

Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch

Qin Liu¹, Sophia Vrontou^{2,3}, Frank L Rice⁴, Mark J Zylka⁵, Xinzhong Dong¹ & David J Anderson^{2,3}

C-fiber tactile afferents are a subpopulation of unmyelinated cutaneous sensory neurons activated by gentle stroking. Using a genetically encoded tracer, we found that Mas-related G protein-coupled receptor B4 marks a rare subpopulation of unmyelinated, nonpeptidergic sensory fibers that exclusively innervate hairy skin. These fibers terminate in large arborizations similar in size and distribution to C-fiber tactile afferent receptive fields, suggesting that *MrgprB4* may provide genetic access to these elusive neurons in mice.

Mas-related G protein-coupled receptors (*Mrgprs*), also called sensory neuron-specific receptors (SNSRs), are specifically expressed in subsets of small-diameter sensory neurons^{1–4}. Neurons expressing *MrgprD* project exclusively to glabrous and hairy epidermis⁵. Other *Mrgprs* are expressed in different subsets of small-diameter neurons^{1,4}, raising the question of whether these subsets show distinct target specificities.

We mapped the projections of *MrgprB4*⁺ neurons by targeting placental alkaline phosphatase (PLAP) to the *MrgprB4* locus in embryonic stem cells (Supplementary Fig. 1 online). Homozygous *MrgprB4*^{PLAP} mice were viable and fertile, and did not show obvious phenotypic abnormalities. Histochemistry showed that PLAP was expressed exclusively in a small subset of dorsal root (DRG) and trigeminal ganglion neurons (Fig. 1a,b), with a postnatal onset (Supplementary Fig. 2 online). Thoracic (T12/T13) DRG contained, on average, 119 ± 21 *MrgprB4*⁺ neurons per ganglion (Fig. 1a, *n* = 6),

corresponding to ~2% of all DRG neurons. There were even fewer *MrgprB4*⁺ neurons in lumbar (L4) and cervical (C6) DRG (Fig. 1b; 45.57 ± 8.96, *n* = 7 and 45.86 ± 8.89, *n* = 8, respectively). *MrgprB4*⁺ neurons were small diameter (average 26.86 ± 1.93 μm), negative for

