A New Model for Facial Nerve Research

The Novel Transgenic Thy1-GFP Rat

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Objective: To introduce a Thy1-GFP transgenic rat model, whose axons constitutively express green fluorescent protein (GFP), in order to study facial nerve regeneration. Facial nerve injury can cause devastating physical and social sequelae. The functional recovery of the facial nerve can result in synkinesis and permanent axonal misrouting. Facial nerve research has been hindered by the lack of available animal models and reliable outcome measures.

Methods: Transgenic Thy1-GFP rats underwent a proximal facial nerve crush injury and were imaged at 0, 1, 2, 4, and 8 weeks after injury. Nerve regeneration was assessed via confocal imaging and fluorescence microscopy.

Results: Uninjured animals reliably demonstrated facial nerve fluorescence and had predictable anatomical landmarks. Fluorescence microscopy demonstrated the loss and reappearance of fluorescence with regeneration of axons following injury. This was confirmed with the visualization of denervation and reinervation of zygomaticus muscle motor end plates using confocal microscopy.

Conclusions: The Thy1-GFP rat is a novel transgenic tool that enables direct visualization of facial nerve regeneration after injury. The utility of this model extends to a variety of clinical facial nerve injury paradigms.


The Face is the Epicenter of Human Emotion and Expression. The inability to move the muscles of facial expression or to endure the contorted movements of synkinesis after facial nerve injury is devastating. In addition, patients with facial nerve injury may have oral incompetence or corneal exposure leading to ocular damage. The improvement of facial nerve function after injury is of the utmost importance to both patients and the physicians who treat them.

Many strategies have been used to improve the recovery of facial function after facial nerve damage. These include the use of nerve guidance conduits at the site of injury, neurotrophic factors, nerve grafts and transfers, electrical stimulation, and stem cells. Despite the numerous strategies to improve nerve regeneration, interventions often result in suboptimal functional outcomes and aberrant reinnervation patterns.

One of the main barriers to understanding aberrant regeneration and in improving functional recovery after injury is the complex anatomy of the facial nerve. The facial nerve has a tortuous path from the brainstem through the temporal bone and then subsequently exits the stylomastoid foramen into the periphery, where it receives and extends multiple communicating branches to other peripheral and cranial nerves. The nerve courses anteriorly to bisect the parotid gland into a superficial and deep lobe, and then terminates in a delicate network of innervating muscular branches distally.

The complex anatomy of the facial nerve has been studied and surgically manipulated in several animal models to afford translation of findings to the clinical setting. One difficulty with this research has been the caliber of the nerve and its branches in small rodent models. In addition, the regeneration of the nerve after injury is difficult to quantify and has been previously done by analyzing nerve cross-section histomorphometry, in addition to retrograde labeling, muscle force and power testing, stereologic testing, and a variety of other techniques. A further challenge in the study of facial nerve regeneration is the measurement of functional recovery.

Direct visualization of facial nerve regeneration is now possible because of a re-
cent breakthrough in transgenic animal technology. The green fluorescent protein (GFP) gene, expressed intrinsically in the native *Aequorea victoria* jellyfish, has been successfully cloned, introduced, and rendered heritable in nonnative organisms.36-38 Furthermore, by understanding the genetics of neural tissue development and expression, investigators have created transgenic mice that express GFP in targeted tissues, including axons and Schwann cells.39-43 These transgenic animals allow nerve reinnervation to be directly visualized after injury. Fluorescence in the nerve may be lost and regained as seen under fluorescence microscopy, and confocal imaging reveals dynamics of motor end plate reinnervation in the target muscle.28,44

To study nerve regeneration in a larger animal model, we collaborated with investigators at genOway (Lyon, France) to engineer a Thy1-GFP rat. These animals express GFP under the regulation of the Thy1 promoter. The Thy1 regulatory element is found in neurons, thymocytes, and other supporting cells.45 By visualizing the neural fluorescent tissues in these animals, we were able to characterize their facial nerve anatomy. We performed a pilot study in which the proximal facial nerve trunk was crushed and then imaged at weekly intervals up to 8 weeks. In addition, we studied the denervation and reinnervation of the long, thin zygomaticus muscle with confocal microscopy to demonstrate regeneration dynamics at the motor end plate.

**METHODS**

**TRANSGENIC RATS**

The construct of the Thy1-XFP mouse lines41 (gift from Jeffrey Lichtman, PhD, and Joshua Sanes, PhD, and colleagues) was used to create transgenic Sprague Dawley rat founder lines in collaboration with genOway (Lyon, France) using pronuclear injection. Further characterization of F1 and subsequent generations was performed.45 The presence of the GFP transgene was confirmed by polymerase chain reaction (PCR) using genomic DNA extracted from rat tail specimens, and expression was verified by examination of the retina under fluorescence microscopy. Primer sequences (Integrated DNA Technologies) were provided by genOway (PCR size, 422 base pairs [bp]; BOR1-C1: 5’-CTGAGGTATTCTCATGTGCTCGCTGG-3’; BOR1D1: 5’GCG-GACTTGAAGAAGTCGT-GCTGC-3’). A total of 8 transgenic animals were used in this study, with 4 used as uninjured control animals for facial anatomy characterization (Figure 1). The relatively recent development and characterization of the Thy1-GFP founder line mandated a low total number of animals for use in this study. The rats are maintained and bred in a central animal housing facility, and all described procedures were performed according to protocols approved by the Division of Comparative Medicine at Washington University School of Medicine.

