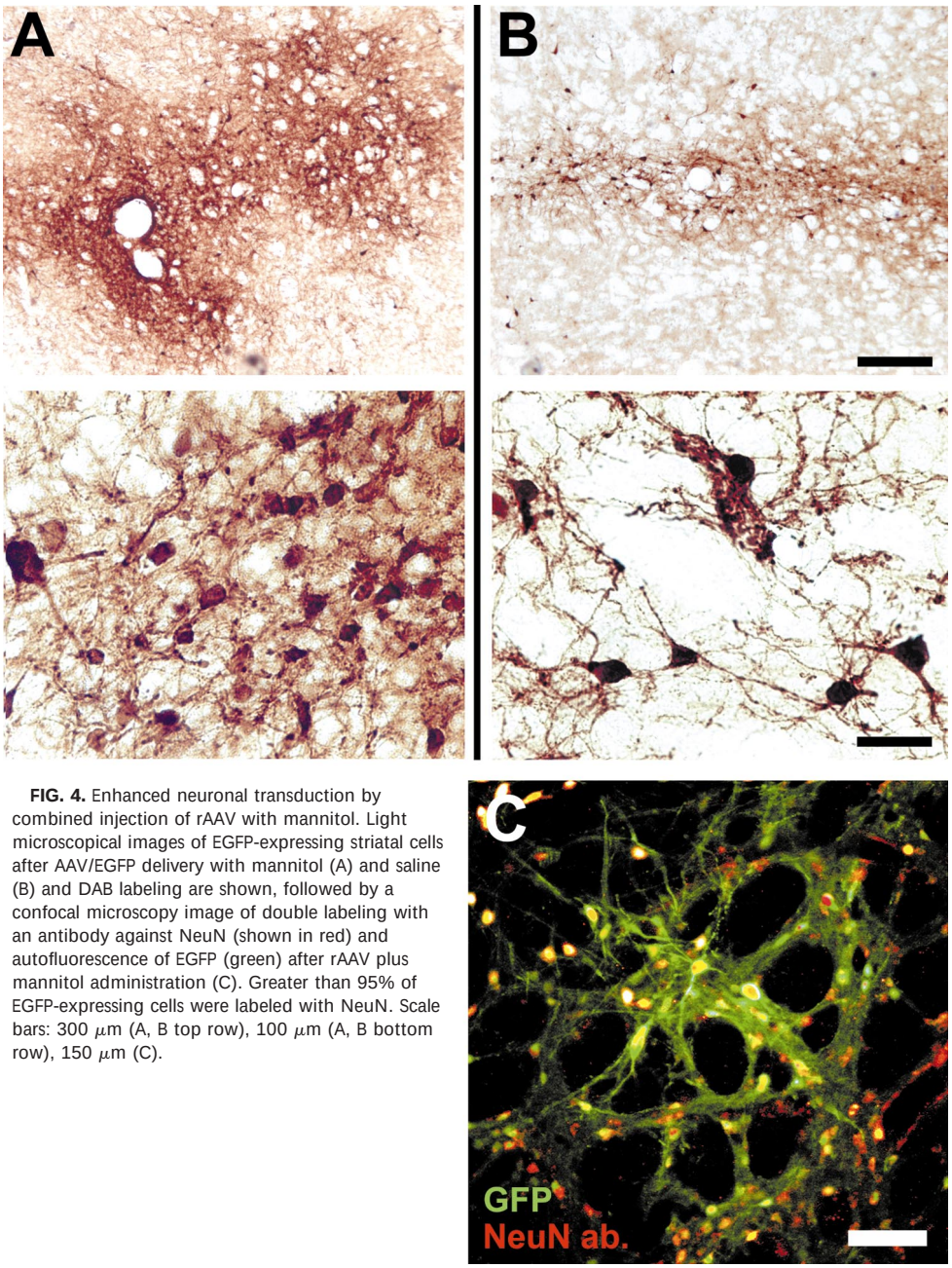

FIG. 4. Enhanced neuronal transduction by combined injection of rAAV with mannitol. Light microscopical images of EGFP-expressing striatal cells after AAV/EGFP delivery with mannitol (A) and saline (B) and DAB labeling are shown, followed by a confocal microscopy image of double labeling with an antibody against NeuN (shown in red) and autofluorescence of EGFP (green) after rAAV plus mannitol administration (C). Greater than 95% of EGFP-expressing cells were labeled with NeuN. Scale bars: 300 μm (A, B top row), 100 μm (A, B bottom row), 150 μm (C).

a distance of only −0.4 to 0.6 mm ($n = 10$, $P = 0.018$). The maximum luciferase enzyme expression was reached at the injection site in both groups (66.09 ± 15.26 pg in the M group compared to 28.83 ± 5.69 pg in the S group), but it was 2.6 ± 0.6 times higher in the M compared to the S group.

