

1 **A neuroskeletal atlas of the mouse limb**

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18

1 **ABSTRACT**

2 Nerves in bone play well-established roles in pain and vasoregulation and have been associated with
3 progression of skeletal disorders including osteoporosis, fracture, arthritis and tumor metastasis.
4 However, isolation of the region-specific mechanisms underlying these relationships is limited by our
5 lack of comprehensive maps of skeletal innervation. To overcome this, we mapped sympathetic
6 adrenergic and sensory peptidergic axons within the limb in two strains of mice (B6 and C3H). In the
7 periosteum, these maps were related to the surrounding musculature, including entheses and
8 myotendinous attachments to bone. Locally, three distinct patterns of innervation (Type I, II, III) were
9 defined within established sites that are important for bone pain, bone repair, and skeletal homeostasis.
10 In addition, we mapped the major nerve branches and areas of specialized mechanoreceptors. This
11 work is intended to serve as a guide during the design, implementation, and interpretation of future
12 neuroskeletal studies and was compiled as a resource for the field as part of the NIH SPARC
13 consortium.

14

1 Peripheral nerves target nearly every organ in the body to communicate afferent and efferent
2 signals to and from the brain; the skeleton is no exception¹. Neurons in and around the bone have well-
3 established roles in the generation and maintenance of pain associated with skeletal pathologies
4 including fracture, tumor progression, and arthritis²⁻⁴. Within cortical bone and bone marrow, sensory
5 and sympathetic nerve fibers track with the arteriolar vasculature⁵⁻⁷ and coordinate vasoregulatory
6 responses⁸⁻¹¹. Additional functions have also been proposed, including modulation of bone formation
7 and turnover in the context of skeletal development¹², adaptation to mechanical loading^{13,14}, repair, and
8 regeneration¹⁵⁻¹⁷, as well as regulation of bone marrow adipogenesis¹⁸, joint disease¹⁹, and control of
9 hematopoietic cell maturation and egress²⁰.

10 Direct neural modulation of skeletal function is dependent upon the precise localization of axons
11 near target cells and the capacity of the neurons to sense the bone microenvironment and/or to release
12 neurotransmitters. Sensory peptidergic axons, often marked by calcitonin gene-related peptide
13 (CGRP), as well as sympathetic axons containing tyrosine hydroxylase (TH), the rate-limiting enzyme in
14 catecholamine synthesis, are distributed in and on the bone^{6,21}. These neuronal subtypes have been
15 shown to innervate the periosteum in a meshwork pattern, traverse the cortical bone through nutrient
16 canals, and to innervate the marrow cavity⁶. Skeletal axon varicosities, presumed sites of
17 neurotransmitter release and receptor clustering, have been observed in close proximity to bony
18 surfaces and resident osteoblasts, osteoclasts, and their precursors, as well as at sites of marrow
19 adipose tissue and hematopoietic cells^{21,22}. While the morphology and molecular phenotype of skeletal
20 axons have been well studied²³, the inherent challenges of visualizing and quantifying neural fibers in
21 calcified tissue have led to a functional and anatomical neuroskeletal map that is incomplete. This is
22 complicated by the heterogeneity of the bone architecture, the complex tissue interactions therein, and
23 the opposing influences of the sensory and sympathetic arms of the nervous system that converge to
24 influence the physiological functions of the skeleton¹.

25 To begin to overcome this, we used thick section immunohistochemistry and tissue clearing, in
26 concert with high-resolution imaging and axon quantification techniques, to map skeletal innervation in

1 the lower limb with emphasis on comprehensive mapping of axons within and around the femur and
2 tibia. In addition, we defined the location, prevalence, and morphology of specialized mechanoreceptor
3 endings. This information has been compiled into a reference atlas of skeletal innervation that can be
4 used to inform future neuroskeletal studies. This resource was generated as part of the SPARC
5 consortium (Stimulating Peripheral Activity to Relieve Conditions), a call by the National Institutes of
6 Health in the United States to generate foundational neuroanatomic maps of axons within peripheral
7 tissues with the aim of supporting the development of novel neuroceuticals for targeted treatment of
8 pain and other conditions.

9

10 **RESULTS**

11 ***Validation of neuroskeletal immunoassay and axon quantification in the C3H and B6 mouse*** 12 ***tibia***

13 Prior to creating the atlas, we validated our axon tracing and immunostaining paradigm relative
14 to previous publications (Table 1)^{6,24,25}. To achieve this, we quantified CGRP+ sensory and TH+
15 sympathetic axons in the periosteum and bone marrow of 12-week-old male C57BL/6J (B6) and
16 C3H/HeJ mice (C3H) at four levels along the length of the tibia. Region-specific bone parameters
17 including cortical and trabecular bone volume fraction (BVF) were also assessed. Levels of the tibia
18 selected for analysis were distinguished by bone morphology at the metaphysis, mid-diaphysis with
19 tibial ridge, the diaphysis proximal to the tibia-fibula junction (TFJ), and the diaphysis just distal to the
20 TFJ. Analyzed levels approximated to 10% (L1), 30% (L2), 50% (L3), and 60% (L4) distal to the knee
21 for B6 mice (Fig 1.a,b). Consistent with previous reports^{26,27}, the diaphyseal cortical BVF was
22 significantly higher in C3H than B6 mice (L2, L3; Fig.1c). However, cortical BVF was matched in the
23 two strains at both the proximal and distal metaphysis (L1, L4; Fig.1c). Trabecular BVF was
24 comparable in both strains at the proximal metaphysis (L1; Fig.1g), also consistent with prior
25 comparisons at this region and age²⁸. Trabecular bone was largely absent in the tibial diaphysis of both
26 B6 and C3H mice (L2-4).

1 At each level, sensory and sympathetic axons were traced and the total fiber length within each
2 compartment was expressed relative to the total volume (Fig.1b). As reported previously for femur
3 (Table 1), the periosteum was the most densely innervated compartment of the tibia for both B6 and
4 C3H mice. Within the periosteum, we found that CGRP+ sensory axons demonstrated level-specific
5 densities that were dependent on mouse strain (Fig.1d). By contrast, TH+ sympathetic axon density
6 was relatively constant along the length of the periosteum. C3H mice had a greater absolute TH+ fiber
7 density at all levels relative to B6 (Fig.1e). Independent of strain, periosteal CGRP+ sensory fibers were
8 more prevalent than TH+ sympathetics at all levels of the tibia (periosteal CGRP:TH ratio > 1; Fig.1f).
9 This ratio was higher in B6 animals than C3H (Fig.1f). Within the marrow cavity, innervation density
10 increased from proximal to distal independent of strain (Fig.1h,i). Converse to the ratio of neural
11 subtypes observed in the periosteum, TH+ sympathetic innervation consistently exceeded CGRP+
12 sensory innervation in all regions of the bone marrow (CGRP:TH ratio < 1; Fig.1j). Overall, our
13 immunolabeling and analysis paradigm corroborates previous reports of compartmentalized innervation
14 density (Table 1), and additionally demonstrates that this varies by strain. Our results further establish a
15 gradient of bone marrow axon density that increases from proximal to distal along the length of the
16 tibia.

