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Efficiencies of Transgene Expression in Nociceptive Neurons Through Different Routes of Delivery of Adeno-Associated Viral Vectors

YA XU,[1] YANPING GU,[1,2] PING WU,[1,2] GUANG-WEN LI,[1] and LI-YEN MAE HUANG[1,3]

ABSTRACT

Transferring therapeutic genes into the nociceptive system, including dorsal root ganglia (DRGs) and the spinal cord, is potentially a powerful approach for the treatment of chronic pain in humans. Adeno-associated viral vectors (AAVs) are particularly useful in delivering foreign genes to targeted tissues because they seldom induce immune responses or produce cytotoxicity. To determine the efficiency of transgene expression and the best route(s) of delivery, a recombinant AAV type 2 vector containing the enhanced green fluorescent protein (EGFP) gene driven by the neuron-specific enolase (NSE) promoter (rAAV-EGFP) was constructed. We injected the vector into subcutaneous tissue, sciatic nerve, DRGs, and subarachnoid space, and examined EGFP expression in the DRG, spinal cord, and nerve fibers. Both sciatic nerve and DRG injection led to strong EGFP expression in a large number of DRG neurons. The expression persisted for more than 6–8 months. We then delivered the μ-opioid receptor (μOR) gene into DRGs through direct DRG or sciatic nerve injection of rAAV-μOR and found a significant increase in morphine efficacy. These results suggest that delivering therapeutic genes to DRGs by the rAAV-NSE vector is a valid strategy for treatment of chronic pain.

OVERVIEW SUMMARY

We studied the expression of EGFP under the control of the NSE promoter in DRGs by injecting rAAV vector into subcutaneous tissue, sciatic nerve, DRGs, and the subarachnoid space. Injections into DRG and sciatic nerve resulted in strong EGFP expression in DRGs, dorsal roots, and peripheral axons. Subarachnoid injection led to EGFP expression in many dorsal horn neurons. The expression occurred exclusively in neurons and lasted for an extended period (6–8 months). Delivery of the μOR gene to DRGs through direct DRG or sciatic nerve injection greatly potentiates morphine analgesia. Therefore rAAV-NSE vector can be used to transfer a variety of therapeutic genes involved in pain processing and should have wide applications in the treatment of chronic pain in humans.

INTRODUCTION

A POTENTIALLY POWERFUL GENETIC APPROACH for treatment of diseases is to deliver genes of therapeutic interest and thus manipulate the expression of proteins in the target tissue (Mulligan, 1993). Virally derived vectors are particularly suited for such purposes because of their ability to penetrate cell membrane efficiently and deliver transgenes into host cells. The nervous system has become an attractive target for *in vitro* and *in vivo* gene delivery because of possible clinical applications for treating neurological disorders, such as Alzheimer's, Parkinson's disease, and chronic pain syndromes. Most viral vectors used to date are derived from herpes simplex virus, lentivirus, adenovirus, and adeno-associated virus (AAV) (Hermens and Verhaagen, 1998). Among these viral vectors, AAV holds substantial advantages. AAV is a nonpathogenic and replication-

[1]Marine Biomedical Institute, [2]Department of Anatomy and Neurosciences, and [3]Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77555.

defective human parvovirus (Berns and Adler, 1972). It can infect nondividing cells and integrate into the host cell genome (Samulski *et al.*, 1989) without signs of cytotoxicity (Kaplitt *et al.*, 1994; McCown *et al.*, 1996; Ferrari *et al.*, 1997; Jooss *et al.*, 1998). Advances in construction of the AAV helper plasmid make it possible to produce high-titer AAV vectors in the absence of infectious helper adenovirus (Xiao *et al.*, 1998; Clark *et al.*, 1999; Zolotukhin *et al.*, 1999). This further reduces possible immune responses to the AAV vector and makes it a useful tool for safe gene transfer.

Recombinant AAV (rAAV) containing the green fluorescence protein (GFP) reporter gene under the control of various promoters has often been used to examine the efficiency of gene delivery. Human cytomegalovirus (CMV) or CMV hybrid (including CMV–chicken β-actin) promoter-driven rAAV-EGFP has been injected into the brain and spinal cord and shown to transduce neurons efficiently (McCown *et al.*, 1996; Bartlett *et al.*, 1998; Dhillon *et al.*, 1999). Compared with the CMV promoter, the platelet-derived growth factor (PDGF) or neuron-specific enolase (NSE) promoter construct could lead to more efficient and stable transgene expression in neurons in the central nervous system (Peel and Klein, 2000). Gene transfer into the peripheral nervous system has been explored. Injection of rAAV-EGFP into dorsal root ganglion (DRG) neurons under the control of the PDGF promoter could result in strong and stable EGFP expression for up to 52 days (Glatzel *et al.*, 2000). Because DRGs and the spinal cord are crucial for transmitting pain and sensory information, we explored the possibility of using rAAV to transfer genes into these target structures and determined whether this approach is a valid therapeutic strategy for the treatment of pain. If rAAVs are to have applications in gene therapy of pain disorders in humans, it is important to determine how efficiently viral vectors, for example, rAAV-EGFP, transduce DRGs, whether the transgene expression is restricted to neurons, how long the expression lasts, and what is the most convenient and effective route(s) for viral vector delivery. The feasibility of the approach was further tested by delivering the μ-opioid receptor (μOR) gene into DRGs, using rAAV-μOR, through various routes of delivery. Changes in morphine doses required for relieving thermal pain in rats with inflammation were examined.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (Galveston, TX).

Adeno-associated viral vector

rAAV vector plasmid pAAV-NSE/EGFP, containing the enhanced green fluorescent protein (EGFP)-encoding gene under the control of an NSE promoter, was constructed by inserting an NSE promoter and an EGFP-expressing cassette between the inverted terminal repeats of pTR-UF2 (kindly provided by N. Muzyczka, University of Florida, Gainesville, FL). The NSE-GDNF/pA fragment (2.9 kb) from pSSV9NSEgdnf.XS was cloned into pTR-UF2 to replace its original CMV promoter, yielding the plasmid pTR-NSE/pA. The 0.7-kb EGFP gene from pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) was then cloned into pTR-NSE/pA to replace the glial cell-derived neurotrophic factor (GDNF) fragment and generate the plasmid pAAV-NSE-EGFP. pTR-NSE-μOR was generated by replacing EGFP with the MOR-1 gene (a gift from H. Akil, University of Michigan, Ann Arbor, MI) (Thompson *et al.*, 1993; Xu *et al.*, 2003).

