Published in final edited form as:

Vet Pathol. 2012 July ; 49(4): 727–730. doi:10.1177/0300985811414033.

Fluorescence labeled reporter gene in transgenic mice provides a useful tool for investigating cutaneous innervation

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Abstract

B6.Cg-*Tg(Thy1-YFP)16Jrs/J* transgenic mice were created to express the yellow fluorescent protein gene driven by a mouse *Thy1* promoter which labeled motor and sensory neurons such that individual nerves could be followed. We used these mice to identify nerves in the skin that innervate the erector pili and panniculus carnosus muscle. Whole mounts demonstrated yellow fluorescent protein (YFP) expression in nerves of the skin which was confirmed by labeling the neuromuscular junction with fluoresceinated α -bungarotoxin. Frozen and paraffin embedded skin sections revealed innervation of the panniculus carnosus muscle. Paraffin sections labeled with an anti-GFP antibody revealed innervation of the panniculus carnosus as well as the erector pili muscle and around the hair follicle bulge, the last two of which were not seen by direct fluorescence.

The skin contains sensory nerve endings located in the dermis for touch while other nerves innervate the erector pili (smooth muscle) which function to raise the hair in involuntary response to environmental conditions such as low temperature, fear, or other stimuli. Below the hypodermal fat layer of the skin is a layer of striated skeletal muscle, the panniculus carnosus, innervated by motor neurons allowing the animal to move or twitch its skin.³

The B6.Cg-*Tg(Thy1-YFP)16Jrs/J* strain was created to express yellow fluorescent protein (YFP) in motor neurons and retinal ganglion cells driven by a *Thy1* promoter.⁴ These mice were used primarily to study neuromuscular junctions using tetramethylrhodamine-conjugated α -bungarotoxin to label acetylcholine receptors. Acetylcholine receptors aggregate at postsynaptic sites of the neuromuscular junction.^{5,6}

Variations in this transgenic construct, for example using the “rainbow mice”, provides tools to follow individual nerves.⁷ This investigation determined that these mice could be used successfully to investigate the innervations of the skin and appendages either by direct visualization of the excited YFP or indirectly by immunohistochemical detection of the YFP protein, the latter of which proved to be more sensitive.

Materials and Methods

Mice

B6.Cg-*Tg(Thy1-YFP)16Jrs/J* mice (hereafter referred to as YFP16, JR#3709, The Jackson Laboratory, Bar Harbor, ME USA) were euthanized by CO₂ asphyxiation using methods approved by The Jackson Laboratory Institutional Animal Care and Use Committee. Dorsal

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Declaration of Conflict of Interest

The authors declare that they had no conflicts of interest with respect to their authorship or the publication of this article.

and ventral skin was depilated (Nair®, Church and Dwight Co., Princeton, NJ, USA). Skin was lightly fixed in 4% paraformaldehyde (PFA) by immersion for 1h at 4°C, stored in phosphate buffer solution (PBS) at 4°C until use, then directly viewed with a dissection microscope (MZFLIII, Leica, Wetzlar, Germany) and imaged (Leica RX300 camera).

Labeling nerves and neuromuscular junctions

The YFP16 skin samples were also incubated with tetramethylrhodamine-conjugated α -bungarotoxin (BTX; 1:1000; Alexa 594 alpha-Bungarotoxin, catalog # B13423, Invitrogen (Molecular Probes), Carlsbad, CA) diluted in PBS/0.3% Triton 100/1% bovine serum albumin (BSA) for 90 min at room temperature in the dark, followed with 3 washes for 15 min each in PBS/0.3% Triton 100. Tissues were placed on a slide and cover slipped with 80% glycerol. The slides were visualized with a confocal microscope (E600, Nikon, Tokyo, Japan) and imaged (RTColor camera, Diagnostic Instruments, Sterling Heights, MI, USA). For frozen sections fresh skin was lightly fixed in 4% PFA for 1h at 4°C and then transferred to 30% sucrose at 4°C overnight. The skin was embedded in Optimal Cutting Temperature compound (Tissue Tek, Torrance, CA, USA) at -30°C and serial 10 μ m sections were placed on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Slides were washed 3 times for 5 min in PBS and then incubated with BTX (1:1000) diluted in PBS/0.3% Triton 100/1% BSA for 90 min at room temperature in the dark. Slides were washed 3 times for 10 min in PBS/0.3% Triton 100 and were subsequently treated with 4',6-diamidino-2-phenylindole (DAPI, 1:10000) for 5 min in the dark. Slides were again washed 3 times for 5 min in PBS and cover slipped with Fluoromount (Sigma, St. Louis, MO, USA). Slides were examined and imaged (SP5 AOBs confocal microscope, Leica).