**SURGICAL PROCEDURE**

Transgenic animals were anesthetized for surgery with subcutaneous injections of ketamine (75 mg/kg) and medetomidine (100 mg/kg). The right side of the face was carefully shaved and then depilated with Nair hair removal cream (Church and Dwight Co, Inc, Princeton, New Jersey) prior to skin preparation with alcohol swabs. Great care was taken to avoid caustic or exposure injury to the eye. The skin was incised 1 mm anterior and inferior to the tragus and carried in an arch posteriorly. Following the tragal pointer medially, the facial nerve trunk was identified at an original magnification of ×16 as it exits the stylomastoid foramen. The main nerve trunk was then crushed with a No. 5 Jeweler’s forceps for 30 seconds,46 and the crush site was marked with a 10-0 nylon stitch. The wound was then irrigated, and skin was reapproximated with interrupted 6-0 nylon sutures. Animals recovered on a heated surface following anesthesia reversal with atipamezole hydrochloride (1 mg/kg). Animals were inspected and weighed weekly to ensure that nerve damage did not impair the ability to eat or cause ocular damage. At the time of imaging evaluation, animals were reanesthetized, and the surgical site was prepared. Following this, they were perfused transcardially with paraformaldehyde, 4%, in 0.1M phosphate-buffered saline (pH 7.4). The superficial subcutaneous tissue of the face was then dissected completely off of the underlying superficial musculoaponeurotic system and imaging of the nerve trunk and its branches. The zygomaticus muscle was removed with careful dissection, placed on a Sylgard resin-coated dish (Dow Corning Corporation, Midland, Michigan) and rinsed with phosphate-buffered saline. It was then stained with α-bungarotoxin Alexa Fluor 594 (10 µg/mL; Invitrogen, Carlsbad, California) for 30 minutes at room temperature. The muscle was then rinsed again with phosphate-buffered saline and mounted under a coverslip in Vectashield (Vector Laboratories, Burlingame, California) for confocal microscopic imaging.
MOTOR END PLATE EVALUATION

The staining of the zygomaticus muscle with α-bungarotoxin conjugated to Alexa 594 (Molecular Probes, Eugene, Oregon)-labeled motor end plates with red fluorescence and allowed the axon and the motor end plate to be viewed together using different lasers specific to separate excitation wavelengths. The entire muscle was placed on a glass slide with a coverslip to facilitate whole-mount imaging, allowing the course of nerves and their branches to be viewed throughout the entire muscle specimen. The muscle was surveyed and imaged under low magnification before higher power imaging of nerve terminals and motor end plates. The confocal microscope (Olympus FV 1000; Olympus America Inc, Center Valley, Pennsylvania) captures GFP-labeled axons with a spectral detection window at 488 nm and motor end plates at 568 nm. Final confocal images were taken with a ×20 objective (numerical aperture, 0.75) and were merged from pictures taken at 5-µm intervals through the depth of the muscle. These were used to create a motor end plate reinnervation map to visualize the branching system of the facial nerve to the zygomaticus muscle. Image data was obtained in uninjured control animals (n=4) and after injury (n=4) at 1, 2, 4, and 8 weeks following nerve crush. At each time point, regions of interest within the image were calibrated to examine the number of denervated and reinnervated motor end plates.

RESULTS

ANATOMY OF THE Thy1-GFP FACIAL NERVE

The rodent facial nerve anatomy has been previously described and studied.12-47 Notable features of the nerve include the lateral location of the lacrimal gland, which is located outside of the bony orbit, and branching patterns of the nerve distally that innervate the rodent vibrissae. To facilitate study of reinnervation patterns after injury, a thin, reliable muscular branch was required. The anatomical study of several control animals (n=4) under a dissecting fluorescent microscope revealed that the zygomaticus muscle had a persistent innervating branch that was reliably imaged under low and high power (Figure 1 and Figure 2). The long, thin anatomy of this muscle was conducive to fluorescence microscopy as well as confocal imaging of the muscle whole mount.