Viral production was conducted according to published protocols, with modification (Clark *et al.*, 1999; Zolotukhin *et al.*, 1999; Wu *et al.*, 2002). The rAAV vector plasmids pXX2 and pXX6 (provided by X. Xiao, University of Pittsburgh, Pittsburgh, PA) were cotransfected with calcium phosphate at a molar ratio of 7:2:4 in 12 culture dishes (150-mm diameter), each containing 6.3×10^6 293 cells. The transfection medium was replaced with fresh medium 18 hr later and the cells were incubated for an additional 30 hr before harvesting. Cells were collected and pelleted by centrifuge at $1140 \times g$ for 15 min at room temperature. Cells were then lysed in 20 m*M* Tris (pH 8.0) and 150 m*M* NaCl at $\sim 5 \times 10^6$ cells/ml and treated with deoxycholate (0.54%) and Benzonase (50 U/ml) for 45 min at 37°C. Cellular debris was removed by spinning at $3000 \times g$ for 15 min at room temperature and at $10{,}000 \times g$ for 30 min at 4°C. The supernatant was collected and passed through a 1-μm pore size disk filter. Purification of the virus was conducted by using a heparin–agarose type I column (Sigma, St. Louis, MO). The eluted rAAV solution was then applied to a Biomax-100 filter (Millipore, Billerica, MA) to be concentrated and desalted to produce the viral stock.

The viral stock was titrated by transgene expression assay (Chamberlin *et al.*, 1998; Xiao *et al.*, 1998). Transducing units per milliliter (TU/ml) was determined by transduction of cultured DRG neurons with serially diluted viral solutions. After 10 days, the transgene-expressing cells were examined under a fluorescence microscope. The titer of rAAV-EGFP viral stock was 4.2×10^8 TU/ml. The titer of rAAV-μOR was 2.6×10^8 TU/ml.

Transduction of cultured DRG neurons

DRGs were taken from 13- to 16-day-old rats and desheathed in Hanks' balanced salt solution (HBSS). DRGs were digested in Earle's balanced salt solution (EBSS) with trypsin (1 mg/ml) and collagenase (1 mg/ml) at 35°C. After 1 hr, additional culture medium was added to stop enzymatic reactions. The cells were pelleted by centrifugation at $50 \times g$ for 6 min. The pellet was resuspended in Eagle's minimum essential medium (MEM; Life Technologies, Rockville, MD) and plated on coverslips in a 24-well culture plate. DRG cells were cultured at 1.2×10^4 cells/well. The cells were incubated at 37°C with 5% CO_2.

Twenty-four to 48 hr after plating, DRG cells were transduced with the virus. The viral stock was serially diluted in OptiMEM (Life Technologies). After 1.5 hr of incubation at 37°C, MEM containing 20% fetal bovine serum (FBS) was added to reach a final concentration of 10% FBS and the cells were incubated overnight. Medium was replaced with 10% FBS medium the next day. Transduction efficiency was checked after 10–14 days treatment.

Microinjection to the hind foot, sciatic nerve, and the DRG

For subcutaneous injection, 1- or 3-week-old rats (male Sprague-Dawley) were used. Ten microliters of viral solution

was injected subcutaneously into the ventral or dorsal skin of the hind foot. Animals were killed 1, 3, or 6 weeks later. Transduction efficiencies in DRGs were assessed.

For nerve injection, 25- to 30-day-old rats were anesthetized with a pentobarbital sodium solution (intraperitoneal, 50 mg/kg). The sciatic nerve was surgically exposed. Two microliters of viral solution was delivered slowly (20 min) into the nerve through a glass micropipette connected to a Hamilton syringe. The pipette was pulled out after a 10-min wait.

For DRG injection, 25- to 30-day-old rats were anesthetized as described above. The left lumbar L4 and L5 DRGs were exposed by removing part of the vertebra. Viral solution (2 μl) was delivered to each DRG, using the same procedure as described for the nerve injection.

