In vivo introduction of transgenes into mouse sciatic nerve cells *in situ* using viral vectors

Sergio Gonzalez, Ruani N Fernando, Claire Perrin-Tricaud & Nicolas Tricaud

INSERM U1051, Institut des Neurosciences de Montpellier (INM), Université de Montpellier 1 and 2, Montpellier, France. Correspondence should be addressed to N.T. (nicolas.tricaud@inserm.fr).

Published online 24 April 2014; doi:10.1038/nprot.2014.073

The myelin sheath is essential for the rapid and efficient propagation of action potentials. However, our understanding of the basic molecular mechanisms that regulate myelination, demyelination and remyelination is limited. Schwann cells produce myelin in the peripheral nervous system and remain associated with the axons of peripheral neurons throughout axonal migration to the target. Owing to the intimate relationship between these cell types it is difficult to fully reproduce their function *in vitro*. For this reason, we developed an approach based on the injection of an engineered virus into the sciatic nerve of mice to locally transduce peripheral nerve cells. This approach can be used as an alternative to germline transgenesis to facilitate the investigation of peripheral nerve biology *in vivo*. The detailed protocol, described here, requires 3 weeks to complete. In comparison with genetic modification strategies, this protocol is a fast, reproducible and straightforward method for introducing exogenous factors into myelinating Schwann cells and myelinated axons *in vivo* to investigate specific molecular mechanisms.

INTRODUCTION

Myelination is essential for the rapid propagation of action potentials along axons in both the central (CNS) and peripheral (PNS) nervous systems. In the PNS, the myelin sheath is formed by Schwann cells[1]. Myelinating Schwann cells (mSCs) wrap around axons so that the molecular machinery required to propagate action potentials is concentrated at regular sites, known as nodes of Ranvier.

The scarcity of data regarding axon-glia physiology in peripheral nerves underscores the complexity of this cell-cell relationship and the relative inadequacy of the current approaches used in peripheral nerve research. Recent data have highlighted the role of subcellular organelles and molecular factors in the interaction between SCs and axons[2]. This is consistent with the emerging opinion in the field that the interdependence between the axon and the myelinating glia is so deep that CNS and PNS neuropathies cannot be correctly addressed and treated without first understanding the extent and complexity of the relationship between neurons and glial cells[3].

Hereditary and acquired peripheral nerve diseases are numerous and affect an increasing number of people[4]. Although some neuronal peripheral neuropathies such as amyotrophic lateral sclerosis are relatively rare, they range in severity from discomfort to severely debilitating conditions with high mortality. For this reason, there is a growing focus on peripheral nerve therapy and repair research. A short analysis of the publications in this domain highlights an exponential increase in the last 3 years. This increased focus is based, in part, on the development of new techniques for the culture of primary SCs and neurons[5], modification of neurons and SCs *in vivo*, and stem cell strategies to replace missing or defective glial cells[6]. The PNS is a particularly attractive system for the development and testing of new biotherapies because of its relative simplicity and accessibility. It is therefore anticipated that new PNS-based cell and gene therapies are likely to emerge[7,8].

With this in mind, we developed and successfully applied a viral strategy to introduce exogenous factors into SCs and neurons of the PNS[9]. The strategy takes advantage of the receptivity of axons and SCs to viral infection. By injecting engineered viral constructs into the sciatic nerve many proteins in the SCs or axons of wild-type or mutant mice can be rapidly silenced or overexpressed *in vivo*. The impact of these modifications on myelination can be analyzed and quantified by light and electron microscopy (**Fig. 1**).

Comparison with mouse genetic approaches

The development of mouse genetic approaches has revolutionized the field of neuroscience with the creation of mouse models for human peripheral pathology[10–12]. Although these mouse models are invaluable to understanding the etiology of diseases, the time and resources invested to obtain a conditional knockout are considerable. In addition, despite the commercial availability of an increasing number of embryonic stem cells (ESCs) harboring specifically floxed genes, it commonly takes 18–24 months to generate a conditional mutant[13]. Moreover, obtaining one mutant requires the use of 10–20 other mice. In this way, the production and characterization of an allelic series in a single gene is an effort requiring several years of concerted and focused experimental biology at a current cost of no less than 1 million US dollars[14] (e.g., funded by a single 5-year grant at roughly € 150,000 per year in total costs). More recently, new technologies, such as adopting zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) to edit the mouse genome, are emerging as valuable and effective alternatives to conventional gene targeting with ESCs, enabling a quicker and cheaper creation of genetically modified models[15,16].

However, although the mouse genetic approach is useful for the analysis of specific disease-related genes, it is less appropriate for the study of fast complex mechanisms such as cell interactions or differentiation. The viral strategy described here enables the reliable and reproducible manipulation of multiple cellular factors *in vivo*[9,17–19]. The total cost of the proposed method is <€ 60 per mouse, excluding equipment setup. This estimate takes into account the fact that virus production, either in-house or from a commercial source, can cost € 400–800 and assumes an animal housing cost of ~€ 50 per mouse for 3 weeks. The protocol is straightforward and less time and resource consuming

