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Somatotopic organization of central arbors from nociceptive afferents develops independently of their intact peripheral target innervation

William Olson^{1,2,*} and Wenqin Luo^{1,*}

¹Department of Neuroscience, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, 19104

²Present address: Department of Neuroscience and Brain Science Institute, Johns Hopkins University School of Medicine, Baltimore, MD, 21201

Abstract

Functionally important regions of sensory maps are overrepresented in the sensory pathways and cortex, but the underlying developmental mechanisms are not clear. In the spinal cord dorsal horn (DH), we recently showed that paw innervating Mrgprd⁺ non-peptidergic nociceptors display distinctive central arbor morphologies that well correlate with increased synapse transmission efficiency and heightened sensitivity of distal limb skin. Given that peripheral and central arbor formation of Mrgprd⁺ neurons co-occurs around the time of birth, we tested whether peripheral cues from different skin areas and/or postnatal reorganization mechanisms could instruct this somatotopic difference among central arbors. We found that, while terminal outgrowth/refinement occurs during early postnatal development in both the skin and the DH, postnatal refinement of central terminals precedes that of peripheral terminals. Further, we used single-cell ablation of *Ret* to genetically disrupt epidermal innervation of Mrgprd⁺ neurons and revealed that the somatotopic difference among their central arbors was unaffected by this manipulation. Finally, we saw that region-specific Mrgprd⁺ central terminal arbors are present from the earliest postnatal stages, before skin terminals are evident. In summary, we find that region-specific organization of Mrgprd⁺ neuron central arbors is present shortly after initial central terminal formation, which likely develops independently of peripheral target innervation. Our data suggest that either cell-intrinsic and/or DH pre-patterning mechanisms are likely to establish this somatotopic difference.

Abstract

Somatotopic organization of a major class of mammalian nociceptors is evident at early postnatal stages. We performed sparse genetic tracing of Mrgprd⁺ nociceptor axonal arbors in P3 mouse spinal cords. The presence of region-specific morphologies at early stages of spinal cord innervation suggests that pre-patterning mechanisms could establish these differences.

*Correspondence should be addressed to William Olson, wolson6@jhmi.edu, and Wenqin Luo, luow@mail.pennmedicine.upenn.edu.

Keywords

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1. Introduction

Animals use distal limb regions (paws/hands) for exploration and object manipulation. To facilitate these behavioral requirements, distal limb somatosensory circuits have several region-specific (somatotopic) organization mechanisms to increase the sensitivity of these skin regions. One such mechanism involves the ‘magnification’ of distal limb representations in the central nervous system, meaning distal limb regions are overrepresented in the spinal cord and cortex circuits (Fitzgerald & Swett, 1983; Millecchia et al., 1991; Penfield & Boldrey, 1937). While other sensory systems use analogous forms of regional magnification in various species (Ahnelt, 1998; Challis et al., 2015; Daniel & Whitteridge, 1961; Suga et al. 1987), the developmental mechanisms used by sensory systems to differentially allocate circuit space in this manner have not been clearly defined.

Similar to light touch, humans have increased sensitivity for pain in the distal limbs and fingertips (Mancini et al., 2014; Mancini et al., 2013). Recently, we used single-cell genetic tracing of *Mrgprd*⁺ mouse nociceptors to characterize the somatotopic organization of mammalian pain neurons (Olson et al., 2017). While we found no obvious mechanisms for increased sensitivity in paw peripheral circuits (these neurons have lower innervation density in the paw glabrous skin compared to the limb hairy skin and have similar terminal areas across skin regions), we identified a novel region-specific organization of the central terminal arbors of these neurons in the spinal cord dorsal horn (DH) (Figure 1A). Specifically, paw and trunk innervating nociceptors have distinct terminal morphologies

(‘round’ vs. ‘long’) in the DH, such that paw nociceptors have a much wider mediolateral spread. Interestingly, this central terminal morphological distinction closely correlates with increased synaptic transmission efficiency in the spinal cord and decreased threshold for activating these afferents in the paw skin. This work suggests that ‘magnification’ of these nociceptors through region-specific central arbors could expand the representation of the paw in the spinal cord and downstream circuits. These results also indicate that *Mrgprd*⁺ neurons, which as a population show very similar molecular markers and anatomical features, differentially direct their central terminal arbor formation based on their somatotopic location during development. Our ability to trace and genetically manipulate single primary afferents from this population offers a unique ability to study the developmental mechanisms underlying region-specific organization of this sensory circuit.