FLUORESCENCE MICROSCOPY

The facial nerve in uninjured control animals reliably expressed GFP in axons (Figure 1C and Figure 2). After nerve crush and marking of the crush site, a clear loss and recovery of a fluorescent regenerative front was not clearly seen at any of the time points chosen for the study. However, fluorescence in the frontal, buccal, and mandibular (*) branches was decreased at 2 weeks (B1). The triangle represents the crush site.
CONFOCAL IMAGING

In studies of uninjured control animals, adult Thy1-GFP rats were noted to have a 1:1 relationship between terminal axons and motor end plates in the zygomaticus muscle (Figure 4A). After nerve crush, this relationship changed as Wallerian degeneration occurred and motor end plates were denervated and then reinnervated over time. Imaged nerve terminals were completely denervated at 1 week following nerve crush injury with only "ovoids" of fluorescing debris visible (Figure 4B). This persisted at 2 weeks after injury. Reinnervation of end plates is visualized at 4 weeks (Figure 4D). The axonal relationship with motor end plates at 4 weeks did not show any hyperinnervation after nerve crush injury, as has been seen in other animal models. However, there was disorganization of the axonal branches, and unmatched axons were seen in addition to fluorescing debris, which may represent phagocytosis of GFP from degenerated axons, or fluorescing lymphoid cells. By 8 weeks after nerve crush injury, the 1:1 axon-to-end plate relationship was reestablished, and images approximated uninjured controls (Figure 4A and E).

Figure 4. Confocal imaging of the zygomaticus muscle. Acetylcholine receptors (AChRs) appear red after staining with α-bungarotoxin. A control animal is shown (A), followed by denervation up to 2 weeks (B and C), and then reinnervation, which is also visualized under fluorescence microscopy (D and F). By 8 weeks, images approximate the appearance of controls (E).

The purpose of this study was to demonstrate the utility of a new transgenic model in facial nerve research. The Thy1-GFP rat, which constitutively expresses GFP in axons, allows the intricate facial nerve anatomy to be studied. The increased nerve caliber of this animal model, compared with other currently available models, allows for more reliable manipulation and use in a variety of surgical paradigms that are translationally powerful. In this study, we performed a proximal facial nerve crush and, for the first time to our knowledge, directly visualized facial nerve regeneration and muscle reinnervation using fluorescence and confocal microscopy in a rat model.

The use of fluorescence microscopy allowed for the direct visualization of nerve regeneration. After nerve injury, axons underwent Wallerian degeneration and fluorescence was lost. As axons expressing GFP regenerated, fluorescence was again visualized and could be used as a marker for regeneration. In the present study, the uninjured Thy-GFP rats revealed expression of GFP in the facial nerve. Following injury, fluorescence was not markedly diminished in the main nerve trunk or immediately distal to the marked crush site at the time points captured. However, evaluation of the nerve branches demonstrated diminished fluorescence at weeks 1 and 2, followed by an increase at 4 and 8 weeks after injury (Figure 3). Specifically, high-power imaging revealed return of fluorescence in the zygomatic muscle branch at 4 weeks (Figure 4F). The persistence of fluorescent protein at earlier time points after nerve injury has been previously documented in other fluorescence-expressing animal models. This persistence of fluorescence has been overcome by using a "double-crush" technique, in which an initial crush is followed by a second nerve injury to allow fluorescent debris to be cleared. The double-crush method was considered in the study design but was decided against in order to establish baseline data in a single nerve crush model using this new transgenic rat model.
line. In future studies, a double-crush technique could be used and coupled with line scanning to quantify the degree of fluorescence intensity over time. In addition, live in vivo imaging techniques with fluorescence microscopy are also possible in these transgenic animals to follow the same nerve branch and its specific morphologic characteristics at serial time points.

The thin, zygomaticus muscle is well suited for confocal imaging after facial nerve injury. We visualized the return of innervation at 4 weeks after the crush injury. The thin nature of the superficial musculoaponeurotic system affords other facial muscles to be used in reinnervation studies as well. By correlating the morphologic characteristics of axonal regrowth in different injury paradigms, the Thy1-GFP model will assist in understanding aberrant reinnervation patterns and the development of synkinesis.

There are a variety of assays that can be used to evaluate facial nerve regeneration after injury; however, few techniques confer the direct visualization of nerve reinnervation or the possibility of a live in vivo study. The outcome measures used to assess nerve regeneration in this study were chosen based on the novel fluorescing properties of this new transgenic tool. The direct visualization of axonal behavior in the Thy1-GFP line may allow questions regarding aberrant nerve regeneration to be answered and improve treatment modalities available for patients with facial nerve injury.

Accepted for Publication: April 29, 2010.
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FinancialDisclosure: None reported.

Funding/Support: This study was made possible by funding awarded to Dr Mackinnon by the National Institutes of Health (grant RO1 NS051706) and to Dr Borschel by the American Society for Peripheral Nerve. The Barnes-Jewish Foundation provided confocal microscopy equipment and support.

Additional Contributions: Alice Tong, MS, taught confocal techniques used in this work, and Andrew R. Magill, BS, assisted with image calibration and the figures.

REFERENCES


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