To determine whether this higher level of transgene expression after combined injection of rAAV with mannitol derived from a higher number of cells expressing the transgene, the EGFP-expressing cells were counted across the striatum (Fig. 5B). This analysis revealed that in the S group, the cells expressing EGFP were detectable between −0.69 and +0.69 mm from the needle track with a maximum number of 98.5 ± 21.5 cells/30 μm (dark area). In the M group, however, these cells were countable up to a distance of 4 mm with a maximum number of 415.5 ± 135.1 cells/35 μm (white area). The total number of EGFP-expressing cells was three to four times higher in the M compared to the S group ($n = 6$, Student's t test, $P < 0.05$). These data show that virus spread and level of expression were more than two times higher with mannitol administration, thus enabling efficient rAAV-mediated gene transfer into neurons *in vivo*. Additional immunohistochemistry was performed in order to exclude an inflammatory re-

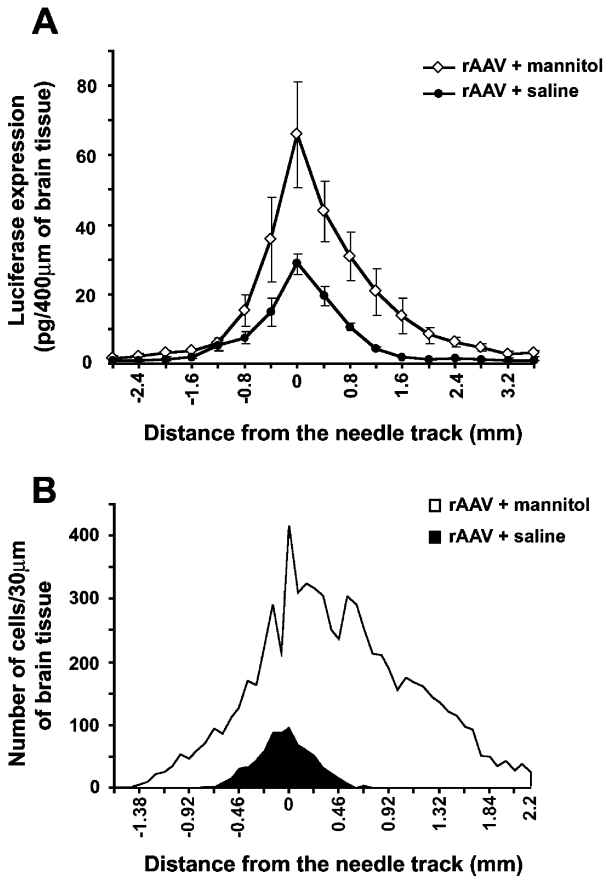

FIG. 5. Virus spread with or without mannitol administration. 3 μl of AAV/Luc (7×10^9 particles/μl) were delivered into rat striatum over 90 min, one hemisphere in combination with mannitol, the other with saline. (A) After 3 weeks, luciferase activity was measured in sequential sections every 400 μm. The maximum enzyme activity was 2.6 $\pm$ 0.6 times higher and the spread of the virus was 3.0 $\pm$ 0.6-fold wider with mannitol administration ($n = 10$, $P = 0.018$). (B) After 3 weeks, the number of EGFP-expressing cells was counted in sequential sections of 35 μm thickness in every other section. The total number of transduced cells as well as the virus spread was 3–4 times higher on the mannitol side ($n = 6$, $P < 0.05$).

sponse using antibodies recognizing the following proteins: isolectin B4, T cell helper/macrophages CD-4 (ox-35), and suppressor/cytotoxic cells CD-8 (Fig. 6). There was no detectable difference between the M and the S group regarding the expression of neuronal versus glial cells in the needle track or an inflammatory response after a single injection, indicating no change in cell tropism nor any pathogenicity following rAAV with or without mannitol administration. In summary, we could show *in vivo* that the local injection of mannitol facilitates gene transfer to neurons increasing both the total numbers of transduced cells and the vector distribution by 200–300%.

DISCUSSION

The majority of applications using viral vectors for CNS gene transfer have efficient and sustained expression of the transgene as a primary objective. Improving long-term expression remains a significant hurdle, with studies to date showing that rAAV can transfer genes to cells within the CNS of mice and rats with expression varying from days to 1 year (4, 29–31). Furthermore, advances in the construction of vectors and methods of administration provide alternative strategies for gene transfer, yet significant limitations remain. In general, further improvements in efficacy and stability of gene transfer to the brain are necessary and this study now provides optimized delivery parameters for rAAV administration to support applications for CNS gene transfer.