Previous research has characterized the development of somatosensory topographic maps and investigated potential mechanisms. Somatosensory afferents, including nociceptors, are dorsal root ganglion (DRG) or trigeminal ganglion neurons that have two axonal branches innervating the skin (or other tissues) and the spinal cord, respectively. Somatosensory circuits of the DH have a ‘flipped’ topographic map: in the lumbar enlargement (innervating the hindlimbs), the distal limbs (foot and toes) are represented in medial DH while the proximal limbs are represented in the lateral DH (Figure 1A) (Brown & Fuchs, 1975; Swett & Woolf, 1985). Nerve tracing experiments have shown that cutaneous sensory topographic maps formed early in development are similar to the mature pattern (Mendelson et al. 1992; Smith, 1983). Based on the rough coincidence of peripheral and central target innervation, and based on the proximal-to-distal progression of hind limb epidermal innervation, it was proposed that peripheral innervation could drive formation of a correct somatotopic map in the DH (Reynolds et al., 1991). However, subsequent studies provided evidence against this model (Mirnics & Koerber, 1995; Sharma et al., 1994; Wang & Scott, 2002). In general, the mechanisms directing the somatotopically-appropriate wiring of somatosensory afferents remain unclear.

Since the previous work could not resolve the single-cell structure of DRG neurons, the mechanisms for the disproportionate representation (magnification) of paw regions in somatosensory circuits have not been examined. Multiple potential models exist for the mechanisms that could instruct the magnification of paw regions among *Mrgprd*⁺ afferents. Peripheral cues from different skin regions (such as region-specific signaling molecules and/or activity-dependent processes) could play a major instructive role for the formation of region-specific central arbor morphologies in the DH (Figure 1B). Alternatively, *Mrgprd*⁺ neurons could form immature, somatotopically homogenous arbors that are postnatally reorganized into region-specific morphologies (Figure 1C). Finally, DRG afferents may form region-specific arbor morphologies during their initial terminal formation, suggesting mechanisms that ‘pre-pattern’ this somatotopic difference prior to DH innervation (Figure 1D). These models are not mutually exclusive of one another. For example, peripheral cues could even be involved in the prepatterned model, if such cues could act very quickly (Jackman & Fitzgerald, 2000).

Here, we used population-level tracing to characterize the postnatal development of *Mrgprd*⁺ nociceptor central and peripheral terminal arbors. In addition, we performed single-cell

ablation of *Ret* to disrupt peripheral target innervation of these neurons and analyze the effect on their central arbor morphology in the DH. Lastly, we performed single-cell tracing of *Mrgprd*⁺ neurons in early postnatal animals, right after their initial innervation of the DH. These experiments show that region-specific arbors are present from the very early postnatal time we examined (supporting the ‘pre-patterned’ model), and that central terminal development slightly precedes, and occurs independently of, peripheral terminal development/refinement. Taken together, our results indicate that the region-specific single-cell organization of mammalian nociceptor central terminal arbors is likely to be dictated through pre-patterning mechanisms that are intrinsic to the DRG neurons themselves and/or by mechanisms within the spinal cord.