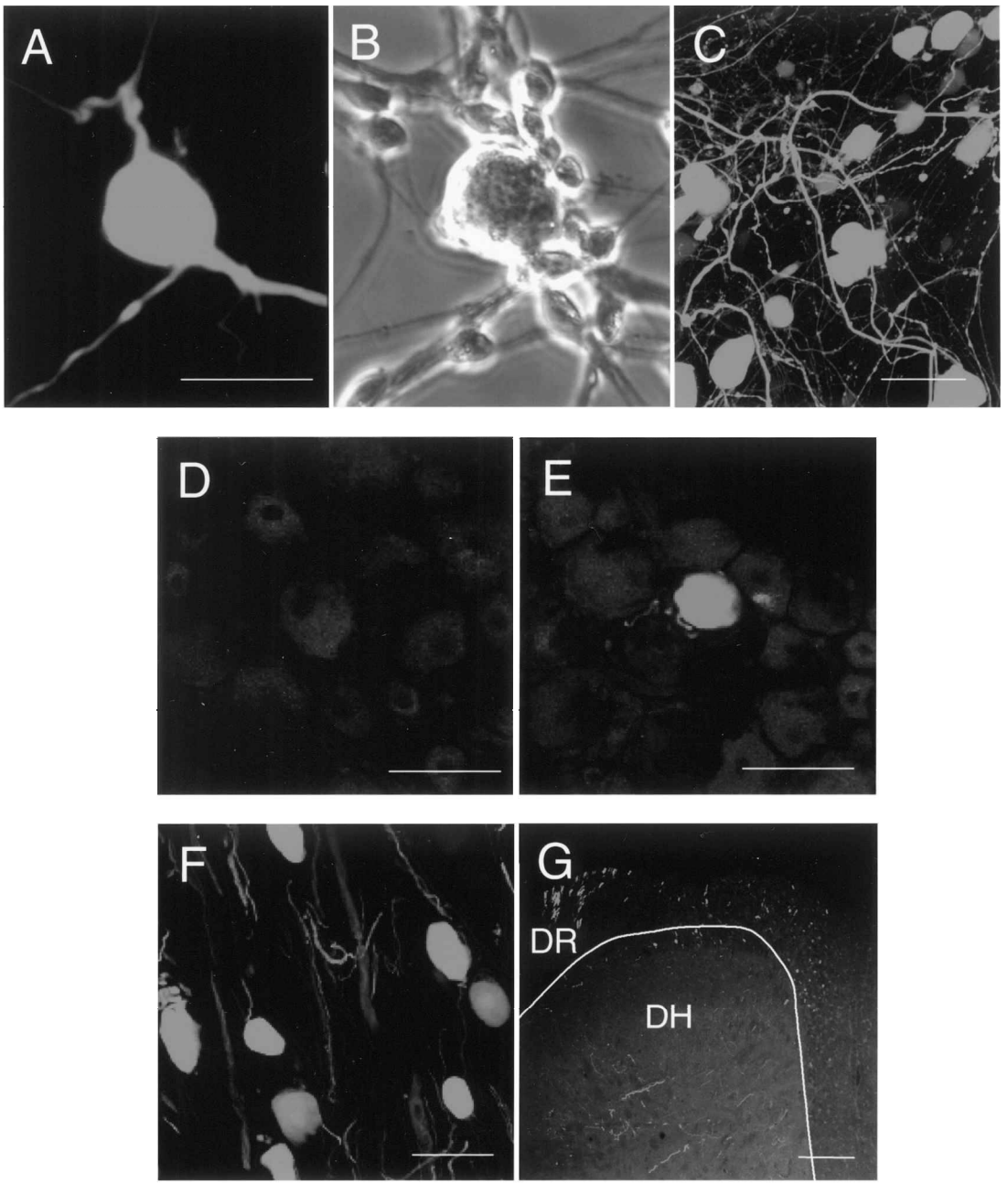

FIG. 1. EGFP expression in primary cultured rat DRG neurons (**A–C**) and the L4 DRG (**D–G**). (**A**) Fourteen days after cultured DRG neurons were exposed to rAAV containing the EGFP gene under the control of the NSE promoter, a DRG neuron showed strong EGFP expression. (**B**) The same cell as in (**A**), viewed under phase optics. (**C**) Twenty-one days after rAAV-EGFP treatment, almost all cultured DRG neurons were intensely labeled. (**D**) As a negative control, without an injection of rAAV-EGFP, there was no EGFP expression in the L4 DRG. (**E**) A neuron in the DRG showed EGFP expression 3 weeks after an injection of 10 μl (titer, 4.2×10^8 TU/ml) of viral solution into the ventral side of the rat hind foot. (**F**) Many neurons in the DRG were fluorescently labeled 3 weeks after 2 μl of viral solution was injected into the sciatic nerve of the rat hind limb. (**G**) EGFP expression was also seen in dorsal roots after injection of rAAV-EGFP into the sciatic nerve. Bars: 50 μm.

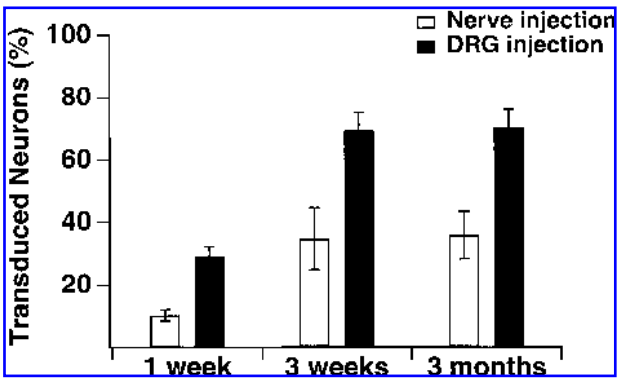

FIG. 2. Efficiencies of EGFP expression after direct DRG or nerve injection. The percentages of neurons transduced by rAAV-EGFP were 10.3 ± 1.8% ($n = 3$), 34.8 ± 9.9% ($n = 3$), and 36.0 ± 7.5% ($n = 5$) 1 week, 3 weeks, and 3 months after sciatic nerve injection, respectively. The percentages of neurons transduced were 29.4 ± 2.9% ($n = 3$), 69.6 ± 5.7% ($n = 3$), and 70.6 ± 5.7% ($n = 3$) 1 week, 3 weeks, and 3 months after direct DRG injection, respectively.

Subarachnoid microinjection to the spinal cord

For intrathecal injection, 25- to 30-day-old rats were used. The left lumbar spinal cord was exposed by partial laminectomy. A 5-μl viral solution was infused into the left side of the subarachnoid space at the lumbar enlargement of the spinal cord, using a glass micropipette as described. Injection usually took 10 min and the pipette was pulled out after a 10-min wait.

Histology

Cultured DRG cells on coverslips were fixed with 4% paraformaldehyde in 0.1 *M* sodium phosphate buffer (pH 7.4) for 1 hr at 4°C. Animals were anesthetized with a pentobarbital sodium solution (intraperitoneal, 50 mg/kg) and perfused by cardiac puncture with normal saline, followed by the same fixative. Dorsal root ganglia (L4–L6) and the lumbar spinal cord were dissected and incubated in the same fixative solution for 2 hr at 4°C. The tissue was then transferred to a 30% sucrose solution and stored at 4°C overnight. For sectioning, the tissue pieces were put in O.T.C. embedding medium and placed on a cryostat stage. DRG tissue sections (10 μm thick) or spinal cord sections (16 μm thick) were serially collected. To visualize EGFP fluorescence directly, cells or tissue sections were rinsed with phosphate-buffered saline (PBS) for 5 min, coverslipped with No-Fade mounting solution (Vector Laboratories, Burlingame, CA), and viewed under a fluorescence or confocal microscope. Untreated cultured DRG neurons, DRGs, and spinal cord were used as controls. In some experiments, rabbit anti-GFP (diluted 1:200; Chemicon, Temecula, CA) was used to label EGFP; Cy3-conjugated IgG (diluted 1:400; Jackson ImmunoReseach, West Grove, PA) was the secondary antibody. Mouse anti-NeuN (diluted 1:200; Chemicon) was used to label neurons. Alex Fluor 546 goat anti-mouse IgG (diluted 1:200; Molecular Probes, Eugene, OR) was used for visualization.

Cell counting

To determine the percentage of rAAV-transduced DRG neurons *in vitro*, EGFP-negative and positive cultured cells on coverslips were counted under a fluorescence microscope. Three coverslips were used for each viral solution. For *in vivo* studies, EGFP labeling was visualized directly on tissue sections (10 μm thick) without further processing. To minimize the possibility of double counting, cells were counted by stereological counting techniques (Coggeshall, 1992). The percentages of EGFP-labeled cells from 3–5 animals in each delivery method were averaged.

Induction of inflammation

Three to 4 weeks after rAAV treatment, 100 μl of complete Freund's adjuvant (CFA) (*Mycobacterium butyricum*; Difco, Detroit, MI) emulsion (peanut oil–saline, 1:1; 10 mg of *Mycobacterium* per milliliter) was injected into the plantar surface of the left hind paw of rats to induce inflammation. Behavioral experiments were performed 5–14 days after CFA injection.