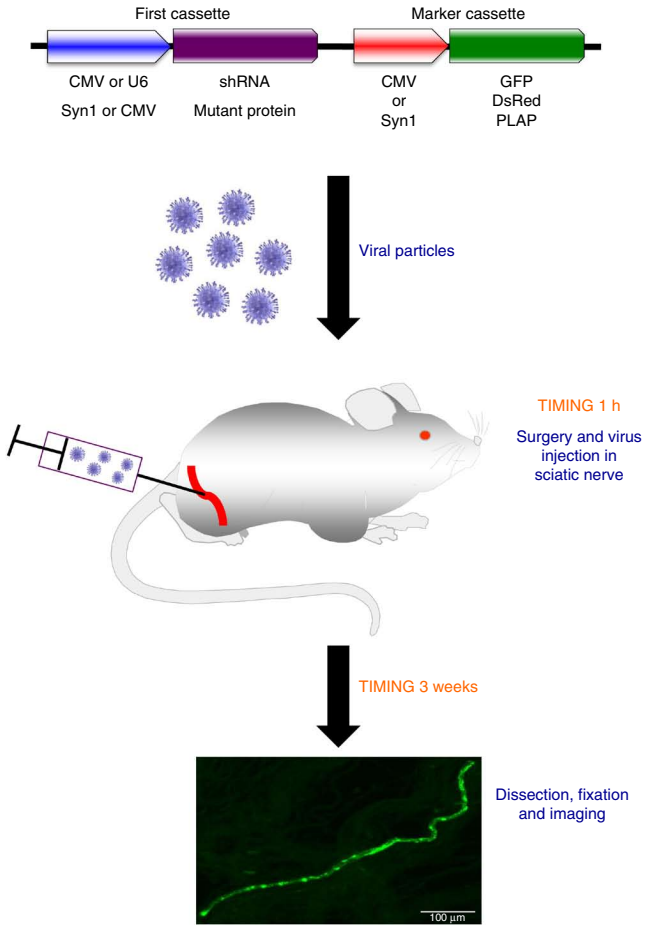

Figure 1 | Schematic overview of the viral transduction approach in the mouse peripheral nerve. Viral vectors contain two cassettes of expression: the first cassette drives the expression of shRNAs or mutant proteins under the control of CMV, synapsin 1 (syn1) or U6 promoters. The second cassette drives the expression of marker proteins such as GFP, DsRed2 or PLAP. Viral particles are produced *in vitro* and injected into the sciatic nerve of adult mice or mouse pups. Three weeks after injection, the infected nerve can be processed for analysis: dissection, fixation and imaging of sciatic nerve.

compared with genetic approaches, taking as little as 2–3 weeks to efficiently modify mSCs *in vivo*. Moreover, virus-based silencing facilitates acute downregulation of gene expression, thus preventing the adaptation and compensation that frequently occur in mice with genetic modifications.

Experimental design

Early studies demonstrated that the injection of adenoviral particles into the sciatic nerve of anesthetized mice infects axons and SCs[17,20,21] (**Fig. 1**). To understand myelination it is extremely important to target or myelinated axons or mSCs. Because adenoviruses infect both SCs and axons when the virus is directly injected into the nerve, we used or the cytomegalovirus promoter (CMV) or the synapsin 1 promoter (Syn1) to restrict expression to SCs and axons, respectively[18] (**Table 1**). In our initial studies, we were limited to viral vectors expressing dominant-negative proteins to perturb the function of endogenous proteins in adult mice[22]. We later broadened this approach, first by using viruses that express short hairpin silencing RNA, and second by developing a strategy for injecting viral particles in the sciatic nerve of pups at postnatal days 3–5, when myelination starts in mice[18]. So the function of genes of interest can be investigated both by reducing the gene expression via silencing or by perturbing the protein function with dominant negative (or active) mutant proteins. In addition, the use of this approach in pups allows one to investigate the function of genes of interest during myelin formation, whereas its use in young adult mice (4–8 weeks old) allows one to investigate myelin maintenance and, after nerve crush injury, demyelination (**Table 1**).

The viral infection itself does not affect myelination[17,18,20–22]; however, because adenoviruses are strongly immunogenic, they can only be used in immunodeficient mice[17,18,20–22]. This restriction does not apply to lentiviral vectors, which can also be used to transduce SCs in the sciatic nerve of mice[9,17]. However, these lentiviral vectors only transduce premyelinating cells in the sciatic nerve of pups at postnatal days 3–5 and not neurons or mature SCs (**Table 1**). Accordingly, this approach can be used with both integrating (for example, lentiviruses) and nonintegrating (for example, adenoviruses) viruses. However, in both cases the expression of the transgene is maintained for the long term in mSCs *in vivo* without a marked decrease, suggesting that the integration is not necessary to maintain expression in these cells[9,17,22].

To summarize, during the development of this protocol, we tested the tropism of various viral vectors after their injection into the sciatic nerves of mice at different ages (**Table 1**). We also found that by choosing a specific promoter, expression can be restricted to a specific subset of cells. It is therefore important to select the correct mouse strain, viral particle and promoter required on the basis of the factor being studied and the cell type you wish to infect (**Table 1**). It is essential to always include parallel control injections. We routinely use viral particles expressing shRNA directed against DsRed2 (5′-AGTTCCAGTACGGCTCCAA-3′) or GFP (5′-CAAGCTGACCCTGAAGTTC-3′) depending on the fluorescence reporter used. We have previously demonstrated that both shRNA controls give similar results, do not vary depending on the viral vector and do not affect myelination or demyelination processes[9,17,18]. An additional caveat is that adenoviral vectors and immunodeficient mice cannot be used to study demyelinating neuropathies or Wallerian degeneration because these biological events require an active immune system. In this context, only lentiviruses can be used to introduce exogenous factors into mSCs and myelinated axons.

For optimal results, we strongly recommend the use of high-titer viral particles (between 10^{10} and 10^{11} colony-forming units (c.f.u.)/ml). It is possible to produce high-titer adenovirus or lentivirus in-house by using the methods proposed by He *et al.*[19] or by Cotter *et al.*[9], respectively. Briefly, for adenovirus production, pAdtrack or pAdtrackCMV vectors containing the constructs are recombined with the pAdeasy1 vector in the Adeasy1 BJ5183 bacterial strain (Stratagene). The isolated adenoviral DNA is cut with PacI and transfected in HEK 293 cells. The first production of adenovirus is followed by three rounds of amplification. Finally, freeze-thaw cycles are used to collect the viral particles from the cells, which are then purified by using cesium chloride gradients. For lentivirus production, HEK293T cells are transfected with psPAX2, pMD2G and the pSICOR lentiviral vector. The supernatant

TABLE 1 | Comparison of viral vectors, mouse strains, age of infection, promoters, infected cells and applications.