17

18 ***Identification of three distinct patterns of periosteal innervation (Type I, II, III)***

19 Next, we applied this validated immunolabeling and tracing paradigm to assess the periosteal
20 axon profiles throughout the entire length and circumference of the tibia and femur of male and female
21 12-week-old C3H and B6 mice. The periosteum is the layer of connective tissue that envelops the bone
22 and provides a supportive microenvironment for vasculature, nerves, and periosteal cells^{29,30}. We
23 identified three consistent patterns of adult periosteal innervation that aligned with the attachments
24 formed between the periosteum and the surrounding tissues, independent of mouse sex or strain.
25 Within each innervation pattern, we observed variations in local axon density and orientation that

1 should be considered for future studies pertaining to the relationships between nerves and bone, as
2 described below.

3

4 *Type I: Aneural*

5 Many regions of the bone surface were aneural, completely lacking CGRP+ sensory or TH+
6 sympathetic innervation (Fig.2a-d). Aneural regions were observed primarily where tendons or
7 ligaments interfaced with the bone at sites called entheses. There are two main classes of entheses:
8 fibrocartilaginous and fibrous^{31,32}. Fibrocartilaginous entheses comprise continuous zones connecting
9 tendinous fibers through unmineralized and mineralized fibrocartilage tissue that blend imperceptibly
10 into bone (Fig.2a). At fibrous entheses, the tendon is continuous with a thick fibrous layer of the
11 periosteum (Fig.2b). The only axons found in these regions were located in the fascial tissue lining the
12 tendon or ligament, often referred to as the epitenon. Type I regions greatly contributed to the
13 discontinuous nature of periosteal innervation.

14

15 *Type II: Diving*

16 In other regions, axons transited directly from the associated muscle tissue through the fibrous
17 and cambium layers of the periosteum at a 90-degree angle to the cortical bone surface (Fig.2e-h).
18 Type II regions contained TH+ sympathetic axons and an abundance of CGRP+ sensory axons that
19 appeared to dive from the sarcolemma or muscle fascia to the bone surface. Regions with a diving
20 innervation pattern aligned most closely with sites where muscle is connected to periosteal bone by
21 partially mineralized fiber anchors, known also as extrinsic fibers or Sharpey's fibers³³.

22

23 *Type III: Meshwork*

24 The most prevalent pattern of innervation observed consisted of a meshwork of CGRP+
25 sensory and TH+ sympathetic axons within the periosteum where fascia attached to the bone (Fig.2i-l).
26 At these sites, the periosteal layer is relatively thin and the axons run close to the bone, parallel to the

1 cortical surface. This Type III meshwork pattern is apparent in periosteal whole-mount preparations
2 taken from regions of the bone where the absence of tendinous or extrinsic fibers allows for separation
3 of the periosteum from adjacent tissues (Fig.2j)^{7,24,34}.

4

5 ***Generation of 2D and 3D maps of Type I-III periosteal innervation patterns in relation to***
6 ***overlying nerves and muscles***

7 *Nerve supply of the limb*

8 Axons innervating the femur are primarily derived from the femoral nerve (Fig.3a). The femoral
9 nerve runs along the anteromedial aspect of the femur and provides innervation to the muscle groups
10 that function as knee extensors (rectus femoris and vastus muscles)^{35,36}. It also provides the
11 innervation to the anterior and medial aspects of the femur bone³⁷. The obturator nerve runs nearby,
12 also anteromedial to the femur, and innervates the hip adductors (gracilis anterior, gracilis posterior,
13 adductor longus, adductor magnus) and some hip rotators in the gluteal region (quadratus femoris,
14 obturator externus). There is no current evidence that the obturator innervates the femur bone. The
15 sciatic nerve runs along the posterior aspect of the femur and innervates the posterior femoral muscles
16 that function as hip extensors (semitendinosus, semimembranosus, caudofemoralis, biceps
17 femoris)^{35,36}. A segment of the posterior femur bone is also innervated by the sciatic nerve³⁸.

18 Slightly above the posterior aspect of the knee, the sciatic nerve branches into the tibial and
19 common peroneal nerves. Innervation of the tibia bone is primarily derived from the sciatic nerve
20 (Fig.3b) with evidence in humans for minor sensory contributions by the terminal branches of the
21 femoral nerve in the medial regions of the proximal tibial epiphysis/metaphysis and distal epiphysis^{37,38}.
22 The tibial branch of the sciatic nerve runs down the leg on the posterior side of the tibia with the
23 peroneal artery and veins and innervates the knee flexors, ankle plantar flexors, hip extensors, and hip
24 motion groups in addition to the tibia bone^{35,36}. By contrast, the common peroneal nerve wraps around
25 the fibula to the front of the tibia. It sends a branch termed the recurrent articular nerve to the region of
26 the proximal epiphysis and joint. Distally, it splits into the superficial peroneal nerve and the deep

1 peroneal nerve. The deep peroneal nerve runs between the tibia and fibula along the interosseous
2 membrane and, in addition to innervating the tibia bone, it sends smaller branches that innervate
3 muscles of the ankle everter and dorsiflexor families, including the tibialis anterior^{35,36} .
4

5 *Two-dimensional muscle maps with Type I-III innervation patterns*

6 The periosteum is a fascial connective tissue that is continuous with other layers of limb fascia
7 including the epitenon and epimysium. To facilitate the consideration of periosteal axon patterns within
8 the context of these boundaries, we created representative schematics showing muscle attachments
9 and muscle labeling (Fig.3c,d). These were adapted from Charles et. al³⁹ with muscle placement and
10 innervation patterns adjusted based on our serial immunostaining analysis. Type I, II, and III innervation
11 patterns were illustrated on the bone-tissue interface in the cross-sectional schematics and color-coded
12 in relation to surrounding muscle attachments. This process was repeated along the length of the femur
13 and tibia, as presented in the atlas compilation (Supplementary Files A-D) and overview section below.
14

15 *Three-dimensional Type I-III innervation pattern maps*

16 In addition to the 2D maps, the localization of each periosteal innervation pattern was mapped
17 onto the surface of the femur and tibia and color-coded to match surrounding muscle groups (Fig.3e,f).
18 Type I aneural regions were localized to the epiphyses and metaphyses of the tibia and femur. Type II
19 regions were primarily localized in the metaphyses and along the diaphyses of the long bones. There
20 were noted differences in the prevalence of Type II regions between B6 and C3H bones; these can be
21 explored in more detail in the atlases, as detailed below (Supplementary Files A-D). Type III innervation
22 was prominent on bone surfaces interfaced with loosely-adherent fascia, such as with extra-skeletal
23 adipose tissue, epitenon, and unanchored areas of the epimysium. A Type III pattern was also found on
24 the intraosseous fascial membranes between connected long bones, such as the tibia and fibula.
25