2. Materials and Methods

2.1 Mouse strains

Mice were raised in a barrier facility in Hill Pavilion, University of Pennsylvania. All procedures were conducted according to animal protocols approved by Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania and National Institutes of Health guidelines. *Mrgprd*^{EGFPf} (RRID:MMRRC_036772-UNC, MMRRC Cat# 036772-UNC), *Mrgprd*^{CreERT2} (RRID:IMSR_JAX:031286, IMSR Cat# JAX:031286), *Rosa*^{iAP} (RRID:IMSR_JAX:009253, IMSR Cat# JAX:009253), and *Ret*^{f(CFP)} (RRID:MGI:4459058, MGI Cat# 4459058) mice have been previously described (Badea et al., 2009; Olson et al., 2017; Uesaka et al., 2008; Zylka et al., 2005). *Ret*^{null} allele mice were generated by crossing a conditional *Ret* line (*Ret*^{f/f}) (RRID:IMSR_JAX:028548, IMSR Cat# JAX:028548) (Luo et al., 2007) with a germline Cre mouse line (*Sox2*^{Cre}) (RRID:IMSR_HAR:3359, IMSR Cat# HAR:3359) (Hayashi et al., 2002).

2.2 Genetic labeling of *Mrgprd*⁺ nociceptors

To sparsely label *Mrgprd*⁺ nociceptors, we set up timed pregnancy matings of *Mrgprd*^{CreERT2} mice with *Rosa*^{iAP} or *Ret*^{f(CFP)} mice. Population-level labeling was achieved through either prenatal or postnatal tamoxifen treatment. For prenatal treatment, pregnant females were given tamoxifen (Sigma, T5648) along with estradiol (Sigma, E8875, at a 1:1000 mass estradiol: mass tamoxifen ratio) and progesterone (Sigma, P3972, at a 1:2 mass progesterone: mass tamoxifen ratio) in sunflower seed oil via oral gavage at E16.5-E17.5, when *Mrgprd* is highly expressed in mouse non-peptidergic nociceptors (Chen et al., 2006).

2.3 Tissue preparation and histology

Procedures were conducted as previously described (Fleming et al., 2012; Niu et al., 2013). Briefly, mice were euthanized with CO₂ and transcardially perfused with 4% PFA/PBS, and dissected tissue (skin, spinal cord, DRG) was post-fixed for 2 hr in 4% PFA/PBS at 4° C. Tissue used for section immunostaining was cryo-protected in 30% sucrose/PBS (4% overnight). Frozen glabrous skin and DRG/spinal cord sections (20–30 µm) were cut on a Leica CM1950 cryostat. Immunostaining was performed as described previously. DRGs for whole mount immunostaining were treated as described directly after post-fixation. The following antibodies were used: chicken anti-GFP (RRID:AB_10000240, Aves Labs Cat# GFP-1020, 1:1000), rabbit anti-GFP (RRID:AB_221569, Molecular Probes Cat# A-11122,

1:1000). The specificity of these commercial antibodies has been well documented in previous literature (Fleming et al., 2015; Niu et al., 2013). Tissue (skin or spinal cord with attached DRGs) for whole mount AP color reaction with BCIP/NBT substrate was treated as previously described. Following AP color reaction labeling, tissue was cleared in 1:2 (v:v) benzyl alcohol + benzyl benzoate (BABB) for imaging (Niu et al., 2013).

2.4 Image acquisition and data analysis

Images were acquired either on a Leica DM5000B microscope (brightfield with a Leica DFC 295 camera and fluorescent with a Leica 345 FX camera), on a Leica SP5II confocal microscope (fluorescent), or on a Leica M205 C stereoscope with a Leica DFC 450 C camera (brightfield). Image quantification was performed in ImageJ. Graphs and statistical analyses were created in GraphPad Prism5.

For growth normalization of skin terminal densities (Figure 2), plantar paws (n=3 animals for each age) were imaged and fitted ellipses were drawn over the six mouse foot pads. Major axis lengths (proximodistal axis) were averaged across animals. Absolute skin terminal densities were multiplied by a normalization factor: Growth-normalized density (Age) = Absolute density (Age) X (Mean paw length (Age) / Mean paw length (3 pw)).

