

GENETIC TOOLS THAT TARGET MECHANORECEPTORS CAN BE USED TO LABEL BLADDER AFFERENTS

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Introduction

- Neural mechanisms of bladder pain remain poorly understood.
- In addition to nociceptive afferents, mechanosensitive afferents are thought to contribute to visceral pain under pathologic conditions.
- Study of mechanosensitive afferents is limited by the historical inability to label mechanosensitive afferents using immunohistochemical (IHC) techniques
- Transgenic mouse lines that label specific subtypes of mechanosensitive skin afferents may help us identify mechanosensitive afferent populations in the bladder.

Methods

- Transgenic mice.** Mice were generated by crossing Rosa26LSL-tdTomato mice to either RetCreERT2 or TrkBCreER mouse lines. Cre expression was induced with PO administration of 4-hydroxytamoxifen (4-HT) in wheat germ oil, administered at 8 weeks (Ret Line crosses) or 3 to 8 weeks of age (TrkB line crosses).
- Immunohistochemistry.** Naïve adult mice were euthanized followed by transcardial perfusion with 4% paraformaldehyde and postfixation in either paraformaldehyde or Zamboni's fixative. Whole-mount bladders were washed in 1% PBST and stained with a multiplex immunohistochemistry protocol over the course of multiple days. Primary antibodies included Sheep anti-CGRP (1:1000, Abcam), Chicken anti-NF200 (1:250, Aves Lab), Rabbit anti-s100 (1:200, Abcam), and Chicken anti-TH (1:1000, Aves Lab). Cre-dependent tdTomato fluorescence was amplified using Rat anti-mCherry antibody (1:400, ThermoFisher) for all images. Chicken anti-NPY (1:250, Aves Lab) staining turned out suboptimal and was therefore omitted from data analysis. Secondary antibodies raised Donkey included anti-Sheep 488 (1:500, Jackson ImmunoResearch), anti-Sheep 405 (1:2500, Rockland), anti-Rat Cy3 (1:250, Jackson ImmunoResearch), and anti-Chicken 647 (1:250, Jackson ImmunoResearch). Bladders were mounted and coverslipped in Fluoromount G (ThermoFisher) without clearing. Low magnification epifluorescent images in Fig 1 acquired with BZ-X700 digital microscope (Keyence Corporation) and stitched using BZ-X Analyzer software (Keyence Corporation). Confocal images in Fig 1 obtained with the Andor Dragonfly Confocal Microscope (Oxford Instruments) and visualized using Imaris software (Bitplane). Higher magnification epifluorescent images in all other figures show a single Z plane acquired with Thunder 3D Tissue microscope (Leica Microsystems) and LAS X software (Leica Microscopes). Image adjustments and deconvolution and optical clearing algorithms (Large Volume Computational Clearing) were applied to images using LAS X and ImageJ software.

Table 1. Sensory neurons subtypes labeled by each mouse line

Mouse line	Target fiber type*
Ret CreER	Abeta mechanoreceptors (rapidly adapting and field subtypes), non-peptidergic nociceptors, C mechanoreceptors
Split Cre	Abeta mechanoreceptors (rapidly adapting subtype)
TrkB CreER	Adelta mechanoreceptors

*Based on afferents found in skin.

Results

Figure 1. Patterns of innervation differ between locations in the bladder. A) Innervation in the neck of bladders across mouse lines is more dense compared to the body and dome. B) Confocal image of nerve fibers in the body of the bladder show simple (single fiber paths; single arrow), to branching (bifurcated fiber paths; double arrows), to complex (multi-forked fiber paths; triple arrows). Scale bar is 70 microns.