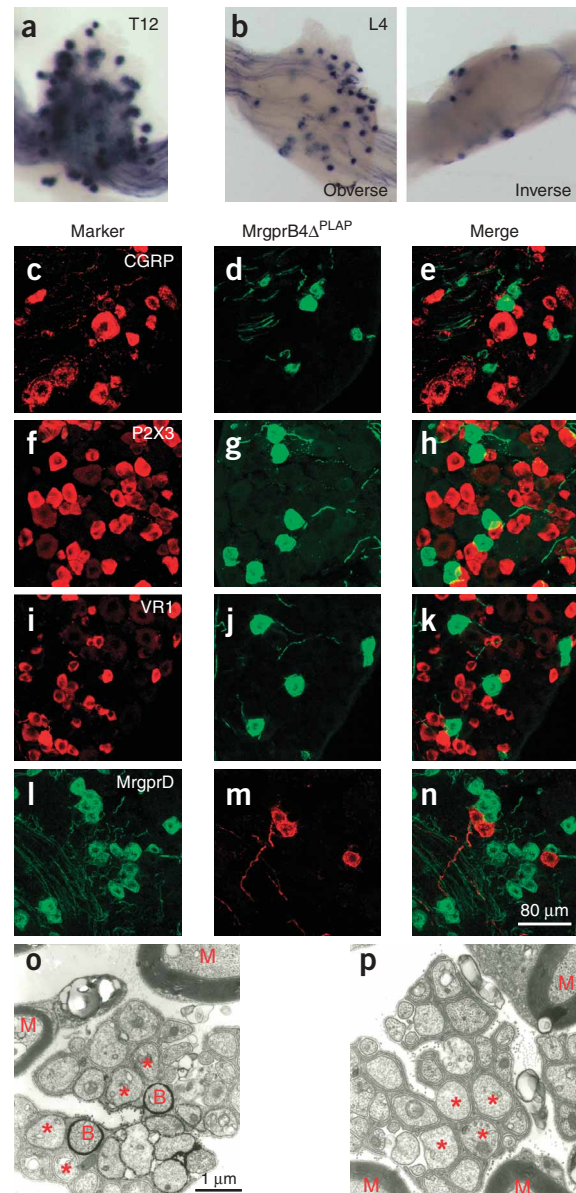


Figure 1 Characterization of *MrgprB4*⁺ sensory neurons. (a,b) Whole-mount PLAP histochemistry of DRG from heterozygous *MrgprB4*^{PLAP} adult mice. (c–k) lumbar level 2 (L2) DRG neurons from *MrgprB4*^{PLAP} mice stained with antibodies to PLAP and the indicated markers. (l–n) DRG from *MrgprB4*^{PLAP}; *MrgprD*^{EGFP} mice stained with antibodies to PLAP and GFP. (o,p) Transverse electron microscopic sections of thoracic nerves from *MrgprB4*^{PLAP} (o) and wild-type (p) mice stained for PLAP activity. Note the dense precipitate on unmyelinated *MrgprB4*⁺ axons (B). * indicates unstained, unmyelinated axons. Myelinated axons (M) were unstained.

¹The Solomon H. Snyder Department of Neuroscience, School of Medicine, Johns Hopkins University, 725 N. Wolfe St., Baltimore, Maryland, 21205, USA. ²Division of Biology, 216-76, California Institute of Technology, 1201 E. California Blvd., Pasadena, California 91125, USA. ³Howard Hughes Medical Institute, 1201 E. California Blvd., Pasadena, California 91125, USA. ⁴Center for Neuropharmacology and Neuroscience, Albany Medical College, 43 New Scotland Ave., Albany, New York 12208, USA. ⁵Department of Cell and Molecular Physiology, UNC Neuroscience Center, University of North Carolina, 105 Mason Farm Road, Chapel Hill, North Carolina 27599, USA. Correspondence should be addressed to D.J.A. (wuwei@caltech.edu) or X.D. (xdong2@jhmi.edu).

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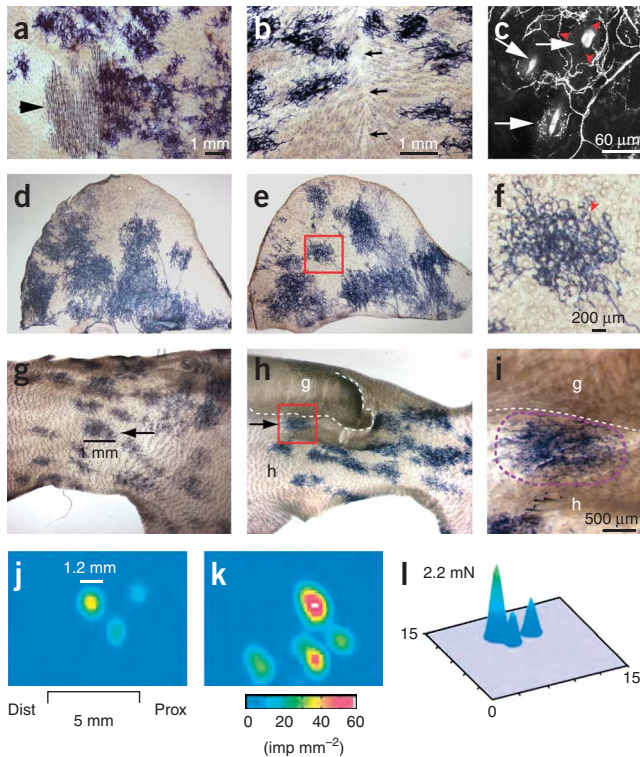


Figure 2 MrgprB4⁺ peripheral terminals exclusively innervate hairy skin and show large arborizations. (a,b) Dorsal (a) and ventral (b) thoracic skin from *MrgprB4 Δ ^{PLAP}* mice stained by whole-mount PLAP histochemistry. Arrowhead indicates hairs (a) and arrows indicate midline (b). (c) Whole-mount immunofluorescent staining of PLAP expression in thoracic skin (flat-mount view); arrows indicate hair follicles. (d,e) Right ears from two *MrgprB4 Δ ^{PLAP}* mice. (f) Higher-magnification view of boxed area in e. (g–i) Whole mounts of total hindlimb skin (proximal to the right). Note the patchy MrgprB4⁺ innervation (arrow). (h,i) MrgprB4⁺ fibers were present in hairy (h), but not glabrous (g), skin. (i) Higher-magnification view of boxed area in h. (j–l) Receptive fields of C-fiber tactile afferents defined by microneurography (from Figs. 5 and 6 in ref. 11, used with permission). Note the similar size and distribution of individual touch-sensitive spots and MrgprB4⁺ arbors (1.2-mm scale bar in j; 1-mm bar in g). (l) Three-dimensional density plot of the receptive field shown in j.