For growth normalization of DH layer thickness (Figure 2), the maximum mediolateral width of the *Mrgprd*^{EGFPf} innervation layer was measured (n=3 sections from separate animals for each age). Absolute layer thickness measurements were multiplied by a normalization factor: Growth-normalized thickness (Age) = Absolute thickness (Age) X (Mean DH width (Age) / Mean DH width (3 pw)).

For single-cell width measurements in sectioned DH tissue (Figure 3), serial DH sections were imaged, and individual arbors were identified by comparing adjacent sections. The DH of lumbar enlargement sections (L3-L5) were divided into thirds based on the maximum mediolateral width of the DH. Cells with most of their width lying in the medial third were classified as “Medial Lumbar”, cells were otherwise classified as “Lateral Lumbar”.

3. Results

3.1 Population-level characterization of postnatal development of peripheral and central terminals of *Mrgprd*⁺ DRG neurons

To investigate whether region-specific arbor development may be driven by peripheral and/or central mechanisms, we used *Mrgprd*^{EGFPf} knock-in mice (Zylka et al., 2005) to characterize the postnatal (P1 – 3 postnatal weeks, pw) innervation of non-peptidergic nociceptors in the paw glabrous skin and the lumbar spinal cord enlargement DH. *Mrgprd* is first expressed at E16.5 in mice and specifically marks the non-peptidergic nociceptor population (Chen et al., 2006). EGFP expression in this knock-in mouse line faithfully indicates expression of *Mrgprd*. This genetic tool offers advantages over previous approaches since it specifically labels non-peptidergic fibers (unlike nerve filling) and avoids issues related to the dynamic expression of immunostaining markers (Jackman & Fitzgerald, 2000; Reynolds et al., 1991).

Peripherally, mature non-peptidergic nociceptor axons travel to the skin in the cutaneous nerves, grow a fiber plexus parallel to the skin surface in the dermis, and send perpendicular terminals out of the subepidermal plexus that penetrate the epidermis (Olson et al., 2017; Zylka et al., 2005). Most of the paw epidermis is not innervated by *Mrgprd*^{EGFPf} fibers at P1, except for a few rudimentary terminals (Figure 2A). From P1 to P7, there is a rapid phase of nerve terminal growth, as indicated by a great increase in density of both primary (leaving the subepidermal plexus) as well as secondary/tertiary (branches off primary terminals) *Mrgprd*⁺ fiber branches (Figure 2A-C, K, M, N). It is then followed by a refinement phase, as indicated by a decrease in density from P7 to 3pw. By 3pw, no secondary or tertiary branches are present (Figure 2G, H, L-N). This pattern is true when quantified as absolute terminal densities or as growth-normalized values (Figure 2M, N).

Centrally, non-peptidergic nociceptor axons travel through the dorsal roots, grow for 0–2 spinal segments rostrally or caudally in Lissauer's tract at the dorsolateral margin of the spinal cord, and then dive ventrally to innervate layer II of the DH (Olson et al., 2017; Zylka et al., 2005). *Mrgprd*^{EGFPf} fibers have established a thick (in the dorsoventral extent) terminal layer by P1 in the DH (Figure 2D). This layer shows a 4-fold (absolute) decrease in layer thickness, reaching the mature layer thickness by P7 (Figure 2D-F, I, J). This decrease of layer thickness is also true when quantified by growth-normalized values (Figure 2O, P). In summary, while *Mrgprd*^{EGFPf} peripheral terminals are still undergoing initial outgrowth in the epidermis (the first postnatal week), their central terminals are in the process of refining to their mature thickness in the DH. These results indicate that, although both central and peripheral terminals show outgrowth and refinement during the postnatal period, central terminal development of *Mrgprd*⁺ neurons precedes peripheral development in this regard.

3.2 Non-peptidergic nociceptor central arbor formation is independent of peripheral terminal innervation

Next, to investigate whether non-peptidergic nociceptors utilize peripheral cues or processes to establish region-specific central arbor morphologies, we took advantage of a genetic manipulation to disrupt their peripheral target innervation. Previous work has shown that, upon DRG-specific deletion of the Glial cell line-derived neurotrophic factor receptor *Ret*, non-peptidergic nociceptors fail to innervate their final peripheral target, the skin epidermis, while their central terminals remain in DH layer II (Luo et al., 2007). However, these experiments did not trace single-cell morphology, so it remains unknown whether their central arbors are altered after this failure in peripheral target innervation.