26 ***Mapping of specialized mechanoreceptor endings***

1 The skeleton is primarily innervated by neurons with small, finely myelinated and/or
2 unmyelinated axons²³. However, in specific regions, large diameter axons with specialized
3 mechanoreceptor endings known as Pacinian corpuscles have also been identified in association with
4 the interosseous membranes and the periosteum of large mammals^{40,41}. This has not been clearly
5 demonstrated or mapped in the mouse. To overcome this, we generated reporter mice with a floxed Ai9
6 allele expressing Cre recombinase under control of the *myelin protein zero* (P_0) gene promoter to label
7 mature myelinating Schwann cells and their precursors⁴². Schwann cells are the main glial cells of the
8 peripheral nervous system. In addition to labeling myelinated axons, we found that the P_0 reporter
9 labels the lamellar Schwann cell layers that make up the outer structure of the Pacinian corpuscle.
10 Whole-bone tissue clearing and light-sheet microscopy of the forearm of the P_0 -reporter mouse
11 revealed major nerve branches and the distribution of Pacinian corpuscles along the lateral aspect of
12 the ulna 50-70% distal to the elbow (Fig.4a-c). In cross-sections, Pacinian corpuscles could be
13 identified morphologically as a single myelinated sensory fiber (NF200+) in the center of a lamellar
14 capsule that was closely approximated to, though not continuous with, the periosteum (Fig4d-f). Serial
15 cross-section examination of Pacinian corpuscles in the limb localized these specialized
16 mechanoreceptors exclusively to the mid-diaphyseal and distal regions of the tibia (Fig4.g-i) as mapped
17 in the atlases (Supplementary Files A,B).

18

19 ***Limb atlases – overview and contents***

20 These observations and outcomes were compiled into four interactive atlases that cover the
21 prevalence, localization, and morphology of the CGRP+ sensory and TH+ sympathetic nerve fibers in
22 and around the femur (Supplementary Files A,B) and tibia (Supplementary Files C,D), in addition to
23 their relationship with the overlying musculature, fascial tissue, and perilipin-positive bone marrow and
24 extraskelatal adipocytes. Unique cross-sections were selected from serial analysis, annotated by
25 relative distance to the knee joint. The longitudinal range of the bone that comprises similar bone
26 morphology, as well as continuous periosteal innervation patterns and common tissue interfaces is

1 indicated on each page to facilitate sectioning of regions of interest. Additional features and proposed
2 use of these atlases are detailed in Figure 5.

3

4 **DISCUSSION**

5 The innervation of the skeleton is not uniform. In this manuscript, we demonstrate that
6 peripheral axon subtype-specific densities and morphology vary by bone compartment and site and are
7 influenced by mouse genetic strain. This dynamic, region-specific innervation of the bone and bone
8 marrow likely influences pain and cellular function in states of skeletal homeostasis and in targeted
9 areas of bone injury and inflammation. The maps provided here are intended to serve as a baseline
10 reference and guide during the design and selection of regions of interest for future neuroskeletal
11 studies in the femur, tibia, and beyond. For example, though not mapped in detail, our light sheet
12 imaging suggests that the nerve patterns and localization of specialized mechanoreceptors within the
13 forelimb, another common site used for skeletal research, mimic those detailed in the tibia. In addition,
14 the tissue preparation, immunostaining, imaging, and nerve quantification methods reported here may
15 be easily applied in any control or transgenic mouse model of targeted injury, pharmacologic
16 intervention, or systemic disease.

17

18 Previous mapping studies in the femur (Table 1) showed that the periosteum is the most
19 densely innervated compartment, where CGRP+ peptidergic sensory axons predominate, while TH+
20 sympathetic axons are more prevalent in the marrow cavity. For the first time, we corroborate these
21 findings in the tibia, and additionally report a strain-dependent innervation density and CGRP:TH ratio,
22 and a strain-independent proximal-to-distal increase in bone marrow axon density. Neural contributions
23 to adaptive loading, as well as fracture pain and healing, have been largely ascribed to local sensory
24 fibers^{17,43,44}, which predominate in the periosteum. Neural contributions to hematopoiesis and skeletal
25 metastasis have been attributed to sympathetic activity^{45,46}, in line with the intimate association of
26 sympathetic fibers with the arteriolar vasculature and their relative abundance in the marrow. Both arms

1 of the peripheral nervous system have been implicated in skeletal homeostasis, suggesting counter-
2 regulatory mechanisms afforded by sensory and sympathetic innervation co-exist to maintain bone
3 homeostasis, for example by coordinating opposing effects on vascular tone or osteoblast function¹. By
4 contrast, non-overlapping sensory and sympathetic axons within the bone marrow may be suggestive
5 of spatially-defined mechanisms regulating unique progenitor cell pools^{18,20}.

6
7 As detailed throughout the atlases, bone is intimately associated with the nervous, vascular, and
8 muscular systems. Periosteal axon patterning, which we have characterized and defined as Type I-III,
9 is aligned to connections of muscle and fascia to the bone. The endocortical surface, by contrast, is
10 relatively aneural. Axonal orientation is less defined in the marrow, but TH+ fibers generally cluster
11 around central arterioles, while CGRP+ axons are more disperse (Supplementary Files A-D).
12 Particularly exciting potential exists in overlaying this neuroskeletal map with whole-bone maps of load-
13 induced strain and adaptation⁴⁷, vascularity⁴⁸, and stem-cell distribution^{49,50}. For example, strain maps
14 could be related to periosteal innervation patterns to give insight to their functional role in load-induced
15 adaptation; Type II axons may integrate muscle cues to the loaded bone while axons in Type III regions
16 might amplify bone formation on surfaces that lack large connective tissue strain. Additionally, this work
17 highlights the need to consider the role of Schwann cells in the bidirectional cross-talk between nerves
18 and bone. Schwann cells not only insulate axons, but can also initiate and modulate neural signaling,
19 as well as contribute to tissue repair⁵¹⁻⁵³, and remain an underexplored component of the neuroskeletal
20 system.

21
22 As we move forward, it is clear that a systematic and comprehensive map of skeletal innervation
23 related to region-specific bone surfaces and bone marrow compartments is fundamental to assigning
24 physiologic functions to local neuronal populations. In addition, the neuroskeletal field needs
25 standardized nomenclature to facilitate more comprehensive and cohesive studies. In this resource, we
26 have provided protocols and easy-to-apply methods for axon visualization and quantification. In

1 addition, we have presented four interactive atlases that can be applied as a reference during study
2 design, implementation, and interpretation. Lastly, based on this work, we propose that future
3 manuscripts report several basic metrics to help to unify the field. Specifically, (#1) section orientation
4 (transverse, longitudinal), (#2) the analyzed level along the length of the bone (e.g. 40% from the knee),
5 (#3) the bone surface (e.g. anterior tibial ridge), and (#4) the axon pattern(s) analyzed (e.g. Types II
6 and/or III). This will help to ensure reproducibility between studies while moving the field toward
7 development of new therapies for treatment of musculoskeletal pain and disease.
8

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6 Jeff Stirman at SmartSPIM for generating the lightsheet image used in this publication.