Behavioral tests

Thermal hyperalgesia to radiant heat was assessed by the method described by Hargreaves *et al.* (1988). Before behavioral experiments, rats were acclimated in Plexiglas boxes placed on a platform for 30 min/day for 5 days. Paw withdrawal latencies (PWLs) were obtained by placing a radiant heat source under the plantar surface of the hind paw and recording the time elapsed from the onset of radiant heat stimulation to the withdrawal of the paw. The heat intensity was adjusted to give a baseline latency of ~5 sec in CFA rats. A cutoff time of 30 sec was set to prevent possible tissue damage. To obtain baseline PWLs, three measurements separated by a 5-min interval were made for each rat's hind paw and scores were averaged. The antinociceptive effects of morphine were evaluated by measuring PWLs before and every 10 min after subcutaneous morphine administration. Morphine antinociceptive responses were expressed as maximum possible effect (MPE), using the relation MPE = [(postdrug PWL − predrug PWL)/(cutoff PWL − predrug PWL)] × 100.

RESULTS

Efficient transduction of DRG neurons in vitro

Cultured DRG neurons 24–48 hr after plating were treated with virions containing rAAV-EGFP under the control of the

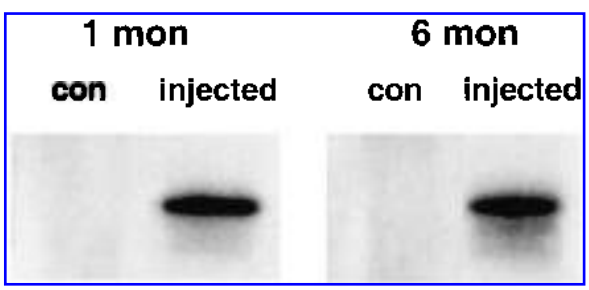

FIG. 3. Western analyses of EGFP expression after nerve injection of rAAV-EGFP. Levels of EGFP expression in L4-6 DRGs 1 and 6 months after the injection were similar. DRGs without any viral injection were used as controls (con).

NSE promoter. Two different viral doses (2.3×10^4 or 2.3×10^3 transducing units [TU]/well) were used. A few EGFP-expressing neurons began to appear 3–4 days postinfection. However, the fluorescence was limited to the cell body and its intensity was low (data not shown). EGFP expression increased with time. By 7 days, neurons became brightly green and fluorescence was seen in proximal and distal processes. At 14 days postinfection, EGFP expression remained high (Fig. 1A). The percentage of EGFP-positive neurons at high transducing units was much greater ($97 \pm 2.3\%$, $n = 4$) than that at low transforming units ($21 \pm 1.7\%$, $n = 4$). Intensive EGFP fluorescence persisted until the end of the period of culture (~3 weeks) (Fig. 1C). Schwann cells, characterized by their spindle bipolar shape and fusiform nuclei, were not labeled.

A few DRG neurons transduced by subcutaneous injection of rAAV in the hind foot

To determine the best route of gene delivery, we attempted to transduce DRG neurons by injecting rAAV-EGFP into the rat hind foot. Viral stock (10 μl) was injected subcutaneously into either the dorsal or ventral surface of the hind foot of 3-week-old rats. No EGFP-positive cells were found in the DRGs 3 or 6 weeks postinjection. When rAAV-EGFP was injected into the dorsal surface of the hind foot of 1-week-old rats, no labeled cells were observed either. However, when viral solution was injected into the ventral surface of the hind foot of 1-week-old rats, one or two EGFP-labeled cells were observed in ipsilateral L4–L6 DRGs 3 weeks postinjection ($n = 2$) (Fig. 1E), although no labeled cells were found 1 week postinjection ($n = 1$). The number of EGFP-positive cells stained with anti-GFP antibody did not increase further when DRGs were examined 3 or 6 weeks postinjection ($n = 2$) (data not shown). No glial cells were transduced.

Efficient transduction of DRG neurons with injection of rAAV into the sciatic nerve

We then examined transduction of DRGs after injection of rAAV-EGFP (2 μl) into the sciatic nerves of 21- to 25-day-old rats. At 1 week postinjection, $10.3 \pm 1.8\%$ ($n = 3$) of DRG neurons were weakly labeled (Fig. 2). At 3 weeks, $34.8 \pm 9.9\%$ of neurons were labeled ($n = 5$) (Fig. 2). The labeled cells showed intensive fluorescence (Fig. 1F) and EGFP expression became visible in dorsal roots and the spinal dorsal horn ($n = 4$) (Fig. 1G). NSE-driven EGFP was selective for neurons because satellite cells were not labeled (Fig. 1). No fluorescence was seen in DRG neurons of uninjected rats (Fig. 1D). EGFP expression plateaued after 3 weeks; $36.0 \pm 7.5\%$ ($n = 5$) of neurons in DRGs were transduced at 3 months (Fig. 2). Western analyses of EGFP expression indicated that EGFP expression reached its peak value 1 month postinfection and persisted for at least 6 months (Fig. 3).

Strong neuronal transduction after direct injection of rAAV into DRG

We then examined EGFP expression after injecting 2 μl of rAAV-EGFP directly into L4–L5 DRGs of 21- to 25-day-old rats. At 1 week postinjection, $29.4 \pm 2.9\%$ ($n = 3$) of neurons were transduced (Fig. 2). Fluorescence was observed in cell