We confirm previous nonquantitative data that used hypertonic mannitol in an attempt to increase the viral accessibility to neurons (19) or to expand the distribution of the transgene product (20) and extend these observations by optimizing the injection parameters and providing quantitative, statistically significant data. Two major findings have emerged from this study: First, our study reports that a rAAV vector expressing luciferase transduces a higher number of neurons accompanied with less injury of the tissue surrounding the injection site when it is administered at rates of 33 nl/min or slower. Second, this ideal flow rate was used to evaluate the effect of mannitol on gene expression and vector distribution and provided strong evidence that mannitol facilitates transduction in the immediate vicinity of the injection site, as well as enhancing the distribution of transduced cells.

Previous reports have demonstrated that rAAV-based vectors can mediate high levels of transgene expression in the CNS (32–34). However, as with other vectors, diffusion of rAAV is limited and was reported to increase only slightly using larger injection volumes at a risk of local injury (35). Additional studies of rAAV vectors administered into the brain showed that transduced cells were present no farther than 1–2 mm from the injection site (4, 5, 30).

However, there are no previous quantitative studies looking at the optimal delivery parameters, nor have data been presented on the use of local administration of a hyperosmotic agent compared to vector alone. The first suggestion that a slower rate of viral vector administration improves transgene expression was provided in a study in which the rate of intracerebral injection into rat brain ranged from 1 to 0.5 μl/min with transduction assessed by immunohistochemical analysis of the transgene (22). Other studies have analyzed transgene expression in rat or monkey brain after delivery of virus at a rate of 1.2 (16), 0.1–0.4 (23), and 0.5 μl/min (19); however, no quantitative data on the transgene expression levels resulting from varied injection parameters have been presented in an independent biochemical assay. Moreover, in none of these studies was a plateau or absolute optimal infusion rate defined, with rates assessed not dropping below 100 nl/min. Furthermore, one study on CNS adenoviral gene transfer reported that varying the infusion rate from 0.3 to 3 μl/min did not improve the volume of brain transduced (12). A few studies have used systemically administered mannitol together with virus administration indicating that blood–brain barrier disruption by mannitol may be an effective way to administer virus particles to