Viral particle	Mouse strain	Age	Promoter	Target cell type	Application
Lentivirus	Swiss or any strain	P3–P5	U6 or CMV[a]	Myelinating SCs (70%)[17,18] Nonmyelinating SCs (30%)	Myelin formation Demyelination
Adenovirus	Immunodeficient strain such as CB17/SCID, or *Rag1*- or *Rag2*-negative strains	P3–P5	U6 or CMV	Myelinating SCs (30%)[b] Nonmyelinating SCs (70%)	Myelin formation
		Adult	U6 or CMV	Myelinating SCs (30%)[b] Nonmyelinating SCs (70%)	Myelin maintenance
		P3–P5 or adult	Synapsin 1[c]	Neurons (100%)	Axonal studies

[a]U6 is a polIII promoter commonly used to express shRNA. CMV promoter is a polII promoter commonly used to express proteins. However, the CMV promoter can be used in specific conditions to express a shRNA[18]. [b]Injection of an adenovirus with the CMV or U6 promoter in the sciatic nerve commonly results in both myelinating and nonmyelinating SCs becoming infected. These cells can easily be distinguished morphologically (**Fig. 3a,b**). [c]The synapsin 1 promoter is specifically expressed in neurons[17].

is collected at 48 and 72 h, pooled, filtered and centrifuged. The pellet is then resuspended in PBS and divided into aliquots. In general, production takes around 1 week for lentivirus and 4 weeks for adenovirus, and all steps are restricted to biosafety level 2 tissue culture facilities. There are also commercial companies that offer high-titer virus production services.

MATERIALS

REAGENTS

- Newborn Swiss strain mouse pups (P3–P5) or adult mice (aged 6 weeks–3 months) from immunodeficient strains such as CB17/SCID or *Rag1*- or *Rag2*-negative strains (Janvier Labs) **! CAUTION** All experiments using mice must be performed in accordance with all relevant institutional and government ethics and animal handling requirements.
- Bactrim, sulfamethoxazole/trimethoprim (Roche, cat. no. 10130293 FR-S FY 1106.1071)
- Fast Green FCF (Sigma, cat. no. F-7252)
- Viral particles (high titer: lentiviral particles $\geq 10^{10}$ c.f.u./ml and adenoviral particles $\geq 10^{10}$ c.f.u./ml) **! CAUTION** Viral particles must be handled carefully ▲ CRITICIAL Avoid freeze-thaw cycles to guarantee the quality of viral particles.
- PBS, pH 7.4, 1× (Gibco Life Technologies, cat. no. 10010-015)
- Isoflurane, 1,000 mg/ml (Virbac Santé Animale, cat. no. 83532901)
- Medical oxygen bottle (Linde Gas, cat. no. 210120) with an oxygen regulator (C300/1, Linde Gas, cat. no. 333333336519)
- Nitrogen gas bottle (Linde Gas, cat. no. 4.5B20)
- Absolute ethanol (VWR, cat. no. 20821.296)
- Betadine solution, Povidone iodine, 10 grams per liter (Vetoquinol, cat. no. 3042413)
- Leibovitz's L15 medium, 1× (Gibco Life technologies, cat. no. 21083-027) **! CAUTION** Store the medium at 4 °C for up to 6 months.
- Eye protection gel (Ocry-gel, TVM, cat. no. 48026T613/3)
- Buprenorphine, 0.3 mg/ml (Axience, cat. no. GTIN03760057151244)
- Disinfectant Relyton Virkon tablets (Antec International, cat. no. 60007)
- Histoacryl tissue glue (B/Braun Aesculap, cat. no. 1050060)

EQUIPMENT

- Tube, 15 ml (Falcon, cat. no. 352096)
- Isoflurane-processing anesthesia system (Datex Ohmeda Isotec 5) with mouse nose cone
- Microinjector (Pneumatic Picopump PV820, World Precision Instruments or Picospritzer III, Parker-Hannifin)
- Function pulse generator, 3 MHz (Langlois, cat. no. GFG8215)
- Dissecting kit comprising scissors (cat. no. 1500-03, 15020-15 and 14094-11), forceps (cat. no. 11294-00 and 11000-18), spatula for adult surgery (cat. no. 10094-13), spatula for pup surgery (cat. no. 10089-11), retractors (cat. no. 17021-13), wound clip system (cat. nos. 12031-09, 12032-09 and 12033-00) (Fine Science Tools)
- Surgery table (S&T, cat. no. PHP-101)
- Animal cage changing hood (Techniplast, CS5 Evo Plus)
- Disposable scalpels (Swann-Morton, cat. no. 0505)
- Instrument sterilizer (Fine Science Tools, cat. no. 18000-50)
- Instrument beaker with silicone bottom (Fine Science Tools, cat. no. 18000-30)
- Cotton buds (Hartmann, cat. no. 967 936/9)
- Borosilicate glass capillaries, 1.0 mm outer diameter × 0.58 mm inner diameter (Harvard Apparatus, cat. no. 30-0016)
- Clipper, Oster Turbo 111
- Micromanipulator, IM-3C (Narishige Japan Group)
- Glass pipette holder (Hamilton, cat. no. 55752-01)
- RN compression fitting, 1 mm (Hamilton, cat. no. p/n55750-01)
- Insulin syringe, 0.5 ml (Terumo, cat. no. BS05M2913)
- Stereomicroscope Zeiss Stemi 2000 (Carl Zeiss Microscopy)
- Cold light source Zeiss KL200
- Flaming/Brown micropipette puller, model P97 (Sutter Instrument)
- Thermal blanket, 230 V–50 W (Solis Model 215)
- Micropipette, P10 µl and P200 µl (Gilson, cat. nos. F144802 and F123601)
- Eppendorf microloader (Eppendorf, cat. no. 5242956003)

REAGENT SETUP

Bactrim solution Add 3 ml of Bactrim (200 mg sulfamethoxazole/40 mg trimethoprim stock) to 250 ml of drinking water. ▲ **CRITICAL** This solution must be freshly prepared per cage and changed every week.

Fast Green FCF solution, 0.1% (wt/vol) Dissolve 0.01 g of Fast Green FCF in 100 ml of PBS (pH 7.4). ▲ **CRITICAL** Store the solution at 4 °C for up to 6 months.

Ethanol, 70% (vol/vol) Add 15 ml of absolute ethanol to 35 ml of sterile deionized water. Store it at room temperature (20–25 °C) for up to 1 year.