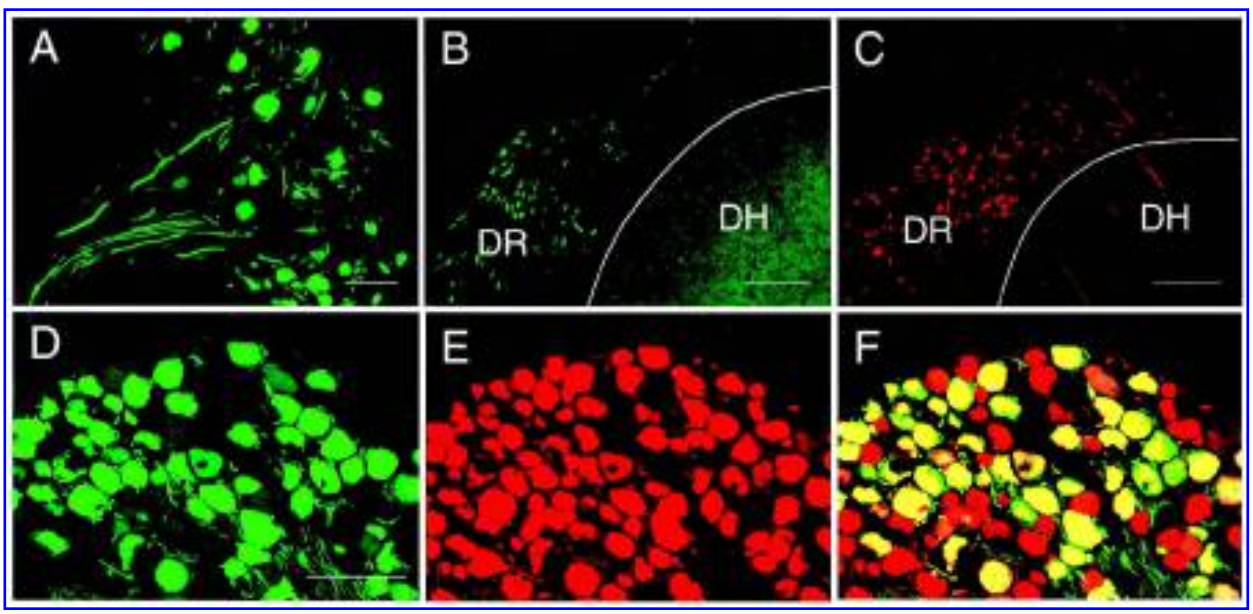

FIG. 4. EGFP expression 1–3 weeks after direct injection of rAAV-EGFP into DRGs. (**A**) One week after injecting 2 μl of viral solution into the L4 DRG, fluorescent neurons of various intensities were found in the DRG. Axons were also fluorescently labeled. (**B**) EGFP labels are seen in dorsal roots (DR) innervating the lumbar enlargement of the spinal cord 1 week after rAAV-EGFP was injected into L4 and L5 DRGs. DH, Dorsal horn. (**C**) Using an anti-GFP antibody, EGFP-labeled nerve fibers could be detected in both the dorsal roots and dorsal horn of the spinal cord 1 week after viral injection. (**D**) Three weeks after DRG injection, many neurons were labeled with EGFP. (**E**) The same tissue section was labeled with anti-NeuN antibody. (**F**) Merging of (**D**) and (**E**) showed that all EGFP-labeled neurons were NeuN positive. Bars: 100 μm.

bodies and axons near the ganglion (Fig. 4A). Dorsal roots innervating the dorsal horn of the lumbar cord were also labeled (Fig. 4B and C). At 3 weeks, the percentage of cells expressing EGFP greatly increased (Fig. 4D). On average, 69.6 ± 5.7% ($n = 3$) of neurons in the ganglia were transduced. All EGFP-labeled were NeuN positive, indicating neuron-specific expression (Fig. 4E and F). At 3 months postinjection, 70.6 ± 5.7% ($n = 3$) of DRG neurons were transduced (Fig. 5A). Intensive EGFP fluorescence was detected in a large number of nerve fibers innervating both the dorsal and anterior horns of the spinal cord ipsilateral to the injection (Fig. 5A, inset). No cell bodies of spinal cord neurons were labeled, suggesting that rAAV-EGFP was not transported across central synapses. Bright fluorescent labels were also seen in sciatic nerve fibers (Fig. 5A). To determine the duration of expression of rAAV, EGFP labels were examined 8 and 12 months postinjection (Fig. 5B and C). At 8 months, DRG neurons stayed brightly fluorescent but sciatic nerves and nerve fibers in the spinal cord ($n = 3$) were not as bright as at 3 months (Fig. 5B). At 12 months, EGFP expression in neurons and sciatic nerves postinjection was somewhat reduced ($n = 3$) (Fig. 5C). We also inspected peripheral tissue in the hind foot ipsilateral to the injected DRGs. Labeled nerve fibers were found around muscle cells (Fig. 6A and B), blood vessels (Fig. 6D), and in subcutaneous tissue (Fig. 6E). No labeled nerve fibers were found in uninjected animals (Fig. 6C and F).