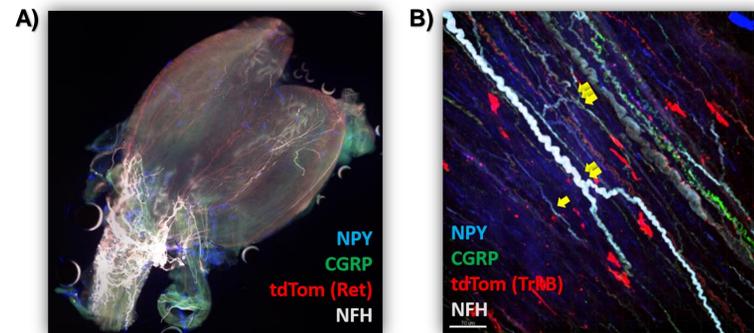


Figure 2. Co-localization and intermingling within large fiber bundles and individual staining of small nerve terminals across mouse lines. An example of individually-labeled fibers (A) and a combination of intermingling and co-localized nerve fibers (B) from a Ret CreER bladder.

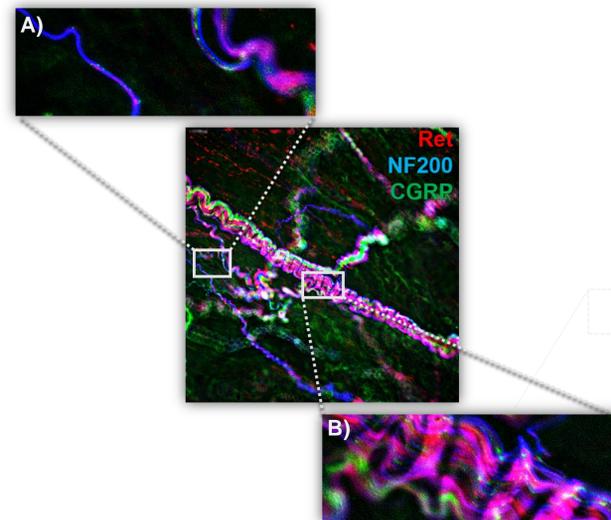


Figure 3. Motoneuron and sensory neuron intermingling in Split Cre bladders without co-localization.

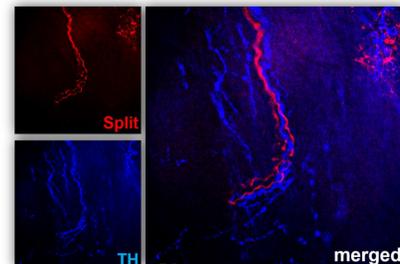


Figure 4. Co-localization of Ret, TrkB, and Split-Cre with mechanosensitive fiber molecular markers. A) Ret partially overlaps with both CGRP and NF200. B) TrkB partially overlaps with NF200 but is more distinct from CGRP. C) Split-Cre appears to overlap with TH in some cases but primarily is distinct. Scale bar is 100 microns.

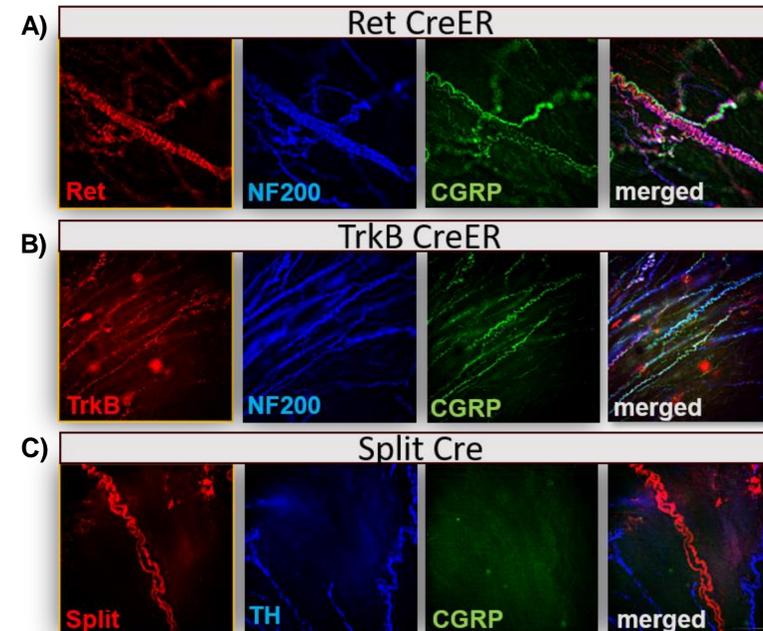


Figure 5. Ret co-localization with NF200 and CGRP along with NF200 and CGRP overlap. A) A merged image of all markers. B) Ret co-localization with NF200. C) Ret co-localization with CGRP. D) CGRP and NF200 partial overlap. Scale bar is 100 microns.