Buprenorphine solution Dilute buprenorphine in sterile PBS (pH 7.4) to a final concentration of 100 µg/ml. ▲ **CRITICAL** Keep the solution away from direct light and store it at 4 °C for up to 1 month.

Disinfectant solution Add one tablet of disinfectant Relyton Virkon to 500 ml of sterile deionized water. ▲ **CRITICAL** Store it at room temperature for up to 1 week.

Viral particle aliquots Make 8-µl aliquots of the viral particles and add 2 µl of 0.1% (wt/vol) Fast Green solution (final concentration 0.01% (wt/vol)) to them. After mixing, the solution will appear blue. ▲ **CRITICAL** Store the aliquots at −80 °C for up to 12 months.

EQUIPMENT SETUP

Pulled glass capillaries Pull the glass capillaries into fine glass needles by using the Flaming/Brown micropipette puller. For pup injections, pull capillaries with the following parameters: heat 580 °C, pull 100 p.s.i., velocity 50 and time 250 ms. For adult injections, the parameters are: heat 550 °C, pull 100 p.s.i., velocity 50 and time 250 ms. ▲ **CRITICAL** As these parameters are highly dependent on the pipette puller, we recommend that the appropriate parameters be determined by successive trials. Take note that the final diameter of the orifice is not important; rather, the glass needle should be long enough to enable freehand trimming to different lengths to achieve the correct diameter and dimensions for each injection.

PROCEDURE

Preparing mice for surgery ● TIMING 1 d

1| The day before surgery, add Bactrim antibiotic to the drinking water of the mice being operated on. If using pups, Bactrim antibiotic should be added to the drinking water of the lactating mothers. Bactrim is a broad-spectrum mix of antibiotics that will be passed to the pups via lactation.

Surgery ● TIMING ~30 min

2| Set up all equipment for surgery in the animal cage changing hood. The use of hood of this style ensures adequate personal protection during viral injections while allowing optimal accessibility to equipment.

3| Switch on the thermal blanket and place it under the surgical table. The homogeneous diffusion of the heat through the table will maintain optimal body temperature during surgery and injection.

! CAUTION Electrical heating blankets can be very hot on localized areas and may cause burns. Don't put the animal directly on the blanket. Take particular care with pups as they are more sensitive to the heating blanket. We recommend using extra layers of absorbent paper or similar absorbent material between the pups and the heating blanket.

4| Open the oxygen bottle valve and set the air compressor to a pressure of 100 bar.

5| For surgery on mouse pups, perform the steps in option A. For surgery on adult mice, perform the steps in option B.

? TROUBLESHOOTING

(A) Pup surgery

(i) Adjust the oxygen flow to 0.6% liters/min and the isoflurane distribution to 5% (vol/vol), and turn on the mask anesthesia system.

(ii) Gently place the pup's head into the nose cone and maintain this position until the animal is immobile (**Fig. 2**).

▲ CRITICAL STEP To adjust the nose cone to optimally fit the pup's head and to reduce the loss of isoflurane, cut a small piece of latex glove and affix it to the nose cone with a rubber band. Cut a small hole with scissors, large enough to comfortably fit around the pup's head without any gaps. It is common for the pup to become immobile before full anesthesia is achieved. It is important to maintain 5% (vol/vol) isoflurane inhalation for at least 5 min as mouse pups are more resistant to isoflurane anesthesia compared with adult mice.

(iii) Check the pup's paw and/or tail reflexes by gently pinching to ensure complete anesthesia.

(iv) Clean the region of incision with Betadine solution by using a sterile cotton bud.

(v) Make the incision with small scissors.

! CAUTION Sterilize all instruments before use.

(vi) Pull apart the skin with small retractors to expose the cavity traversed by the sciatic nerve (**Fig. 2b**).

(vii) By using small scissors, gently clear the connective tissue between the gluteus superficialis and biceps femoris muscles, the intersection of which is commonly found just behind the top edge of a small deposit of fat. This will open a small cavity beneath which lies the sciatic nerve. The appearance of the sciatic nerve will differ depending on age. At P2, the nerve will appear thin and translucent and is difficult to inject accurately. At P3, the nerve is thicker, opaque and easier to distinguish. At P4, the nerve is thicker again and the trunks of the sciatic nerve can be clearly identified. Use forceps to gently clear any connective tissue surrounding the nerve, but avoid rupturing any blood vessels. With a thin spatula, gently lift out the sciatic nerve.

▲ CRITICAL STEP To avoid nerve lesions, don't force the sciatic nerve. If the nerve is accidentally broken, then the animal must be killed.

(viii) Keep the cavity and surrounding tissue moist with L15 medium by using a P200 micropipette.

(ix) Reduce isoflurane to 3–2% (vol/vol) to maintain anesthesia.

! CAUTION Anesthesia of mouse pups is tricky at this early age. Although pups are more resistant to isoflurane anesthesia, an overdose is lethal. If the pup stirs during the surgery do not hesitate to increase the anesthesia to 5% (vol/vol) isoflurane again for a few minutes, but reduce it to 3–2% (vol/vol) once strong anesthesia is re-achieved. The most common cause of pup mortality during injection is an overdose of isoflurane. To avoid this, do not maintain 5% (vol/vol) isoflurane for too long and be sure to visually monitor the pup's respiration rate. Decrease the anesthesia to 3–2% (vol/vol) if the diaphragm movements become intermittent.

(B) Adult mouse surgery

(i) Weigh the mouse before starting anesthesia and note the weight (this is necessary to dose the analgesic in the coming days). Adjust the oxygen flow to the anesthesia induction box to 1.5% liters/min and turn on the isoflurane system to 5% (vol/vol). Put the mouse into the box for 2–3 min.

(ii) Switch off the isoflurane flow to the box, adjust the oxygen flow to 1% liters/min and turn on the mask anesthesia system.