For immunohistochemistry YFP16 skin was fixed overnight in Fekete's acid-alcohol-formalin solution, transferred to 70% ethanol, embedded routinely, and sectioned at 6 μ m. Using an autostainer (Discovery XT, Ventana Medical Systems, Tucson, AZ), slides were double labeled using rabbit anti-green fluorescent protein (GFP, 1:6000, ab290, Abcam, Cambridge, MA, USA) and rabbit or mouse anti-smooth muscle actin (SMA, 1:200, NB600-531, Novus, Littleton, CO, USA or 1:400, A 2547, Sigma). Detection kits used for labeling were UltraMap™ anti-Rabbit Alkaline Phosphatase with Naphthol-Fast red chromogen (red) and either OmniMap™ anti-Rabbit Horse Radish Peroxidase or DABMap™ anti-Mouse Horse Radish Peroxidase with diaminobenzidine chromogen (brown) (Ventana Medical Systems, Tucson, AZ). When the mouse anti-smooth muscle actin was used, the MoMap kit (Ventana) was also used to block non-specific binding of anti-mouse antibodies. Additional sections were stained with hematoxylin and eosin (HE), phosphotungstic acid hematoxylin (PTAH), and Bodian's stains, the latter two to emphasize striated muscle with cross striations.

Results

Direct examination of lightly fixed dorsal and ventral skin confirmed YFP expression in both the motor axon and the neuromuscular junction (Fig. 1). Frozen sections verified YFP expression of the motor axon and the neuromuscular junction which was labeled with α -bungarotoxin (Fig. 2, 3). The panniculus carnosus muscle was identified by its striated structure and subcutaneous location to the dermis associated with a nerve bundle, an axon, and a neuromuscular junction clearly innervating the muscle (Fig. 4, 5). The HE barely identified an axon while the PTAH stain highlighted cross striations in the panniculus carnosus muscle and axons (Fig. 6, 7). Immunohistochemistry, using paraffin embedded sections of YFP16 dorsal skin, identified nerves in the panniculus carnosus (Fig. 8) and innervating the erector pili muscle and surrounding the bulge region (Fig 9). Double labeling with anti-GFP and anti-SMA using both horse radish peroxidase-diaminobenzidine and

alkaline phosphatase in either combination confirmed innervation of the erector pili muscle (Fig. 10, 11).

Discussion

Whole mount YFP expression in skin is similar to motor axon and neuromuscular junction expression in large muscle bellies.^{4,5,11} The tetramethylrhodamine-conjugated α -bungarotoxin effectively labeled the neuromuscular junction in the panniculus carnosus muscle. Vertical frozen skin sections, using light acetone fixation coupled with short processing time, caused the fluorescent protein to precipitate which in turn resulted in no fluorescence when excited (data not shown). Tissue lightly fixed in 4% paraformaldehyde and then incubated overnight in 30% sucrose¹⁰ effectively preserved the fluorescence of the reporter protein and the integrity of the skin structure while allowing visualization of YFP expression in the motor axon in conjunction with tetramethylrhodamine-conjugated α -bungarotoxin labeling of the neuromuscular junction. Nerves of various sizes could be detected by this method as they could by using classical histologic stains. However, when YFP was detected using immunohistochemistry, very small nerves were easily detected innervating the erector pili muscles and traversing the dermis. These were not seen when using the fluorescence of YFP under excitation. Double labeling for smooth muscle actin and YFP confirmed innervation of the erector pili muscle.

While these labels are very specific in the skin, one must be careful to not misinterpret mast cell reactivity when using avidin-biotin-peroxidase methodologies as these present as false positives.^{1,2} Expression of the YFP15 is driven by the neuronal enhancer of the Thy1 (CD90.2) promoter. Thy1 has expression outside the nervous system but with this transgene most expression outside the nervous system is suppressed. There may be nonspecific labeling of inflammatory cells in response to injury based on studies investigating nerve regeneration *in vivo* following injury.^{8,9}

Acknowledgments

The authors thank Dr. R. Burgess for providing the mice and advice on their use, C. Rosales and N. Gott for technical assistance, and J. Hammer for assistance with figures.

Financial Disclosure/Funding

This work was supported by grants from the National Institutes of Health (AR49288, AR047204, and AR054407 to JPS).

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Figure 1. Fluorescence of YFP in whole mounts demonstrates nerves and end plates.

Figure 2. When labeled with alpha bungarotoxin, the neuromuscular junction is evident (yellow).

Figure 3. Higher magnification of Fig. 2.

Figure 4. Transverse skin sections illustrates nuclei in the epidermis and hair follicle (DAPI, blue), direct fluorescence of nerves (green) and neuromuscular junction (yellow).

Figure 5. Higher magnification of Fig. 4.

Figure 6. Small nerves innervating the panniculus carnosus muscle (arrows) can also be visualized with H&E.

Figure 7. A Bodian stain reveals small nerve innervations of the panniculus carnosus muscle (arrow).

Figure 8. Immunohistochemistry directed against YFP reveals innervations of the panniculus carnosus muscle (arrows).

Figure 9. Immunohistochemistry localized YFP expressing nerves (brown, arrows) innervating the erector pili muscle and surrounding the bulge of hair follicles.

Figure 10. Double labeling confirms that the nerves (red, arrows) do innervate the erector pili muscle (brown, arrows).

Figure 11. These can be reversed where the nerves are now labeled brown (arrow) and the erector pili muscles are red (arrows).