7

1 **AUTHOR CONTRIBUTIONS** (CRediT taxonomy)

2 Conceptualization: M.R.L, J.M.B., A.B., E.L.S.

3 Data curation: M.R.L, J.M.B., A.B., I.S., E.L.S.

4 Formal analysis: A.B., I.S., E.L.S.

5 Funding acquisition: E.L.S.

6 Investigation: M.R.L, J.M.B., A.B., I.S., E.L.S.

7 Methodology: M.R.L, J.M.B., A.B., E.L.S.

8 Project administration: E.L.S.

9 Resources: E.L.S.

10 Software: M.R.L, J.M.B., A.B., E.L.S.

11 Supervision: E.L.S.

12 Validation: M.R.L, J.M.B., A.B., E.L.S.

13 Visualization: M.R.L, J.M.B., A.B., E.L.S.

14 Writing – original draft: M.R.L, J.M.B., A.B., E.L.S.

15 Writing – review & editing: M.R.L, J.M.B., A.B., I.S., E.L.S.

16

1 METHODS

2 Table of key resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MOUSE MODELS		
C57BL6J	Jackson Laboratories	Strain 000664
C3H/HeJ	Jackson Laboratories	Strain 000659
C57BL6J: Schwann Cell Reporter Mouse	Dr. Jeffrey Milbrandt	P ₀ -Cre (JAX Strain 017927) and Ai9-flox/flox (JAX Strain 007909)
CHEMICALS		
10% neutral buffered formalin	Fisher Scientific	23-245684
EDTA	Sigma-Aldrich	E5134
DAPI	Sigma-Aldrich	D9542
OCT mounting media	Fisher HealthCare	23-730-571
Triton X-100	Sigma-Aldrich	9002-93-1
Fluoromount-G	Thermo Fisher Scientific	00-4958-02
Donkey serum	Sigma-Aldrich	D9663
Acrylamide/bis-acrylamide, 40% solution	Sigma-Aldrich	A7802
Paraformaldehyde	Electron Microscopy Sciences	15710
Histodenz	Sigma Aldrich	D2158-100G
NNN'N'-Tetrakis(2-Hydroxypropyl)ethylenediamine-Quadrol	Sigma Aldrich	122262-1L
VA-044	Wako	27776-21-2
SDS Micropellets	Thermo Fisher Scientific	BP8200-500
Antibodies for immunostaining and western blot	Detailed in Table S1	
SOFTWARE		
GraphPad Prism	GraphPad	v8.4.3
SCANCO Medical microCT systems	Scanco Medical AG	
FIJI	ImageJ	
Microsoft Excel	Microsoft	
Adobe Acrobat Pro DC	Adobe Creative Cloud	
Adobe InDesign	Adobe Creative Cloud	
Adobe Illustrator	Adobe Creative Cloud	
Adobe Photoshop	Adobe Creative Cloud	
ORS Dragonfly	Object Research Systems	
IMAGING SYSTEMS		
Scanco μ CT 40	Scanco Medical AG	N/A
Spinning Disk Confocal Microscope	Nikon CSU-X1	N/A
Smart SPIM Lightsheet Imaging System	Life Canvas Technologies	
Leica Confocal Microscope	Leica TCS SPEll confocal	
OTHER		
PicoLab Rodent Diet 20	LabDiet	5053
Colorfrost Plus Microscope Slides	Fisher Scientific	12-550-18
Cryostat	Leica Biosystems	

1

2 **Mice**

3 All work was performed as approved by the animal use and care committee at Washington University
4 (Saint Louis, MO, USA). Mice were housed on a 12-hour light/dark cycle and fed *ad libitum* (PicoLab
5 5053, LabDiet). C57BL6J (JAX Strain #000664) and C3H/HeJ (JAX Strain #000659) mice were
6 obtained from Jackson Laboratories. Schwann cell reporter mice were a kind gift of Dr. Jeffrey
7 Milbrandt and were generated by crossing the following strains: P_0 -Cre (JAX Strain 017927)⁴² and Ai9-
8 flox/flox (JAX Strain 007909)⁵⁴.

9

10 **Histology and Immunostaining**

11 *Tissue isolation and embedding.* Mice were anesthetized with Ketamine/Xylazine and perfused through
12 the left ventricle of the heart with 10 mL phosphate buffered saline followed by 10 mL 10% neutral
13 buffered formalin (NBF, Fisher Scientific 23-245684). For all experiments, collected tissues were post-
14 fixed in 10% NBF for 24-hours. After fixation, tissues were washed for 2 h in diH₂O. Bones were fully
15 decalcified in 14% EDTA (Sigma-Aldrich E5134), pH 7.4 and equilibrated in 30% sucrose solution prior
16 to embedding in OCT mounting media (Fisher HealthCare 23-730-571).

17

18 *Frozen immunostaining and imaging.* Embedded tissues were cut at 50 μ m on a cryostat (Leica). For
19 immunostaining, cut sections on Colorfrost Plus glass slides (Fisher Scientific 12-550-18) were blocked
20 in 10% donkey serum in TNT buffer prior to incubation for 48-hours with primary antibodies at 4°C
21 (Table S1). After washing 3x 5 min, secondary antibodies in TNT buffer were applied for 24 h at 4°C
22 (Table S1). The sections were then washed 3x 5 min in TNT buffer, incubated in DAPI (Sigma-Aldrich)
23 for 5 min, and washed again prior to mounting with Fluoromount-G (Thermo Fisher Scientific 00-4958-
24 02). For Fig.1,2 and Supplemental Files A-D, serial tiled images were taken with a 10x objective on a
25 Nikon spinning disk confocal microscope (μ m/px = 0.650, step size = 2.5 μ m Number of steps = 21).
26 Stitching of the nd2 multipoint files was done using the FIJI Stitching plugin “snake by rows”. For Fig.5,

- 1 serial tiles images were obtained using a Leica confocal at 10x ($\mu\text{m}/\text{px} = 0.6968$, step size = $2.5 \mu\text{m}$,
2 number of steps= 41) with automatic stitching through the LASX Software.

3

Primary Antibody (Vendor, Cat. No)	Dilution	Secondary Antibody	Fluorophore	Dilution
Anti- Calcitonin Gene Related Peptide (Bio-rad, 1720-9007)	1:1000	Donkey Anti-Goat (Jackson ImmunoResearch, 705-606-147)	AlexaFluor-647	1:500
Anti-Tyrosine Hydroxylase (Abcam, UK, ab152)	1:1000	Donkey Anti-Rabbit (Jackson ImmunoResearch, 711-546-152)	AlexaFluor-488	1:500
Anti-Perilipin (Progen Biotechnik, Germany, GP29)	1:500	Donkey Anti-Guinea Pig (Jackson ImmunoResearch, 706-165-148)	Cy3	1:500
Neurofilament 200 (NF200) (Millipore, AB5539)	1:5000	Donkey Anti-Chicken (Jackson ImmunoResearch, 703-545-155)	AlexaFluor-488	1:500

4 Confocal Image Segmentation and Analysis

5 *Selection of quantification regions.* From the longitudinal array of slides created for each bone, four
6 sections were chosen corresponding to the metaphysis, mid-diaphysis with tibial ridge, diaphysis
7 proximal to the TFJ, and diaphysis just distal to the TFJ. For each of the above regions, sections were
8 chosen based on morphological similarity, including bone shape and attachment sites.