$0.838 \pm 0.068 \text{ mm}^2$ (ear skin) (mean \pm s.e.m., $n = 5\text{--}8$). Given $\sim 4,000$ MrgprB4⁺ neurons per animal and $\sim 60 \text{ cm}^2$ of skin, these measurements suggest that each MrgprB4⁺ fiber terminates in $\sim 1\text{--}3$ such arbors.

In the skin, MrgprB4⁺ fibers appeared to encircle and penetrate the necks of hair follicles and the adjacent epidermis (Fig. 2c, red arrowheads, Fig. 3). Similar to MrgprD⁺ fibers⁵, MrgprB4⁺ fibers were absent from all specialized cutaneous sensory structures, as well as from muscle and blood vessels. MrgprB4⁺ fibers appeared to be aligned with subsets of MrgprD⁺ and CGRP⁺ fibers (Fig. 3a,d). In homozygous *MrgprB4 Δ ^{PLAP/PLAP}* mice, the pattern of MrgprB4⁺ fiber innervation was unchanged, arguing against a requirement for *MrgprB4* in axon guidance.

The central projections of MrgprB4⁺ fibers coterminated with IB4⁺ fibers in spinal lamina II_o, in a patchy distribution mirroring their cutaneous organization (Fig. 3b, arrowheads and Supplementary Fig. 4 online). At limb levels, MrgprB4⁺ fibers were restricted to the lateral spinal cord (Fig. 3e, arrowheads), which is consistent with the proximal bias of MrgprB4⁺ fibers, whereas they occupied the entire medio-lateral extent of lamina II_o in segments rostral and caudal to the L3–L6 (limb) region (Fig. 3b). A direct comparison of MrgprD–farnesylated enhanced green fluorescent protein (fEGFP)⁺ and MrgprB4–PLAP⁺ central fibers in double-heterozygous mice showed

neurofilament 200kD (Supplementary Fig. 2), calcitonin gene-related polypeptide (CGRP), purinergic receptor X3 (P2X3) and transient receptor potential cation channel V1/vanilloid receptor 1 (TrpV1/VR1) (Fig. 1c–k), and positive for c-RET and *Griffonia simplicifolia* isolectin B4 (IB4) (Supplementary Fig. 2), indicating that they are nonpeptidergic, but were distinct from the MrgprD⁺ subset (Fig. 1l–n)⁴. Electron microscopic analysis confirmed that MrgprB4⁺ fibers were unmyelinated (Fig. 1o,p).

MrgprB4⁺ fibers exclusively innervated hairy skin, among all tissues examined (Fig. 2 and Supplementary Table 1 online). Innervation was not detected in the glabrous plantar skin of either the hindpaws or forepaws (Fig. 2h,i; ‘g’ and Supplementary Fig. 2). Scattered MrgprB4⁺ nerve terminals were observed in the dorsal skin of the paw (Fig. 2h,i and Supplementary Fig. 3 online); therefore, the lack of fibers in glabrous skin does not simply reflect a lack of distal limb innervation (although the density of MrgprB4⁺ terminals increased proximally; Supplementary Fig. 3). MrgprB4⁺ fibers were absent in the genitalia, consistent with the lack of hairy skin in these structures.

Notably, MrgprB4⁺ fibers innervated the skin in large, discontinuous patches covering $\sim 50\text{--}60\%$ of the skin surface area (Fig. 2a,b,d,e,g). This broad coverage reflects extensive branching in individual terminal arbors (Fig. 2e,f). The pattern of MrgprB4⁺ arbors was bilaterally asymmetric (Fig. 2b) and distinct between different animals (Fig. 2d,e). The average area of these arbors ranged from $0.27 \pm 0.04 \text{ mm}^2$ (body skin) to