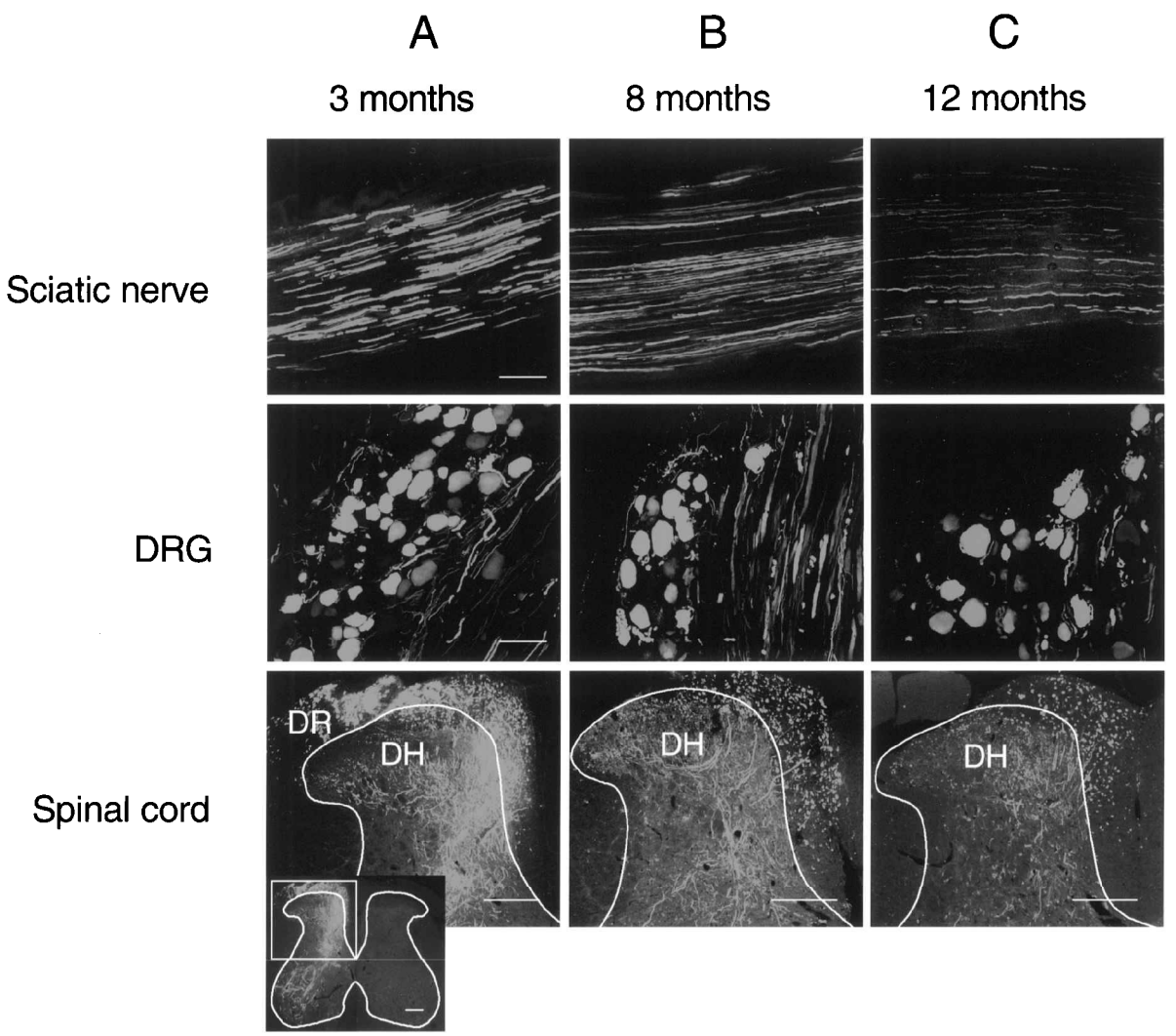

FIG. 5. EGFP expression 3, 8, and 12 months after injection of rAAV-EGFP directly into DRGs. (**A**) EGFP expression 3 months after injection of rAAV-EGFP (2 μl) into L4 and L5 ganglia. The sciatic nerves (*top*) and DRG neurons (*middle*) were brightly fluorescent. Intense EGFP expression was seen in dorsal roots and nerve fibers of the dorsal and anterior horns of the spinal cord (*bottom, inset*). An enlarged picture of the indicated square in the inset is also shown (*bottom*). (**B**) EGFP expression 8 months after direct DRG injection was strong in peripheral nerves, DRGs, and the spinal cord. The fluorescence intensities were somewhat weaker than those seen at 3 months. (**C**) EGFP expression 12 months after injection of rAAV-EGFP into DRGs. Fluorescent neurons and fibers were still detectable. Intensities of the fluorescence were noticeably weaker than those found at 3 months. Magnification bars: for sciatic nerves, 500 μm; for DRGs, 50 μm; for spinal cord, 200 μm; for inset, 100 μm. DR, Dorsal roots; DH, dorsal horn.

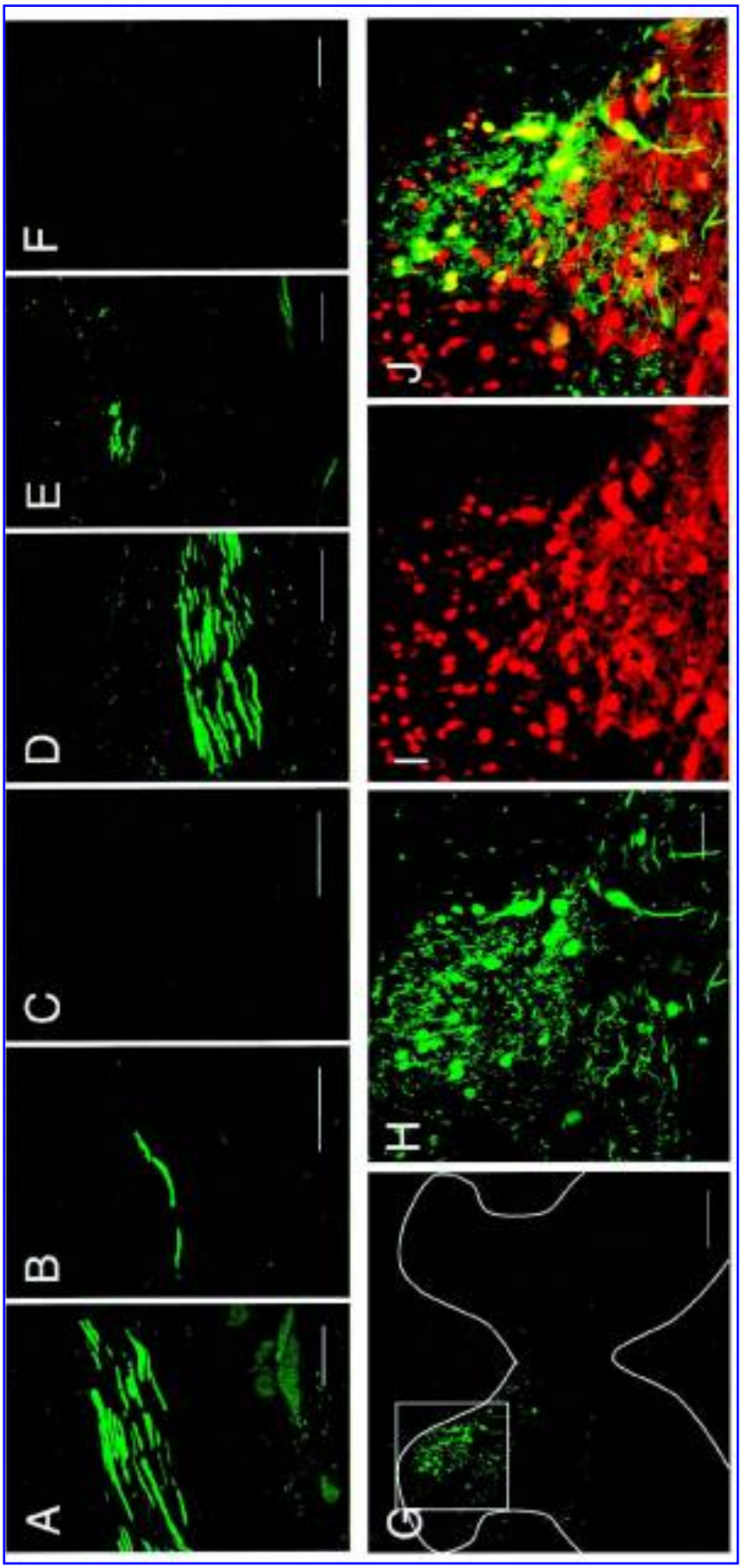

FIG. 6. (**A–F**) EGFP expression in peripheral tissue after direct DRG injection. Three months after injecting rAAV-EGFP (2 μl) into L4 and L5 ganglia, EGFP expression could be found in nerve fibers (**A**) near muscle cells, (**B**) surrounding muscle cells, (**D**) near a blood vessel, and (**E**) in the subcutaneous tissue. (**C** and **F**) Negative controls. Without rAAV-EGFP injection, no EGFP could be found in nerves surrounding muscle cells (**C**) or in the subcutaneous tissue (**F**). (**G–J**) EGFP expression in spinal neurons in the lumbar enlargement of the spinal cord 3 weeks after subarachnoid injection of 5 μl of viral solution. (**G**) EGFP expression was found in dorsal horn neurons. The transduced neurons were mainly in laminae I–III. Note that there was no EGFP label in the contralateral dorsal horn. (**H**) Enlargement of the area shown in the square in (**G**). (**I**) The same tissue section was labeled with anti-NeuN antibody. (**J**) Merging of (**H**) and (**I**). All EGFP-labeled cells were NeuN positive, indicating that neurons, but not glial cells, were transduced. Thus, rAAV-EGFP under the control of the NSE promoter is neuron specific. Bars: (**A–F**) 100 μm; (**G**) 100 μm; (**H–J**) 50 μm.