9
10 *Generation of tissue masks.* A combined cancellous and cortical bone mask was generated
11 simultaneously by thresholding max projections of FITC channel (green) in FIJI⁵⁵. Optimal upper and
12 lower thresholds were chosen to remove muscle, connective tissue, marrow and other background
13 cellularity (some speckle and small holes in bone were allowed at this stage). After thresholding, bone
14 masks were manually corrected based on the source file in ImageJ by clearing non-bone elements
15 using the freehand selection tool. A median filter (radius = 11) was applied to smooth the mask.
16 Medullary masks were generated by inverting the bone mask in ImageJ, drawing a circle inside the
17 cortical ring using the freehand selection tool, and clearing outside the selection. Cancellous and
18 cortical bone masks were produced using the medullary mask, clearing inside or outside the marrow
19 cavity. Lastly holes in the cancellous and cortical bone masks were filled using the FIJI binary operation

1 Fill Holes. In cases where cortical bone completely enclosed the marrow cavity a small path was
2 cleared from the cortex prior to performing the binary operation, and subsequently repaired.
3 The periosteum was defined as a thin, densely cellular layer adjacent to cortical bone. Based on the
4 max projections of the FITC channel in FIJI, upper and lower thresholds were chosen to segment the
5 periosteum. Using previous masks, the bone and marrow were deleted from the periosteal mask.
6 Masks were manually corrected in ImageJ using the freehand selection tool. The DAPI channel was
7 frequently referenced to differentiate periosteum from other connective tissues and fascia. A median
8 filter (radius = 6) was applied to smooth the mask and lastly holes in the periosteum were filled using
9 the FIJI binary operation Fill Holes to create a continuous layer. In cases where the periosteum made a
10 complete ring a small path was cleared from the periosteum prior to performing the binary operation,
11 and subsequently repaired.

12

13 *Cortical and cancellous BV/TV calculation.* The area of the cortical bone, cancellous bone, and marrow
14 cavity were calculated in FIJI from the masks created by thresholding-based segmentation described
15 above. Cortical BV/TV was calculated as cortical bone area/(cortical bone area + marrow area) and
16 cancellous BV/TV was calculated as cancellous bone area/marrow area.

17

18 *Axon tracing and quantification.* TH+ and CGRP+ axons were independently traced with the Simple
19 Neurite Tracer plugin to obtain total axon length. The volume of the periosteum and marrow cavity were
20 calculated as the area multiplied by a section thickness of 50 μm . Volumetric density of fiber lengths
21 was calculated as the total length of fibers traced divided by the corresponding volume for the region
22 traced. Identification of nerves within the bone marrow is more challenging than at other sites due to
23 non-specific autofluorescence, poor light penetration, and the presence of antibody-dependent cellular
24 staining. This can result in staining artifacts, as noted with an information icon (“i”) when they appear in
25 the atlas (Supp Files A-D). Strategies to overcome these limitations include technical optimization,

1 tissue clearing, tiled confocal images of thick frozen sections to capture large volume datasets, and
2 strict morphologic criteria for nerve selection.

3

4 *Axon tracing image overlays.* TH+ and CGRP+ axon tracings were exported from the Simple Neurite
5 Tracer plugin in FIJI⁵⁶. This exported file preserves the coordinates of the traced axons and assigns
6 each traced segment a line with a width of one pixel. Exported nerve masks were dilated four times
7 using the binary processing tool, increasing the width of each traced axon to 9 pixels for the purposes
8 of visualization. After this, bone and nerve masks were imported into Adobe Photoshop and overlaid on
9 a grayscale max projection of the FITC and DAPI channels from the original image with cortical bone
10 and bone marrow removed. This results in a visually accurate map of axon length, branching,
11 orientation and relative density. However, features such as absolute nerve diameter are not preserved.

12

13 **MicroCT and Maps**

14 Representative two and three-dimensional maps of the Type I, II, and III periosteal innervation
15 patterns were generated after comprehensive review of serial images generated every 150 μm along
16 the length of the femur and tibia from four independent mice (12-week old B6 male, B6 female, C3H
17 male, C3H female). A representative 3D model, color-coded to match surrounding functional muscle
18 groups, was then reconstructed in Adobe Photoshop using scans of the tibiae of 12-week-old B6 and
19 C3H male mice performed on a Scanco μCT 40 (Scanco Medical AG, Bassersdorf, Switzerland; 55
20 kVp, 145 μA , 300 ms integration time, 16 μm voxel size). 2D Schematics showing muscle attachments
21 and muscle labeling were generated in Adobe Illustrator. These were adapted from Charles et. al³⁹
22 with muscle placement and innervation patterns adjusted based on our serial section analysis. Type I,
23 II, and III innervation patterns were illustrated on the bone-tissue interface in the 2D schematics and
24 color-coded in relation to surrounding muscle attachments and fascial structures. The final atlas was
25 compiled with Adobe InDesign.

26

1 **Whole Mount Tissue Clearing and Imaging**

2 Tissue clearing was performed as previously described with minor modification⁵⁷. Briefly, mice were
3 intracardially perfused with 20 mL 0.02M PBS followed by 20 mL of fresh 4% paraformaldehyde.
4 Immediately after perfusion, dissected tissues were post-fixed in 4% PFA at 4° overnight. The post-
5 fixed limb was then incubated in A4P0 hydrogel overnight (5 mL 40% acrylamide, 45 mL of PBS, and
6 0.125 g of VA044) and polymerized at 38° for 6 hours. The forelimb was then incubated in 8% SDS at
7 37° for 5 days to fully clear muscle and bone tissue. After 2 days of thorough PBS washing, the forelimb
8 was incubated in 25% quadrol at 37° for 2 days. After another 2 days of thorough washing in PBS, the
9 bones were incubated in 1.42 and 1.48 RIMS of Histodenz for one day each before imaging. Images
10 were taken with 4x objective on the SmartSPIM imaging system through the full length of the bone and
11 3D reconstructed with ORS Dragonfly Software for visualization of the P₀⁺ Schwann cells.

12

13 **Statistics**

14 Statistical analyses were performed in GraphPad Prism. Specific tests are indicated in the figure
15 legends. A p-value of less than 0.050 was considered statistically significant.

16

17 **DATA AVAILABILITY**

18 All relevant data are available from the authors upon reasonable request. Raw images will be made
19 available on the SPARC data portal (<https://sparc.science/>) – to be released with final publication.