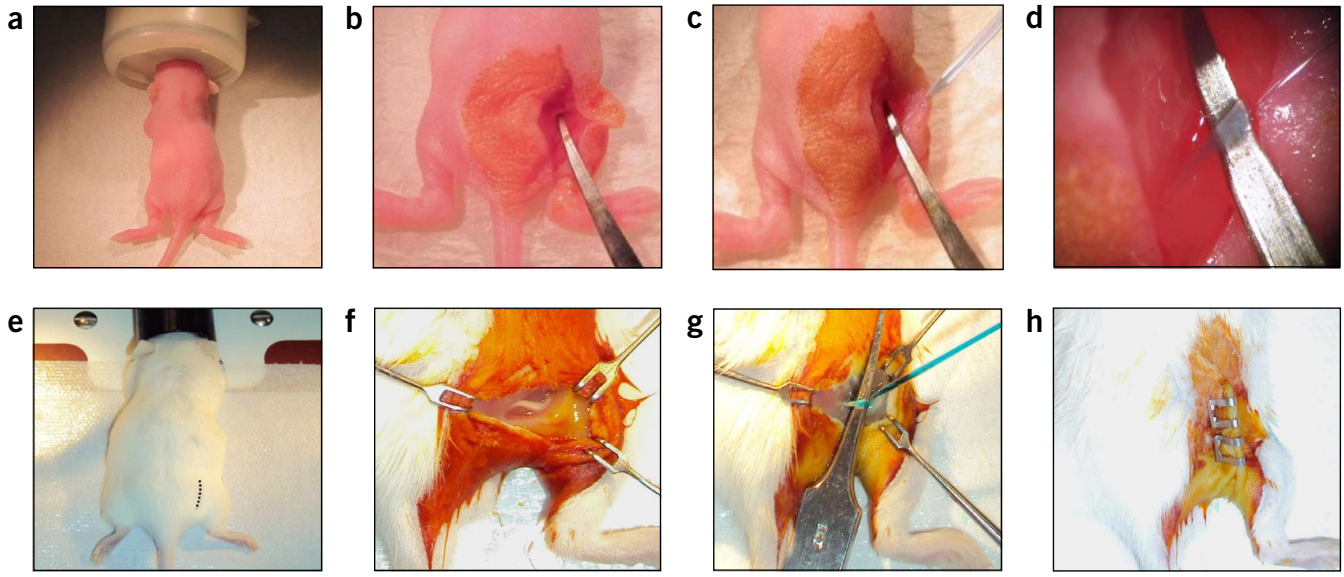

Figure 2 | Key events of the sciatic nerve injection in mouse pups and adult mice. (**a**–**h**) Key events in pups are shown in panels **a**–**d** and in adults in panels **e**–**h**. (**a**) The nose of the pup is placed in the mask cone before surgery. (**b**) A small vertical incision is made in the thigh of the mouse pup with scissors. (**c**) The sciatic nerve is lifted out by using a fine spatula and the tip of the glass needle is carefully introduced into the nerve at a <45° acute angle to the nerve surface via the micromanipulator. (**d**) After injection of the colored viral solution, the sciatic nerve appears blue. (**e**) The nose of the adult mouse is placed in the mask for anesthesia. A dotted line illustrates the orientation of the incision. (**f**) The right thigh is shaved and prepared for surgery. The skin is incised along the dotted line shown in **e**. The skin is retracted to expose the nerve area. (**g**) The colored viral solution (in blue) fills the nerve. (**h**) Staples close the wound after surgery. All experiments using mice were performed under institutional guidelines (approval number CEEA-LR-11032) and according to all relevant regulations.

(iii) Place the mouse's nose into the mask cone and check its tail and paw reflexes by pinching to ensure that complete anesthesia has been reached (**Fig. 2e**).

(iv) Apply a drop of eye protector gel on each eye.

(v) For presurgical analgesia, administer 0.5 ml of buprenorphine/PBS solution (100 µg/kg) i.p. via an insulin syringe.

(vi) Prepare the incision region for surgery: shave the incision area using the clippers. Apply Betadine to the shaved area to clear loose hair. Next, clean the area with 70% (vol/vol) ethanol and finally reapply Betadine to the specific area of incision with sterile cotton buds.

▲ **CRITICAL STEP** CB17/SCID mice are immunodeficient, therefore sterile conditions are recommended to avoid infection.

(vii) Reduce isoflurane to 2–1.5% (vol/vol) to maintain anesthesia and adjust flow oxygen to 0.2% liters/min.

! **CAUTION** If the animal stirs during the surgery, do not hesitate to increase the anesthesia to 5% (vol/vol) isoflurane again for a few minutes, but reduce it to 2–1.5% (vol/vol) once strong anesthesia has been re-achieved.

(viii) Cut the skin with a scalpel along the line indicated in **Figure 2e**.

▲ **CRITICAL STEP** The scalpel has to be handled firmly and applied to the skin with some force for a clean cut.

(ix) Expand the incision with retractors to expose the gluteus superficialis and biceps femoris muscles. A small fat deposit commonly indicates the junction of the two muscles. Use a scalpel or small scissors to cut the connective tissue that connects the muscles, revealing a small cavity traversed by the sciatic nerve. In adult mice, the sciatic nerve appears thick and white (with a wavy pattern typical of myelin) and rests on muscles at the bottom of the cavity. Carefully enlarge the cavity by cutting the connective tissue between the muscles. Use a closed scissor along the edges of the sciatic nerve (top and bottom) to free it from the surrounding tissue.

! **CAUTION** Do not enlarge the cavity too much, as large blood vessels transverse the muscle junction and you may cut them.

(x) Use the spatula for adult mouse surgery to gently lift the sciatic nerve out (**Fig. 2f**). Make sure that you do not lift up the fine muscle that lies beneath the nerve.

▲ **CRITICAL STEP** Be extremely gentle when lifting the nerve out or you may break or crush it.

(xi) Keep the nerve and cavity and the surrounding tissue moist with L15 medium by using a P200 micropipette.

Injection ● TIMING ~25–45 min

6| Use microloader tips and a P10 micropipette to load pulled glass needles with the colored viral particle solution. Use 3 µl for pup and 8 µl for adult injections. To avoid bubbles it is crucial to insert the loaded microloader tip to the base of the glass needle (where it begins to narrow) before slowly releasing the viral solution into the glass needle while carefully withdrawing the microloader tip. Tap the glass needle gently to remove any air bubbles. You may use one glass needle loaded

with 6–7 µl of viral particles to inject two pups in a row. In this case, note the halfway point of the viral solution on the glass needle with a marker.
! CAUTION Avoid freeze and thaw cycles of viral aliquots.