Subarachnoid delivery of rAAV-EGFP results in EGFP expression in the dorsal horn

To determine whether rAAV viral vectors can be efficiently delivered to the spinal cord, we injected viral solution (5 μl) into the subarachnoid space on the lateral side of the lumbar enlargement of the spinal cord of 25- to 30-day-old rats. EGFP-labeled neurons started to appear in the dorsal horn at 1 week postinjection ($n = 1$, data not shown). Fluorescent spinal neurons nearly doubled by 3 weeks. Most of them are located in laminae I to III of the dorsal horn ($n = 2$). Some were in lamina IV (Fig. 6G and H). Caudal–rostral spread of the EGFP expression was examined. Labeled cells were found in spinal cord sections up to 2 mm away from either side of the injection site; EGFP-labeled fibers could be seen up to 6 mm away from the injection site. In contrast, the lateral spread of viral solution was limited because EGFP-positive neurons were not found on the contralateral side of the spinal cord. To ascertain that the labeled cells were neurons, the spinal cord sections were double labeled with anti-NeuN antibody. All EGFP-labeled cells were NeuN positive (Fig. 6I and J), indicating again that the NSE promoter selectively drove EGFP expression in neurons. Because none of the L4–L6 DRGs on either side of the spinal cord were labeled (data not shown), AAV-EFGP did not appear to retrogradely cross spinal cord-afferent synapses.

Transduction of μOR genes in DRGs enhances morphine analgesia

To determine whether the gene transfer strategy can be used in the treatment of nociception, we increased μOR expression in DRGs by delivering rAAV-μOR into DRGs and studied changes in morphine analgesia. The two most effective routes of gene delivery, that is, direct DRG and sciatic nerve injection of rAAV-μOR, were used to deliver the μOR gene. The antinociceptive effects of morphine on inflammatory pain were examined. We injected rAAV-μOR or rAAV-EGFP (as controls) into the left L4–L5 DRGs (Fig. 7A) or into the left sciatic nerve (Fig. 7B). Three to 4 weeks later, paw inflammation was induced by an injection of complete Freund's adjuvant (CFA) into the plantar surface of the left hind paw. Within 12 hr of CFA treatment, the paw showed signs of inflammation, that is, edema and redness, which lasted for more than 2–3 weeks (Guo and Huang, 2001). Five to 14 days after CFA treatment, behavioral tests, that is, paw withdrawal latencies (PWLs) or maximum possible effects (MPEs) in response to a radiant heat source placed under the hind paw, were used to study the effect of morphine analgesia. Studying the rat groups injected with rAAV into DRGs directly (Fig. 7A), we found that the MPEs increased (i.e., analgesia) after subcutaneous injection of morphine. The MPEs peaked at 30–40 min, and dissipated within 100–120 min of morphine treatment. Interestingly, the MPEs in rAAV-μOR-injected rats were significantly greater, clearly indicating an increase in morphine analgesia. Injection of rAAV-μOR into the sciatic nerve also resulted in similar enhancement of the antinociceptive effects of morphine (Fig. 7B). The enhancement persisted for 3 months after the injection of rAAV-μOR (data not shown).

DISCUSSION

We determined the efficiency of EGFP expression *in vitro* under the control of an NSE promoter and found that 97% of

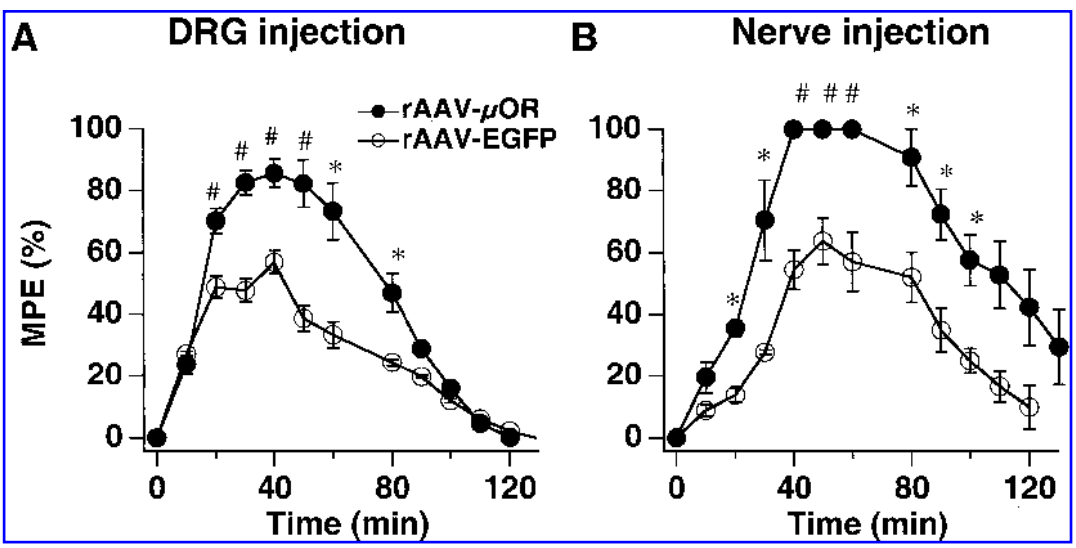

FIG. 7. Morphine antinociceptive effects are enhanced in inflamed rats injected with rAAV-μOR. (**A**) The antinociceptive responses of morphine (8 mg/kg), expressed as units of maximum possible effect (MPEs), in CFA-treated rats injected with either rAAV-μOR (solid circles) or rAAV-EGFP (open circles) into the L4 and L5 DRGs. The antinociceptive effects reached a maximum at about 30–40 min and then slowly dissipated in 100 min. The MPEs obtained from the paw ipsilateral to rAAV-μOR injection ($n = 6$) were greater ($^*p < 0.05$; $^\#p < 0.01$) than those obtained from the paw ipsilateral to rAAV-EGFP injection ($n = 5$). (**B**) Antinociceptive responses of inflamed rats to morphine (10 mg/kg) after either rAAV-μOR (solid circles) ($n = 4$) or rAAV-EGFP (open circles) ($n = 4$) was injected into the sciatic nerves. The antinociceptive responses of rAAV-μOR rats to morphine were significantly higher than those of rAAV-EGFP rats ($^*p < 0.05$; $^\#p < 0.01$).