20

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3

4

1 **Table 1. Previous quantification of TH+ and CGRP+ axon density in mouse bone.**

Publication	Mouse Information	Bone	Compartment	Immunostain	Summary of validated results
Mach et al., 2002	C3H/HeJ male 6-7 weeks, 20-25 g	Femur proximal head, diaphysis, distal head	Periosteum, cortical bone, bone marrow	CGRP TH	<ul style="list-style-type: none">• Nerve density: periosteum > bone marrow• In periosteum, CGRP>TH (1.3 to 2.4-fold)• In bone marrow, CGRP<TH (0.3 to 0.5-fold)
Castañeda-Corral et al., 2011	C3H/HeJ male 20-25 g	Distal femur	Periosteum, cortical bone, bone marrow	CGRP	<ul style="list-style-type: none">• Nerve density: periosteum > bone marrow• TH not assessed
Chartier et al., 2018	C57Bl/6J male 10 days, 3 months, 24 months	Distal femur	Periosteum, bone marrow, cortical pores	CGRP TH	<ul style="list-style-type: none">• Nerve density: periosteum > bone marrow• 3-month periosteum CGRP>TH (1.3-fold)• 3-month bone marrow CGRP<TH (0.7-fold)

2

3

1 **FIGURE LEGENDS**

2

3 **Figure 1. Neuroskeletal axon density varies by bone compartment, tibial level, and mouse strain.**

4 (a) 3D rendering of a μ CT scan of a 12-week-old male B6 tibia, lateral view. L1-4 represent levels
5 analyzed as distinguished by cross-sectional bone morphology. Percentages demarcate the
6 approximate distance from the knee as a percentage of total tibia length. Scale bar, 1 mm. (b) L1 Left:
7 Representative confocal micrographs through a 50- μ m-thick transverse cross section with
8 immunolabeled CGRP+ sensory axons (magenta) and TH+ sympathetic axons (green), as well as
9 DAPI+ nuclei (blue). L1 Right and L2-L4: Bone mask (black), periosteal mask (dotted line), CGRP+
10 axon traces (magenta) and TH+ axon traces (green) overlaid on a grayscale max projection to visualize
11 tibial innervation in the context of the musculoskeletal system at the metaphysis (L1), the diaphysis with
12 tibial ridge (L2), the diaphysis proximal to the TFJ (L3), and distal to the TFJ (L4). Scale bars, 200 μ m.
13 (c-j). Quantification of compartmentalized bone volume fraction (BVF) and innervation density from B6
14 and C3H mice. (c) BVF of cortical bone. (d,e) CGRP+ and TH+ axon density (mm/mm^3) within the
15 overlying periosteum. (f) Periosteal CGRP:TH ratio. (g) BVF of trabecular bone. (h,i) CGRP+ and TH+
16 axon density (mm/mm^3) within the marrow cavity. (j) Marrow CGRP:TH ratio. Data represent the mean
17 \pm SD; n = 3 for each strain and level; Two-way ANOVA with Sidak's multiple comparisons test; * p <
18 0.050

19

20 **Figure 2. Peripheral axons innervate the periosteum in three distinct patterns (Type I, II, III). (a-d)**

21 *Type I: Aneural* patterns are found at entheses, sites where tendons or ligaments attach to the cortical
22 bone (CB) surface. Aneural regions of the periosteum are devoid of CGRP+ (magenta) and TH+
23 (green) axons. (a) Tendinous fibers connect seamlessly to bone through unmineralized and mineralized
24 fibrocartilage tissue (FC) at fibrocartilaginous entheses. (b) The tendon or ligament (T/L) is continuous
25 with a thick fibrous layer of the periosteum (P) at fibrous entheses. (c) Confocal micrograph through a
26 50- μ m-thick transverse cross section a tibia from a 12-week-old C3H male with immunolabeled CGRP+

1 sensory axons (magenta) and TH+ sympathetic axons (green), as well as DAPI+ nuclei. **(d)**
2 Corresponding neuroskeletal traced/masked analysis overlaid on a grayscale max projection
3 demonstrating an aneural enthesis at the tibia metaphysis where the semimembranosus tendon
4 attaches to the bone. Trabecular bone (TB) is visible in the marrow cavity (M). **(e-h) Type II: Diving**
5 innervation patterns are found at sites where axons transit directly from the associated muscle tissue
6 through the fibrous and cambium layers of the periosteum at a 90-degree angle to the cortical bone
7 surface as visualized here in the C3H tibia where the popliteus muscle attaches to the bone. **(i-l) Type**
8 **III: Meshwork** innervation patterns are found at sites where fascia attach to bone. Axons are woven
9 through the thin periosteal layer at these sites and run parallel to the bone surface as evident in cross-
10 section **(i,k,l)**. The mesh-like network of periosteal axons at these sites is clearly visualized by
11 periosteal whole-mount preparation **(j, see ^{7,34})**. Scale bars, 100 μ m.

12

13 **Figure 3. Relationships between peripheral nerves and periosteal axon patterns and the**
14 **musculoskeletal system. (a-b)** Schematic of major nerve branches in the anterior and posterior thigh
15 **(a)** and leg **(b)** overlaid on 3D-rendered- μ CT scans of the left femur and tibia–fibula, respectively. Scale
16 bars, 1 mm. **(c,d)** Representative 2D maps of transverse cross-sections through **(c)** the thigh at the
17 level of 10% of the total femur length proximal to the knee and **(d)** the leg at 10% of the total tibial
18 length distal to the knee. The bone is indicated in gray and the superimposed periosteal innervation
19 patterns are distinguished by pattern-fill and are color-coded to the interfacing muscle group indicated
20 by the key. Detailed maps including labels of individual muscles are available in the atlases (Supp Files
21 A-D). **(e,f)** Three-dimensional periosteal innervation maps overlaid on the lateral, posterior, medial, and
22 anterior aspects of the μ CT-rendered **(e)** femur and **(f)** tibia.

23

24 **Figure 4. Specialized mechanoreceptor endings are associated with the skeleton of the forearm**
25 **and lower leg. (a)** Schematic of major nerve branches in the forearm overlaid on a 3D-rendered- μ CT
26 scan of the radius–ulna. Scale bar, 1 mm. **(b)** Light-sheet image of the Ai9 reporter signal in a cleared

1 forearm from an adult Schwann cell reporter mouse (P_0 -cre; Ai9) revealing the ulnar nerve (filled
2 arrowhead) and median nerve (empty arrowhead) and the distribution of Pacinian corpuscles on the
3 ulnar surface. (c) High-magnification, intensity-pseudocolored image of the boxed region in b. Scale
4 bar, 200 μ m. (d) Drawing of a Pacinian corpuscle demonstrating the lamellar structure derived from
5 Schwann cells surrounding a single unmyelinated ending of a large, myelinated sensory axon. (e)
6 Representative confocal micrograph through a 100- μ m-thick transverse cross section through the
7 radius (R) and ulna (U) and surrounding musculature visualized by DAPI (blue) and NF200 (green)
8 immunolabeling at 60% of the ulnar length. Scale bar, 200 μ m. (f) High-magnification of the corpuscles
9 on the lateral aspect of the ulna of the boxed region indicated in e. (g) Representative confocal
10 projection of a 50- μ m-thick transverse cross section through the leg with immunolabeling for TH+ axons
11 (green) and CGRP+ axons (magenta) along with DAPI staining (blue) at 60% of the tibial length distal
12 to the knee. Scale bar, 200 μ m. (h) High-magnification of the Pacinian corpuscles on the posterior
13 aspect of the tibia of the boxed region indicated in g. (i) 2D map corresponding to the confocal image in
14 g indicating the location of Pacinian corpuscles of all strains and sexes at this level of the tibia.