We ablated *Ret* from individual non-peptidergic nociceptors and quantitatively measured their regional single-cell width (mediolateral) in the DH. We used a mouse line in which Cre-dependent inactivation of *Ret* also leads to expression of CFP (*Ret*^{f(CFP)}) (Uesaka et al., 2008). In *Mrgprd*^{CreERT2/+}; *Ret*^{f(CFP)/null} mice (*Ret* CKO), which carry the *Ret*^{f(CFP)} allele along with a *Ret* null allele, low-dose prenatal tamoxifen (0.5 mg at E16.5-E17.5) generated sparse *Ret*-null non-peptidergic nociceptors that are labeled with CFP (Figure 3C). In *Mrgprd*^{CreERT2/+}; *Ret*^{f(CFP)/+} littermate controls (Control), this same treatment sparsely labeled *Ret* heterozygous non-peptidergic neurons with CFP (Figure 3A). As expected for

Ret deletion, sparsely labeled CFP⁺ DRG cell bodies were smaller in *Mrgprd*^{CreERT2/+}; *Ret*^{fl(CFP)/null} mutant mice (data not shown), but the number of CFP⁺ neurons was not decreased (average CFP⁺ neurons/DRG: control = 58.7 ± 10.3, n = 14 DRGs from 3 animals, mutant = 70.4 ± 24.6 n = 22 DRGs from 3 animals) (Figure 3A, C, I). In addition, while sections of glabrous skin from control mice showed sparsely labeled terminals with mature epidermal endings, sections from mutant skin showed axon bundles in the dermis but no mature endings in the epidermis, consistent with previous work (Luo et al., 2007) (Figure 3B, D).

Lastly, serial sectioning of control and mutant thoracic (T6-T12) and lumbar (L3-L6) spinal cords showed that the round-vs.-long distinction in central terminal morphology was unaffected by *Ret* deletion (Figure 3E-H). We imaged through serial sections and measured the maximum mediolateral width of sparse-labeled neurons. The medial lumbar neurons were roughly twice as wide, on average, as either lateral lumbar or thoracic neurons (thoracic = 28.7 ± 5.2 μm, lateral lumbar = 33.5 ± 6.5 μm, medial lumbar = 64.7 ± 14.2 μm, n = 287 neurons from 3 mice) in control mice (Figure 3E&F, J). A similar difference was also seen in mutant spinal cords (thoracic = 29.0 ± 7.0 μm, lateral lumbar = 33.1 ± 8.7 μm, medial lumbar = 68.6 ± 18.1 μm, n = 239 neurons from 3 mice (Figure 3G&H, J), indicating that the round-vs.-long distinction is maintained in these mutant neurons. Additionally, no difference in the average width of round or long terminals was observed between control and *Ret* null non-peptidergic neurons (Figure 3J), suggesting that *Mrgprd*⁺ neurons grow normal central terminal arbor morphologies in the absence of *Ret*. In summary, this genetic disruption experiment suggests that *Mrgprd*⁺ afferent peripheral target (epidermal) innervation is dispensable for the formation of region-specific central arbors.