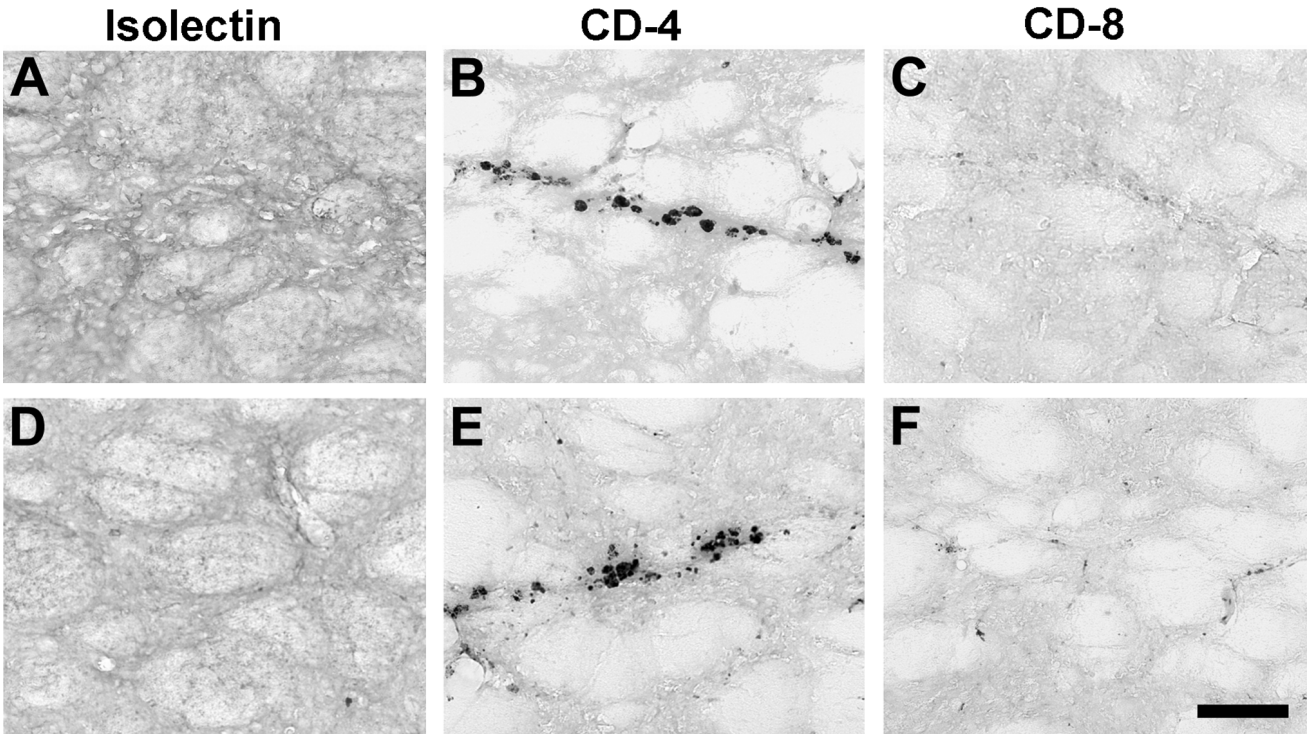

FIG. 6. Lack of significant tissue damage and inflammatory responses after rAAV delivery with or without mannitol. 4 weeks after rAAV injection rat sections from each group were immunohistochemically analyzed using antibodies against isolectin B4, T cell helper/macrophages CD-4 (ox-35), and suppressor/cytotoxic cells CD-8. Light microscopical images of DAB-labeled sections showed no detectable inflammatory response after a single injection and no difference between the M (A, B, C) and the S group (D, E, F). Scale bar, 150 μm.

the CNS (16, 17, 19, 20); however, gene expression in the CNS of these animals is poor compared to the transduction efficiency of new-generation, high-titer, highly purified AAV vector stocks.

The significance of the current study includes an analysis of flow rates as low as 16 nl/min and a comprehensive, blinded analysis of the effect of mannitol administered together with the rAAV. We now report that the region of transduction by local rAAV injection is extended more than twofold by local mannitol. This adjuvant is often applied by intracarotid administration to transiently open the blood–brain barrier (16). The mechanism for this increase is not established, but it is possible that mannitol injection induces shrinking of the capillary endothelial cells that form the blood–brain barrier and disruption or opening of the tight intercellular connections thus enabling viral access to neurons. The exact mechanism by which mannitol facilitates both transduction efficiency and viral spread in our study is unknown, but may include a local osmotic effect with shrinkage of cells thereby increasing interstitial space, facilitating diffusion as well as a direct effect on virus binding and/or uptake or influencing transgene expression indirectly. The receptor responsible for transduction of neurons is not known; however, heparan sulfate proteoglycans (HSPG) have been identified as primary viral receptors (36) and are detected abundantly on neurons (37–39). A more mechanistic interpretation is the potential for mannitol to block the rAAV binding to HSPG thus facilitating spread of the virus. In this study, rAAV serotype 2 was used; however, it would be interesting to determine whether spread and transduction efficiency of rAAV serotypes 4 and 5 is similarly facilitated, as these serotypes do not appear to use HSPG as a primary receptor (40).

The immune response in the CNS to rAAV has not been studied extensively, although it is becoming clear that immune-tolerated vectors are ideal for most gene therapy applications. Brockstedt and colleagues (41) have reported that the cellular immune response to rAAV appears to depend on the route of administration. However, additional studies show in general a limited cellular immune response in brain (35) as well as in muscle (42–45). Our immunohistochemical data support these findings demonstrating that due to the mechanical injury of brain tissue caused by rAAV injection, an immediate local immune response was detectable, but this led neither to a loss of transgene expression nor to a widespread immune reaction. There was no widespread tissue damage at the injection sites or pathogenicity indicated by changes in astrocytic or microglial markers supporting previous studies (46). A comprehensive study of repeated injections of rAAV in the brain including an analysis of the immune response is being prepared for publication (M. Y. Mastakov *et al.*, in preparation).

In summary, we have demonstrated that rAAV vector infusion rates much slower than those previously re-

ported lead to higher transduction efficiency, with no further gain from reducing infusion rates below 22 nl/min. Furthermore, we could show that the local injection of mannitol facilitated gene transfer to neurons, increasing both the total numbers of transduced cells and the vector distribution by 200–300%. Optimization of vector infusion rates and addition of mannitol as an adjuvant together with improved vector stocks and expression cassettes should be considered for the successful use of rAAV in studies of neuronal physiology. Moreover, our findings have positive therapeutic implications for CNS disorders using gene transfer vectors and will help ensure that the full potential of this new technology is realized.

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