15 Additional information can be found in the supplemental tibial atlases (Supplementary Files C,D).

16

17 **Figure 5. Mouse limb atlases – guide for use.** (a) The four supplemental atlases comprise systematic
18 analyses of serial, transverse cross-sections down the length of the femur and tibia in B6 and C3H
19 mice, where each page details a section of the of the bone at a particular % distance from the knee.
20 This includes (b) representative confocal projections of immunohistochemically labeled CGRP+
21 sensory and TH+ sympathetic nerve fibers, along with perilipin+ adipocytes and DAPI-stained nuclei, in
22 and around the bone and (c) corresponding bone-masked, axon-traced overlays to highlight and
23 visualize the neuroskeletal features. (d) In addition, the atlas contains 2D reference maps with pattern-
24 coded periosteal innervation patterns superimposed on the bone interface and related to color-coded
25 muscle groups and individual muscle identities. (e) Lastly, these 2D maps served as the basis for 3D
26 mapping of periosteal innervation patterns and color-coded muscle groups onto the lateral, posterior,

- 1 medial, and anterior aspects of the corresponding bone. (f) Instructions for accessing interactive
- 2 features are located at the bottom of the page, such as displaying innervation pattern legend, muscle-
- 3 group color legend, and full muscle identifiers, or toggling through confocal channels.
- 4

Figure 1

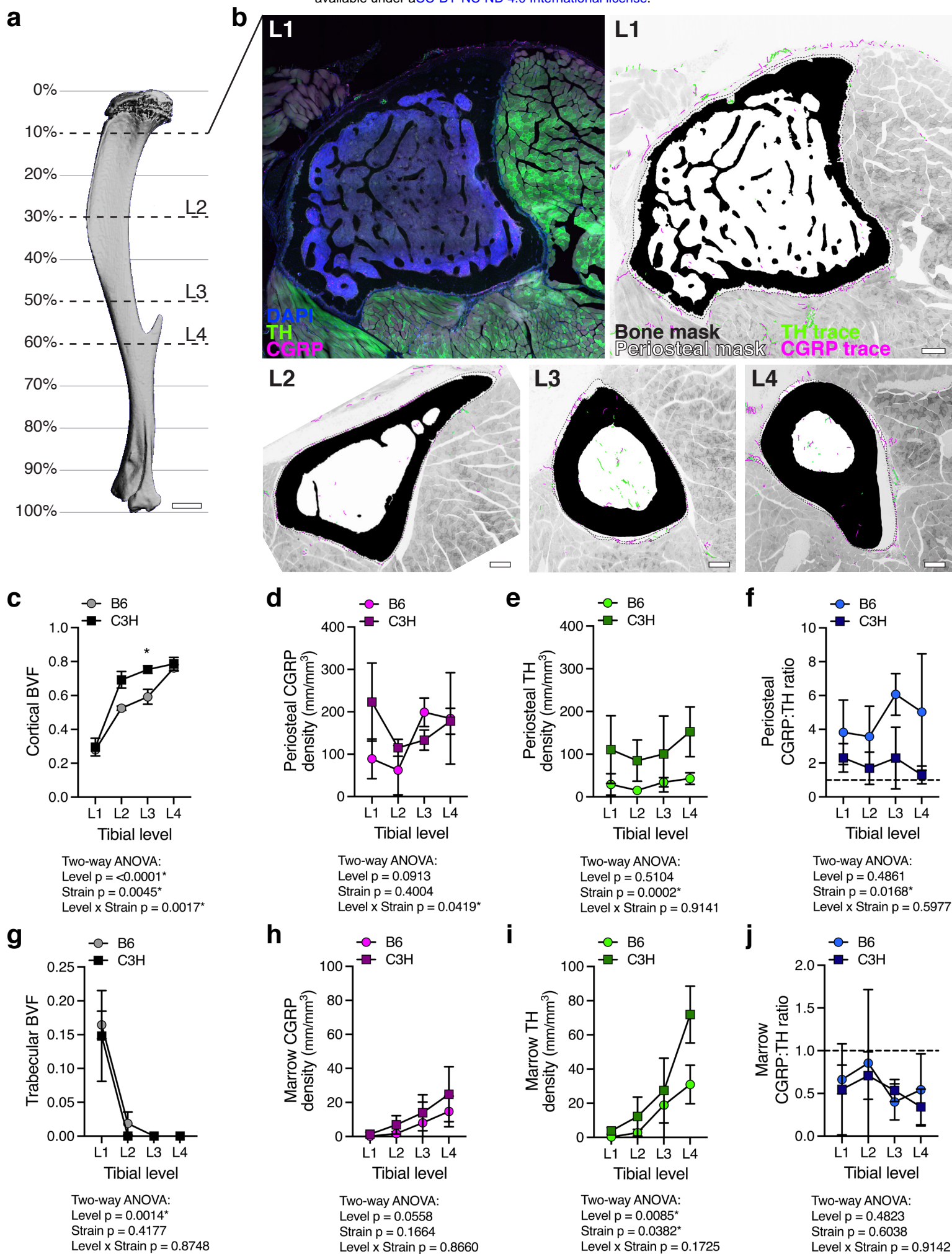
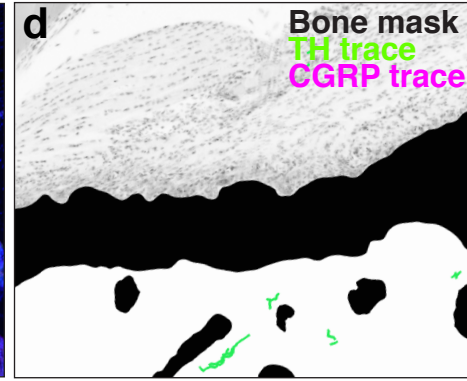
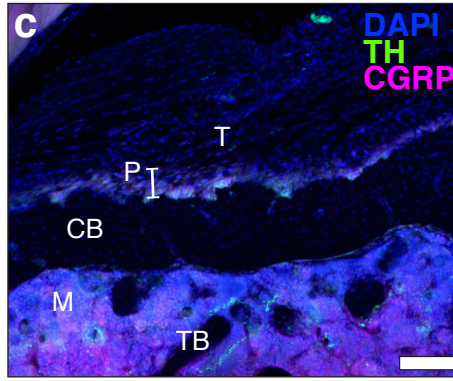
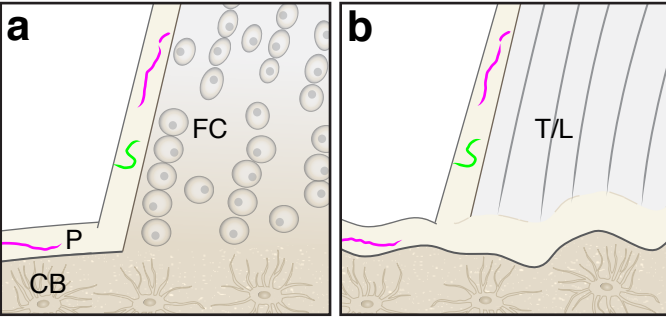
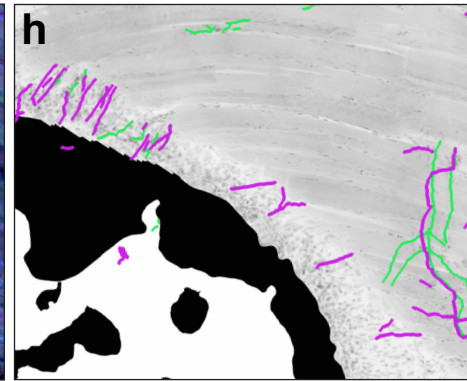
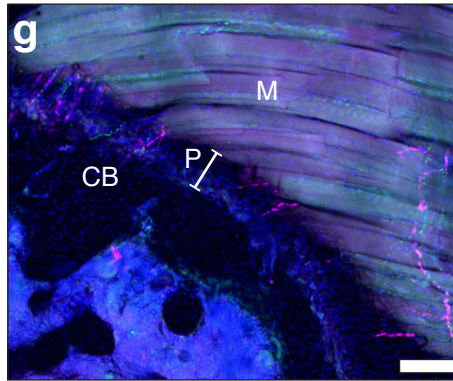
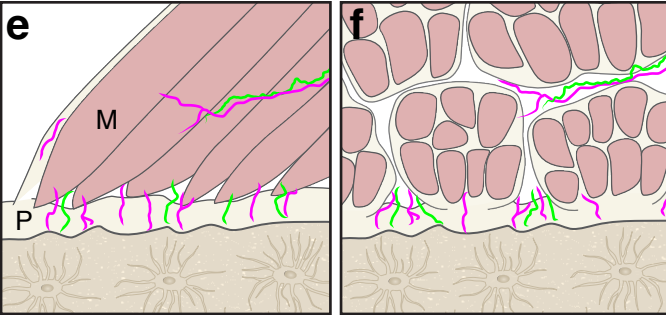