3.3 Sparse labeling reveals region-specific *Mrgprd*⁺ central arbors from the earliest stages of central innervation

Lastly, we asked whether the region-specific central terminal arbors of non-peptidergic DRG neurons might be established through postnatal reorganization (Figure 1C), or instead are present from the earliest stages of DH innervation (Figure 1D). We performed sparse genetic tracing of non-peptidergic neurons at early postnatal stages by crossing tamoxifen-dependent *Mrgprd*^{CreERT2} mice with *Rosa*^{iAP} alkaline phosphatase reporter mice (0.25 mg of tamoxifen was given at E17.5) (Olson et al., 2017). Sparse terminals were seen after AP staining of skin and spinal cord tissue. Immature skin terminals could be seen at P3 but not P1 (Figure 4E&F), consistent with the few *Mrgprd*^{EGFP} fibers in the epidermis at P1 (Figure 2). In addition, while their central arbors still appear immature at P1 and P3, region-specific arbor morphologies (the somatotopic organization of central arbors) are seen in both P1 and P3 spinal cords (Figure 4A-D). Like the mature DH organization of these afferents (Olson et al., 2017), medial lumbar enlargement (paw representation, outlined in Figure 4B, D) regions contain mediolaterally wide arbors while lateral lumbar enlargement and thoracic (proximal hindlimb, trunk) regions contain mediolaterally thin arbors (Figure 1A). While terminals at this stage still appear immature, this result indicates that these neurons form region-specific arbor morphologies during their initial innervation.

4. Discussion

In summary, we found that (1) postnatal development/refinement of central terminals precedes development/refinement in the periphery, (2) genetic disruption of peripheral target innervation had a negligible effect on region-specific central arbors, and (3) region-specific arbors are present from the earliest postnatal stages. While non-peptidergic central terminal arbors do show very clear postnatal layer thickness refinement (in the dorsoventral axis) (Figure 2), and while early postnatal arbors have a somewhat immature morphology (Figure 4), their region-specific structure is apparent from the earliest stages of DH innervation. Our results suggest that, while clear postnatal refinement does occur for both central and peripheral terminals, these neurons do not rely on postnatal refinement in order to establish their region-specific central arbor morphologies.

Trophic factor signaling plays important roles in neurite arbor morphogenesis. For example, *Ret* signaling is absolutely required in rapidly adapting A β low-threshold mechanoreceptors for the growth of central collaterals innervating the spinal cord DH (Fleming et al., 2015; Luo et al., 2009). *Ret* is also the main neurotrophic factor receptor expressed by Mrgprd⁺ DRG neurons (Luo et al., 2007). For these neurons, however, *Ret* deletion did not affect their DH arbor morphology at all, despite the obvious deficit of peripheral epidermis innervation. Given that (1) severely disrupting epidermal innervation had a negligible effect on central arbor morphologies and that (2) region-specific arbors are evident at P1, before distal limb Mrgprd⁺ nociceptors even innervate epidermis, we think that peripheral epidermal cues play a minor role in instructing formation of somatotopic central arbors of Mrgprd⁺ neurons. Past work has established that molecular cues from the dermis induce the development of hair follicles in hairy skin during embryogenesis, thereby playing critical roles in regional skin patterning (Millar, 2002; Sengel, 1990). Many molecular players in the dermis and epidermis have been identified, including the Wnt/ β -catenin pathway, although the exact identity of the initial dermal signal remains to be determined (Millar, 2002). While our developmental characterization and *Ret* deletion experiments suggest that pre-patterning mechanisms are likely to control regional Mrgprd⁺ central arbor growth, our results do not rule out that *Ret* deleted neurons may receive dermally or epidermally-derived cues in the dermis. Future studies could examine whether mutant mice with severe skin patterning deficits, such as *Lef1* mutants (van Genderen et al., 1994), might still display region-specific Mrgprd⁺ morphologies.

Earlier spinal nerve backfilling experiments indicated that DRG central projections form topographically appropriate innervation patterns from the earliest stages of innervation (Mendelson et al., 1992; Smith, 1983) and that spinal cord somatotopic map formation of DRG afferents does not rely on cues from the periphery (Sharma et al., 1994; Wang & Scott, 2002). Here we used single-cell tracing and genetic disruption to show that development of region-specific arbor morphologies, an important mechanism for magnifying paw representation in the central nervous system, occurs early and independent of peripheral target innervation. Our results suggest that DRG-intrinsic and/or DH-intrinsic pre-patterning mechanisms may instruct this difference. While much is known regarding the mechanisms differentiating lower motor neuron groups innervating various muscles (Dasen & Jessell, 2009; Dasen et al., 2005), very little is understood regarding mechanisms that might

differentiate cutaneous somatosensory afferents innervating various regions. These could include transcription factor cascades and/or progressive neurogenesis, and future studies could use RNA sequencing at various developmental stages and/or neuronal birth dating techniques to investigate these mechanisms.