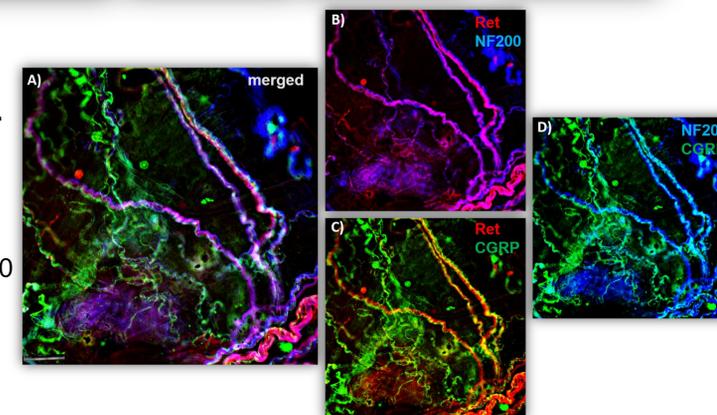


Figure 6. TrkB labeling resembles patterns of labeling in the skin that is largely NF200+ and CGRP-. A) Merged image of TrkB, NF200, and CGRP showing some overlap of all markers. B) TrkB and NF200 co-localization demonstrated in places where CGRP is negative. C) CGRP labeling largely independent of TrkB. D) Partial overlap of NF200 and CGRP. Scale bar is 100 microns.

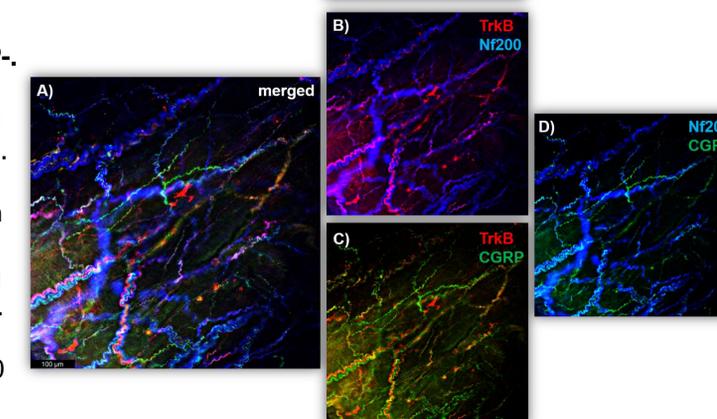
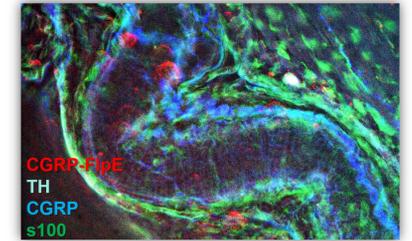


Figure 7. s100 appears throughout a single bladder plane and may surround sensory and motor neurons.



Conclusions

- Autonomic motoneurons and sensory neurons intermingle in the large nerve bundles but smaller nerve fibers are often individual.
- Ret overlap with NF200 suggests that a subset of these labeled afferents are myelinated Abeta fibers.
- Co-localization of NF200 and CGRP suggests that terminals labeling CGRP may not only consist of C-fibers but also myelinated Adelta fibers.
- TrkB nerve terminals are distinct from CGRP, suggesting genetically labeled TrkB positive afferents are a useful tool to study fibers that are distinct from peptidergic nociceptors.
- Various branching patterns are seen across fiber types.

Future Directions

- Determine if any mechanosensitive nerve terminals are associated with spindle-like structures or terminal Schwann cells as we see in the skin.
- Determine if the features labeled by S100 are actually Schwann cells or other structures.
- Perform 3-D reconstruction of Z stacks to analyze innervation patterns throughout the layers of the bladder wall.
- Quantify co-localization of IHC markers within mechanosensitive afferents and compare across mouse lines.
- Characterize alterations in mechanosensitive afferents in models of somatic and visceral pain.

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