cultured DRG neurons are transduced for the duration of the cultures (>3 weeks). The time course and high level of transgene expression are similar to those observed in cultured mouse DRG neurons using rAAV-EGFP with the CMV promoter (Fleming *et al.*, 2001). The major difference between the two studies is that the NSE promoter drove EGFP expression exclusively in DRG neurons (Fig. 1) and the CMV promoter directed EGFP expression in neurons as well as in Schwann and fibroblast-like cells. We also showed that the NSE promoter generated neuron-specific expression of EGFP in DRGs *in vivo* (Figs. 1 and 4). This is consistent with previous studies in which the NSE promoter efficiently drove transgene expression in neurons but not in glial elements in the brain and spinal cord (Peel *et al.*, 1997; Klein *et al.*, 1998).

To determine the best route of vector delivery, we injected rAAV-EGFP into afferent terminals (Fig. 1E), the sciatic nerve (Fig. 1F), DRG (Fig. 4), and the subarachnoid space of the spinal cord (Fig. 6G–J) and compared the efficiencies and durations of EGFP expression. We found that direct injection into DRG gave the strongest EGFP expression. Both the somata of DRG neurons and sciatic nerves are efficiently transduced, similar to those observed by Glatzel *et al.* (2000). In addition, we examined EGFP expression in both central and afferent terminals. Intense EGFP labeling was found in a large number of nerve fibers that targeted the dorsal and anterior horns of the spinal cord (Fig. 5). Furthermore, EGFP expression was also seen in peripheral axons, including nerves surrounding muscle cells, blood vessels, and axons innervating the skin of the ventral surface of the hind foot (Fig. 6A–F). We also documented the time course of EGFP expression (Fig. 2). About 30% of cells became EGFP positive by 1 week postinjection. This differs from the observations made by Glatzel *et al.* (2000), who could not detect any EGFP expression in mouse DRG 16 days after injection, but found strong EGFP expression 45 days later. It will be of interest to determine whether different promoters used in the two studies (i.e., PDGF in mice [Glatzel *et al.*, 2000] versus the NSE in this paper) contribute to the large difference in the time course of EGFP expression. We further showed that EGFP expression reached its peak level (~70%) at 3 weeks and persisted for 6 months (Fig. 3). Expression started to diminish at 8 months and became weaker 12 months after injection. This rapid and long-lasting neuron-specific transgene expression in DRGs, driven by the NSE promoter, suggests that the rAAV vector would be a superior tool for gene transfer in the nociceptive system.

One drawback to direct DRG injection of viral vector is that a small piece of vertebra must be removed to gain access to DRGs. Less invasive procedures for viral vector delivery were explored. We found that injection of rAAV into subcutaneous tissue resulted in only a few EGFP-expressing DRG cells (Fig. 1E). This observation is at variance with the effective transduction of DRGs after infecting cutaneous nociceptive afferents with herpes simplex virus (HSV) vectors (Wilson *et al.*, 1999; Wilson and Yeomans, 2000). The disadvantages of using HSV vectors for gene transfer include relatively short periods of expression (<7 weeks) and apparent cytotoxicity (Wilson *et al.*, 1999; Braz *et al.*, 2001).

Injection of rAAV into the sciatic nerve, although not as effective as direct DRG injection, did give strong expression (Figs. 1F and 3). This observation is in contrast to sciatic nerve injection of EGFP with adenovirus-based vectors, which transduce mostly Schwann cells (Glatzel *et al.*, 2000). Earlier studies of rAAV vectors indicate that retrograde transduction rarely occurred in neurons of the brain (Chamberlin *et al.*, 1998; Zolotukhin *et al.*, 1999), although transgene expression was observed in the spinal cord after peripheral nerve injection (Boulis *et al.*, 2000). It is as yet unclear whether the efficient retrograde axonal transport in afferent axons observed arises from unique axonal transport properties of the peripheral nervous system. We also showed that subarachnoid injection of rAAV gave rise to effective gene transfer to the dorsal horn. Because there is no extensive spread of *de novo* genes to the contralateral side or DRG, this could be a feasible way to transfer genes into the spinal cord. Because the rAAV-EGFP vector leads to strong labeling of nerve fibers in the spinal cord and subcutaneous tissue, this vector would be a useful neuroanatomical tool for studying nociceptive pathways.

Our results indicate that rAAV with the NSE promoter gives rise to neuron-specific expression in the DRG and spinal cord. Both direct DRG and sciatic nerve injections result in strong transgene expression that remains robust for at least 6–8 months. To test the feasibility of this approach in the treatment of pain, we further showed from behavioral studies that transferring the μOR gene into DRGs through either direct DRG injection or nerve injection increases the efficacy of morphine (Fig. 7). Despite the somewhat less efficient level of gene transfer via sciatic nerve injection (Fig. 3), the increase in morphine analgesia is similar to that achieved by direct DRG injection (Fig. 7). Because nerve injection involves a relatively innocuous procedure, it would be a more promising route for gene delivery into DRGs. Opiates are widely used for treating patients with severe pain. However, their use is often hampered by side effects, including nausea, constipation, and tolerance (Yaksh, 2000). With the enhancement of the morphine antinociceptive effect after rAAV-μOR injection, lower doses of opiates would be required for antinociception. This will reduce the risk of morphine side effects, thus indicating the therapeutic potential of this approach. These results plus the lack of cytotoxicity and immunoreactivity produced by rAAV-mediated transduction suggest the exciting possibility of using AAV-NSE vectors to deliver therapeutic genes to the nociceptive system. Our studies show that with a proper choice of promoter and vector delivery route, introduction of appropriate genes into DRGs with rAAV vectors offers a potentially powerful therapeutic strategy for the treatment of nociceptive disorders and chronic pain.

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Address reprint requests to:
Dr. Li-Yen Mae Huang
Marine Biomedical Institute
University of Texas Medical Branch
Galveston, TX 77555-1069

E-mail: lmhuang@utmb.edu

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