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Figure 1. Models of potential developmental mechanisms for region-specific Mrgprd⁺ central arbors.

A, Mature somatotopic organization of Mrgprd⁺ afferents. Proximal hind limb afferents (purple) grow 'long and thin' central arbors in the lateral DH, while plantar paw afferents (orange) grow 'round and wide' central arbors in the medial DH. DH is drawn as a transverse section. B, Peripheral cues model. Upon innervation of the skin around birth, cues from different skin regions direct region-specific developmental of central terminal arbors. DH is drawn from a top-down view. C, Central reorganization model. Afferents grow immature, somatotopically homogenous arbors that are postnatally reorganized into region-

specific arbor morphologies. D, Pre-patterned model. Afferents grow somatotopically distinct arbor morphologies during their initial innervation of the DH. These models are not mutually exclusive.

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Figure 2. Postnatal central and peripheral terminal development of *MrgprdEGFPf* non-peptidergic nociceptors.

A-J, GFP immunostaining of glabrous skin (A-C, G, H) and DH (D-F, I, J) sections from *Mrgprd^{EGFPf}* mice at the indicated ages. K, L, Higher magnification views of peripheral terminals, indicating secondary/tertiary branches (pink asterisks) growing off primary branches (white arrows) in P7 but not 3pw skin. M, N, Quantification of densities of absolute and growth-normalized (N, see Methods) glabrous skin primary, secondary and tertiary branches during postnatal development. Skin terminals show overgrowth during the first week. Dotted yellow lines (A-C, G, H) indicate dermal/epidermal border. O, P,

Quantification of absolute (O) and growth-normalized (P, see Methods) DH layer thickness at the indicated ages. DH terminals show a refinement during the first week, at which point they remain at their mature thickness. $n = 3$ animals per stage. Scale bars = 50 μm (A-J), 20 μm (K, L).

Figure 3. Genetic disruption of peripheral target innervation does not affect region-specific central arbor morphologies.

A, C, Whole mount CFP immunostaining of sparse labeled 2–3pw *Mrgprd*^{CreERT2}; *Ret*^{f(CFP)/+} (control, A) and *Mrgprd*^{CreERT2}; *Ret*^{f(CFP)/null} (mutant, C) DRGs (0.5 mg tamoxifen at E16.5–E17.5). B, D, CFP immunostaining of sectioned glabrous skin shows epidermal endings in control (B) but not mutant (D) mice, indicating a lack of peripheral terminals in Ret null nociceptors. White arrows, mature epidermal endings. White arrowheads, dermal axonal bundles. E–H, CFP immunostaining of serial DH sections from control (E&F) and mutant (G&H) mice shows sparse labeled terminals. I, Quantification of

the number of CFP+ neurons / DRG. n = 14–22 DRGs from 3 animals per genotype. J, Maximal mediolateral width of sparse labeled neurons from control and mutant mice shows that the round-vs.-long distinction is still present in mutant mice. n = 239 (mutant), 287 (control) neurons from 3 mice per genotype. Scale bars = 100 μ m (A&C), 20 μ m (B&D), 50 μ m (E-H).

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Figure 4. Region-specific Mrgprd⁺ DH arbors are evident during the earliest stages of innervation.

A-F, AP color reaction of spinal cord (A-D) and skin (E&F) tissue from P1 or P3 *Mrgprd^{CreERT2}; Rosa^{iAP}* mice (0.25 mg tamoxifen at E17.5). Sparse skin terminals were not seen at P1. While early postnatal nociceptors are still immature, the round vs. long distinction can be seen in B & D. Scale bars = 250 μm (A-D), 100 μm (E&F).