7| Place the loaded glass needle into the Hamilton glass pipette holder and adjust it by using the RN compression fitting.

8| Adjust the stereomicroscope to focus on the tip of the glass needle. Cut the flexible part of the needle tip in order to get a fine tip hole.
! CAUTION If you break the glass needle tip or cut it too much, remove the viral solution by using a fresh microloader tip and a P10 micropipette. Discard the defective glass needle and load another one.
▲ CRITICAL STEP Cut just the flexible part of the tip to control the injection volume. If too much is cut, the orifice diameter will be too large, resulting in a high injection volume and causing most of the viral solution to leak out and not enter the nerve.
? TROUBLESHOOTING

9| Use the microinjector to start the flow of the viral solution and check for a good flow from the tip (a fine drop should form at the tip of the needle).
? TROUBLESHOOTING

10| Adjust the stereomicroscope to focus on the sciatic nerve at the highest possible magnification.
▲ CRITICAL STEP A good view of the injection site at high magnification is essential to control the injection and to detect any leak out of the nerve.
? TROUBLESHOOTING

11| By using the micromanipulator, carefully introduce the tip of the glass needle into the nerve at <45° acute angle to the nerve surface. Puncture the perineurium and penetrate the nerve. Withdraw the glass needle slightly (but not completely) and advance again, to open an injection place. Repeat if necessary.
▲ CRITICAL STEP It is better to avoid multiple injection sites.
? TROUBLESHOOTING

12| Start the injection of the viral particles. Virus should be injected between 200 and 300 nl/min over a 20–40-min period and for up to 10 p.s.i. of pressure to avoid the generation of high pressure in the nerve and subsequent nerve damage. We use the function pulse generator set to frequency 2.1 with an output of 50 Ω, as well as the pneumatic Picopump with amplitude of 100 ms and 10 p.s.i. of pressure. The regulator dial of the Picopump can be used to control the pressure with which the viral solution is expelled.
▲ CRITICAL STEP Repetitive pumping of nanoliters of viral solution through the same injection site is crucial in order to obtain a fully loaded sciatic nerve. The loading of the nerve by using pulsed microinjection takes time, but this process allows a good spread of the viral particles along the sciatic nerve, which is crucial for optimal transduction efficiency.
? TROUBLESHOOTING

13| Adjust the stereomicroscope to visualize the glass needle inside the nerve to control the rate and volume injected. Ideally, you should be able to visualize the solution entering and filling the nerve, and the sciatic nerve should appear blue during and after injection (**Fig. 2c**,**d**,**g**). You should ideally see flow of blue liquid into nerve upstream and downstream of injection site.
? TROUBLESHOOTING

14| Although it is harmless, do not allow any nitrogen gas to enter the nerve and stop pumping just before the glass needle is completely empty.

Closing ● TIMING ~10 min
15| Clean the injection area with L15 medium and remove excess liquid with a sterile cotton bud.

16| Replace the sciatic nerve at the bottom of the cavity and replace the muscles on top of the nerve. The muscles should sit easily back in place, covering the nerve. As they were not cut, they do not need sutures.

17| For closure surgery on mouse pups, perform option A. For closure surgery on adult mice, perform option B.

(A) Pup closure surgery

(i) Use forceps to lightly re-align the skin on either side of the incision. Apply a small amount of histoacryl glue along the incision to seal the wound.
▲ **CRITICAL STEP** The histoacryl glue often attracts the interest of the mother. Occasionally, the mothers will try to 'clean' the glue away from the wound, which in rare instances can result in pup mortality. To minimize this risk, always use fresh glue and limit the application to a minimum.

(ii) Reduce isoflurane flow to 0% and allow the pups to inhale 100% oxygen for 1–2 min to facilitate good revival.

(iii) Allow the pup to fully recover before placing it back with the mother (allow 5–10 min in warm conditions to allow the pup to become fully awake and to recover mobility). It is possible to prep the next pup to be injected during this time.
▲ **CRITICAL STEP** Keep enough littermates (injected or not) to occupy the mother. We inject as many as eight pups of the same litter and keep them together. In addition, some mouse strains display poorer maternal care than others, which can affect the survival of the weakened pups. In this situation, we recommend the use of a lactating foster mother from the Swiss strain.

(B) Adult mouse closure surgery

(i) Use blunt forceps to realign the skin of the incision together and staple with two clips along the wound (**Fig. 2h**).
▲ **CRITICAL STEP** Before closing the incision, remove all air from the point of closure.

(ii) Reduce isoflurane flow to 0% and allow the adult mice to inhale 100% oxygen for 2 min to facilitate good revival.
! **CAUTION** Keep the adult mouse warm until fully awake and then house it in a new cage.

18| Switch off the anesthesia system. Sterilize dissecting tools with the disinfectant solution Relyton Virkon.

Maintenance ● TIMING at least 3 weeks

19| For adult mice, administer i.p. injections of buprenorphine/PBS solution (100 μg/kg) every 12 h for 2 d. Owing to their small weight, pups cannot be treated safely with i.p. injections of buprenorphine. Maintain Bactrim antibiotic in the drinking water for 3 weeks for both pups and adults. At 7–10 d after surgery, remove clips from the injected adult mice.
? TROUBLESHOOTING

20| At 3 weeks after injection, if desired, collect the nerve for analysis and immunohistochemistry by using standard techniques. An example of the analysis we have performed can be found in Tricaud *et al.*[22]. Before this time point, the long mSCs may not be fully filled with the fluorescent marker protein. However, demyelination phenotypes may appear and be detectable as early as 2 weeks after injection. We estimate that the transgene expression (shRNA or reporter protein) can be detected a few days after the injection but requires about 1 week to be fully efficient.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Poorly anesthetized mouse	Loss of isoflurane in the anesthesia system	Mask hole diameter is too big. Reduce the isoflurane leak from the mask by attaching a section of latex glove to the nose cone mask and making a small hole with scissors to comfortably fit the mouse head
		Loss of oxygen in the anesthesia system	Recheck the oxygen flow
	Death of mouse during surgery	Fatal loss of body temperature	Use a thermal blanket to control the mouse's temperature during surgery and injection
		Isoflurane overdose	Carefully control the isoflurane flow down to 2% (vol/vol) for pups and 1% (vol/vol) for adults during injections