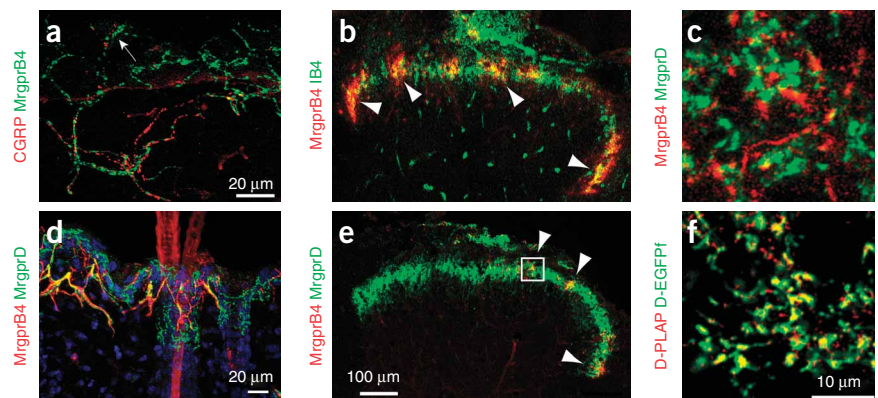


Figure 3 MrgprB4 peripheral and central projections. (a–e) Sections through dorsal thoracic hairy skin (a,d), and L2 (b) and lumbar (L3–L6, e) dorsal spinal cord. MrgprB4⁺ fibers appeared to be aligned with subsets of CGRP⁺ (a) or MrgprD⁺ (d) fibers (arrow in a marks intertwined fibers). A longitudinal section through hair follicle is shown in d; hairs are autofluorescent. Nuclei were counterstained with DAPI (blue). Arrowheads indicate patches of MrgprB4⁺ fibers in lamina II_o in b and e. A higher-magnification view of the boxed region in e is shown in c. Panels (c,d,e) are from *MrgprB4 Δ ^{PLAP},MrgprD Δ ^{fEGFP}* double-heterozygous mice stained with antibodies to PLAP and GFP. (f) High-magnification view of lamina II_o in *MrgprD Δ ^{fEGFP/PLAP}* trans-heterozygous mice.

that they are overlapping, but distinct (Fig. 3c,e). Control experiments using trans-heterozygous *MrgprD*^{fEGFP/PLAP} mice indicated that this distinction does not simply reflect different axonal distributions of the two reporter proteins (Fig. 3f). Whether this difference indicates common or distinct postsynaptic targets for *MrgprB4*⁺ and *MrgprD*⁺ afferents, remains to be determined.

C-fiber tactile afferents were first identified almost 70 years ago⁶, but have never been directly visualized. Like C-fiber tactile afferents, *MrgprB4*⁺ axons are unmyelinated⁶, express neither CGRP nor TrpV1^{7,8}, and exclusively innervate hairy skin^{9,10}. Notably, the size (~0.3–1 mm²), scattered distribution, and number of *MrgprB4*⁺ arborization fields per terminal (~1–3) are remarkably similar to those of touch-sensitive spots in human C-fiber tactile receptive fields (see Fig. 2g,h,j,l and ref. 11). Like C-fiber tactile afferents, *MrgprB4*⁺ central projections terminate in lamina II^{12,13}, but are nonoverlapping with *MrgprD*⁺ fibers that are likely nociceptive (unpublished data). These and several additional notable similarities, including their proximo-distal distribution in the limb (see **Supplementary Discussion** online), suggest that *MrgprB4* may mark C-fiber tactile afferents in mice. Nevertheless, we cannot exclude that *MrgprB4* marks a rare population of nociceptors, such as C mechano-insensitive cells¹⁴. It is possible that *MrgprB4*⁺ fibers are involved in controlling vasodilatation in hairy skin, but this process involves CGRP¹⁵, and *MrgprB4*⁺ fibers are negative for CGRP. Electrophysiological analysis of *MrgprB4*⁺ neurons in culture has thus far failed to reveal responses to any thermal or mechanical stimuli tested, although such neurons do respond to ATP (data not shown). Because *MrgprB4*⁺ fibers represent a small subset of hair follicle afferents (see **Supplementary Fig. 4**), their functional characterization will require genetically targeted activity measurements *in vivo*. The role of *MrgprB4* itself is not clear. Although some other *Mrgpr* family members are known to encode neuropeptide receptors^{1–3}, ligands for *MrgprB4* have not yet been identified.

Studies in humans have suggested that C-fiber tactile afferents might detect 'caress-like, skin-to-skin contact' between individuals that is associated with affiliative emotional behaviors¹⁰. The availability of genetic tools for functionally manipulating neuronal activity in mice should permit investigation of whether *MrgprB4*⁺ neurons indeed

correspond to C-fiber tactile afferents, and to determine their role in such behaviors.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

X.D. generated the *MrgprB4*-PLAP mice, Q.L. carried out the bulk of the immunohistochemical characterization and analysis of these mice, S.V. contributed to the initial analysis and participated in the electron microscopic PLAP experiments, M.J.Z. generated and analyzed *MrgprD*^{PLAP/EGFP} spinal cord, F.L.R. provided expert advice on interpretation of epidermal innervation and D.J.A. supervised the project and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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