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
8–13	No visible flow of virus particles from the glass needle	Glass needle diameter hole is too small	Completely withdraw the glass needle from the sciatic nerve and cut the tip with small scissors
		The glass needle is clogged	Remove the viral particles from the glass needle. Resuspend a new aliquot, taking care to avoid precipitate, and load a new glass needle
		Nerve tissue obstructs viral particles' flow	Completely withdraw the glass needle and reinject at a different site
11	The glass needle bends and doesn't puncture the perineurium	The glass needle tip is too flexible	Withdraw the glass needle and cut the tip with small scissors to attain a firmer tip
19, 20	Death after injection	Immunogenic reaction in the mouse Possible post-surgery infection	Adenovirus is strongly immunogenic, so use immunodeficient mice if you inject adenoviral particles Carefully sterilize the surgery kit and administer antibiotic for 3 weeks after injection
	Low number of infected cells (**Fig. 3f**)	Titer of viral particles is too low	Re-produce virus to obtain a high titer of particles
		Not enough viral particles are injected	During and after injection, monitor the color of sciatic nerve If an adequate amount of viral solution is injection the nerve should appear blue
		Adenoviral infection in an immunocompetent strain	Use a lentiviral vector or an immunodeficient strain
	Low number of fluorescent cells	The marker protein is weakly expressed or not stable Fast and robust demyelination induced by the transgene remove labeled cells	Change the marker protein or kill animals earlier to catch healthy fluorescent cells
	Infected cells not fully filled with the marker	Animals were killed too early before the marker completely fills the cell	Wait longer before euthanasia and analysis

● TIMING

Step 1, preparation for surgery: 1 d
Steps 2–4, anesthesia: 5 min
Step 5A and 5B, surgery: 20–25 min
Steps 6–8, needle loading and setup: 5 min
Steps 9–14, injection of viral particles: 20–40 min
Steps 15–17, closure surgery: 10 min
Step 18, recovery: 5 min
Steps 19 and 20, antibiotic administration and maintenance: at least 3 weeks

ANTICIPATED RESULTS

By using viral particles with a corresponding promoter, this protocol enables rapid and straightforward silencing or overexpression of a target gene in SCs of wild-type or mutant mice. Ultimately, it allows analysis of the effect of the selected gene modification on myelination and myelin maintenance, the main processes affected in peripheral nerve diseases. Our previous publications using this protocol demonstrate that protein expression can be efficiently silenced in mSC by using engineered lentivirus[9,17] or adenovirus[18] expressing shRNAs under U6 or CMV promoters, together with GFP or DsRed fluorescent markers, to detect infected cells (**Fig. 3**). We also demonstrate that lentiviral particles, pseudotyped with

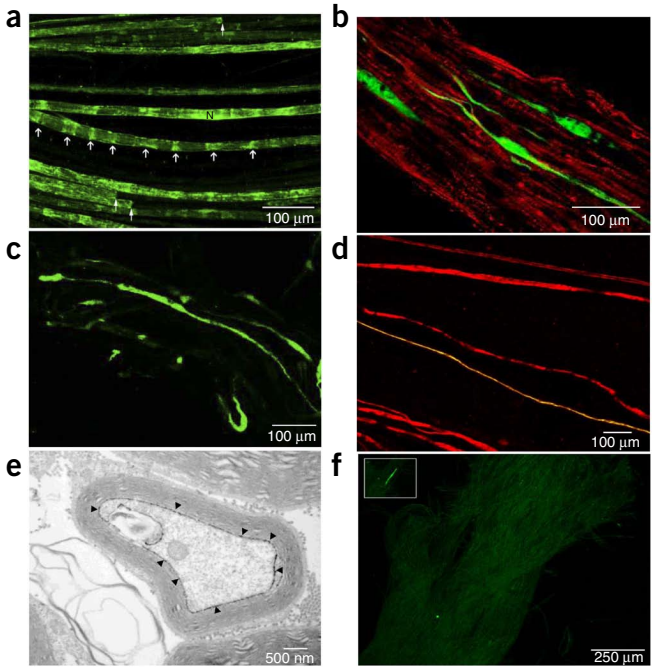

Figure 3 | Examples of different types of infected cells after adenoviral or lentiviral injection into the mouse sciatic nerve. (**a**) Myelinating SCs. These cells are infected with an adenovirus expressing GFP after a viral particle injection into the sciatic nerve of a 6-week-old immunodeficient CB17/SCID mouse. The nerve was collected 3 weeks after infection. Accumulation of GFP in the nucleus in the middle of the infected cell (N), accumulation of GFP at regular intervals indicating cytoplasmic accumulation in the Schmidt-Lanterman incisures (thick arrows), and clear indications of the node of Ranvier at the interface between infected and noninfected cells (thin arrows). (**b**) Nonmyelinating SCs. Six-week-old immunodeficient CB17/SCID mouse sciatic nerve infected with adenovirus expressing GFP. Nerve collected 3 weeks after infection. The nonmyelinating SC, infected with adenovirus (green), shows no colocalization with E-cadherin (red), a marker of mSCs. (**c**) Demyelinating SC. A P4 Swiss mouse pup was injected with a lentivirus expressing GFP. One month later, the injected nerve was crushed to induce demyelination. At 4 d after crushing, the nerve was collected. (**d**) Peripheral nerve axon. The neuron was infected with an adenovirus expressing GFP (green) under the promoter Synapsin 1 three weeks after a viral particle injection in the sciatic nerve of a 6-week-old immunodeficient mouse CB17/SCID. The nerve was collected 3 weeks after viral infection. After fixation and teasing, the fibers were immunostained for neurofilaments (red). Colocalization is shown in yellow. (**e**) An electron microscopy image showing the myelin sheath of a mSC from a 6-week-old immunodeficient CB17/SCID mouse sciatic nerve infected with an adenovirus expressing PLAP. The nerve was collected 3 weeks after infection. PLAP enzyme produces very fine black precipitates (arrowheads). (**f**) Inefficient infection of mSC from a 6-week-old immunodeficient CB17/SCID mouse sciatic nerve injected with a low-titer adenovirus expressing GFP. The nerve was collected 3 weeks after viral infection. The insert shows an infected cell from an adjacent region for comparison. All experiments using mice were performed under institutional guidelines (approval number CEEA-LR-11032) and according to all relevant regulations.

the human vesicular stomatitis virus G protein (VSV-G), infect mSC only at early stages[17]. The combined use of these vectors allows maximum flexibility in experimental design (**Table 1**). Unlike adenoviruses, lentiviruses are not immunogenic and can therefore be used in immunocompetent mice. This allows the use of genetically modified mice to set up a background with a specific gene deletion or with a gene-encoded fluorescent probe upon which the virally induced modification can be added. The use of adenovirus, although requiring the use of an immunodeficient mouse strain, allows flexibility in the timing of experimental investigation, as mSC in both pups and adults can be efficiently infected.

In addition to its use in studying myelination and myelin maintenance, this local viral transgene introduction approach can be used to study other biological processes in peripheral nerves. We have successfully applied this protocol to evaluate the effect of protein silencing on the demyelination process. To this end, we injected the sciatic nerve of pups with lentiviral particles expressing shRNA of proteins implicated in this process, and we have evaluated the impact of this modification after the induction of demyelination by nerve crush (**Fig. 3c**). Moreover, as the synapsin 1 promoter drives the expression in myelinated axons (**Fig. 3d**) but not in glial cells, this local viral transgene introduction approach can also be used to specifically modify axons in peripheral nerves. Finally, to optimize the analysis of molecular changes of myelin, we designed viral vectors expressing placental alkaline phosphatase (PLAP), an enzyme whose activity is preserved after strong fixation[9]. This enables detection of infected cells with the aid of an electronic microscope without perturbing the infected cell's ultrastructure (**Fig. 3e**).

ACKNOWLEDGMENTS We acknowledge Montpellier RIO Imaging Platform and the Animal Facility of the INM for help and technical assistance. This work was supported by the European Research Council (grant no. FP7-IDEAS-ERC 311610) and an INSERM-AVENIR grant. All animal experiments were conducted in accordance with institutional (approval no. CEEA-LR-11032) and French governmental regulations.

AUTHOR CONTRIBUTIONS S.G., R.F. and N.T. wrote the paper. C.P.-T., S.G., R.F. and N.T. performed experiments and analyzed data. N.T. developed the protocol and supervised the project.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

1. Sherman, D.L. & Brophy, P.J. Mechanisms of axon ensheathment and myelin growth. *Nat. Rev. Neurosci.* **6**, 683–690 (2005).
2. Viader, A. *et al.* Schwann cell mitochondrial metabolism supports long-term axonal survival and peripheral nerve function. *J. Neurosci.* **31**, 10128–10140 (2011).
3. Nave, K.A. Myelination and support of axonal integrity by glia. *Nature* **468**, 244–252 (2010).
4. Hughes, R.A. Peripheral neuropathy. *BMJ* **324**, 466–469 (2002).
5. Kaewkhaw, R. *et al.* Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue. *Nat. Protoc.* **7**, 1996–2004 (2012).

6. Walsh, S. & Midha, R. Practical considerations concerning the use of stem cells for peripheral nerve repair. *Neurosurg. Focus* **26**, E2 (2009).
7. Zacchigna, S. & Giacca, M. Chapter 20: gene therapy perspectives for nerve repair. *Int. Rev. Neurobiol.* **87**, 381–392 (2009).
8. Lehmann, H.C. & Höke, A. Schwann cells as a therapeutic target for peripheral neuropathies. *CNS Neurol. Disord. Drug Targets* **9**, 801–806 (2010).
9. Cotter, L. *et al.* Dlg1-PTEN interaction regulates myelin thickness to prevent damaging peripheral nerve overmyelination. *Science* **328**, 1415–1418 (2010).
10. Suter, U. & Scherer, S.S. Disease mechanisms in inherited neuropathies. *Nat. Rev. Neurosci.* **4**, 714–726 (2003).
11. Höke, A. Animal models of peripheral neuropathies. *Neurotherapeutics* **9**, 262–269 (2012).
12. McGoldrick, P. *et al.* Rodent models of amyotrophic lateral sclerosis. *Biochim. Biophys. Acta* **1832**, 1421–1436 (2013).
13. Davey, R.A. & MacLean, H.E. Current and future approaches using genetically modified mice in endocrine research. *Am. J. Physiol.* **291**, E429–E438 (2006).
14. Williams, R.W. *et al.* The math of making mutant mice. *Genes Brain Behav.* **2**, 191–200 (2003).
15. Joung, J.K. & Sander, D.J. TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol.* **14**, 49–55 (2013).
16. Sung, Y.H. *et al.* Mouse genetics: catalogue and scissors. *BMB Rep.* **45**, 686–692 (2012).
17. Ozcelik, M. *et al.* Pals1 is a major regulator of the epithelial-like polarization and the extension of the myelin sheath in peripheral nerves. *J. Neurosci.* **30**, 4120–4131 (2010).
18. Perrin-Tricaud, C., Rutishauser, U. & Tricaud, N. P120 catenin is required for thickening of Schwann cell myelin. *Mol. Cell Neurosci.* **35**, 120–129 (2007).
19. He, T.C. *et al.* A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95**, 2509–2514 (1998).
20. Glatzel, M. *et al.* Adenoviral and adeno-associated viral transfer of genes to the peripheral nervous system. *Proc. Natl. Acad. Sci. USA* **97**, 442–447 (2000).
21. Guenard, V. *et al.* Effective gene transfer of *lacZ* and *P0* into Schwann cells of *P0*-deficient mice. *Glia* **25**, 165–178 (1999).
22. Tricaud, N. *et al.* Adherens junctions in myelinating Schwann cells stabilize Schmidt-Lanterman incisures via recruitment of p120 catenin to E-cadherin. *J. Neurosci.* **25**, 3259–3269 (2005).