Figure 2

Type I: Aneural



Type II: Diving



Type III: Meshwork

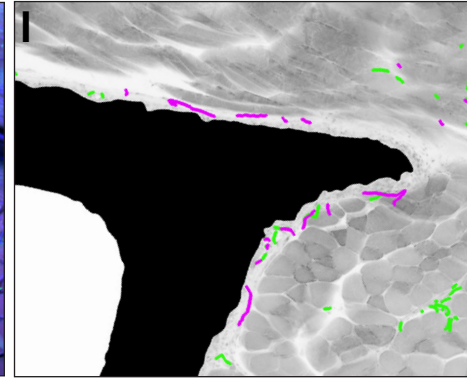
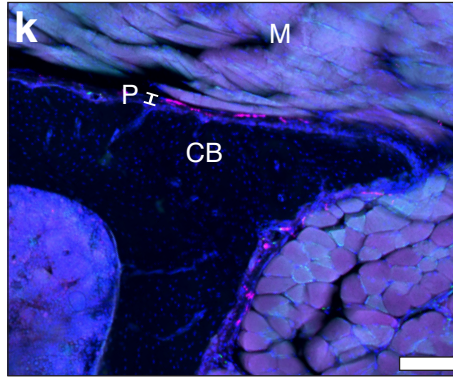
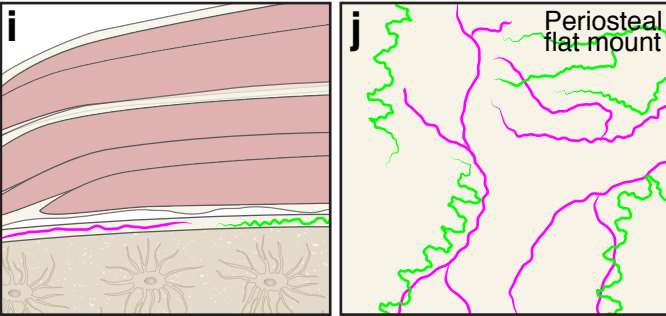


Figure 3

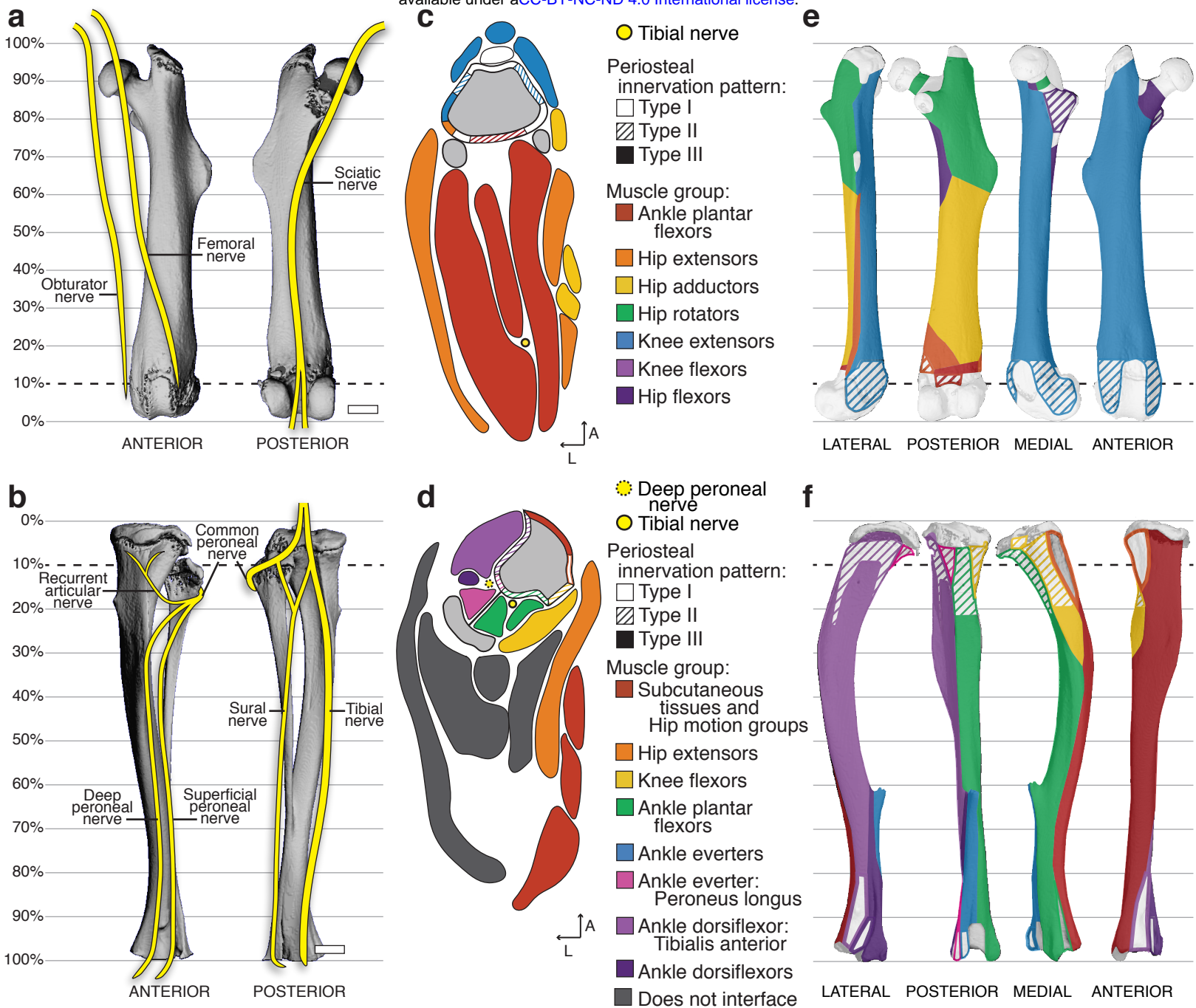


Figure 4

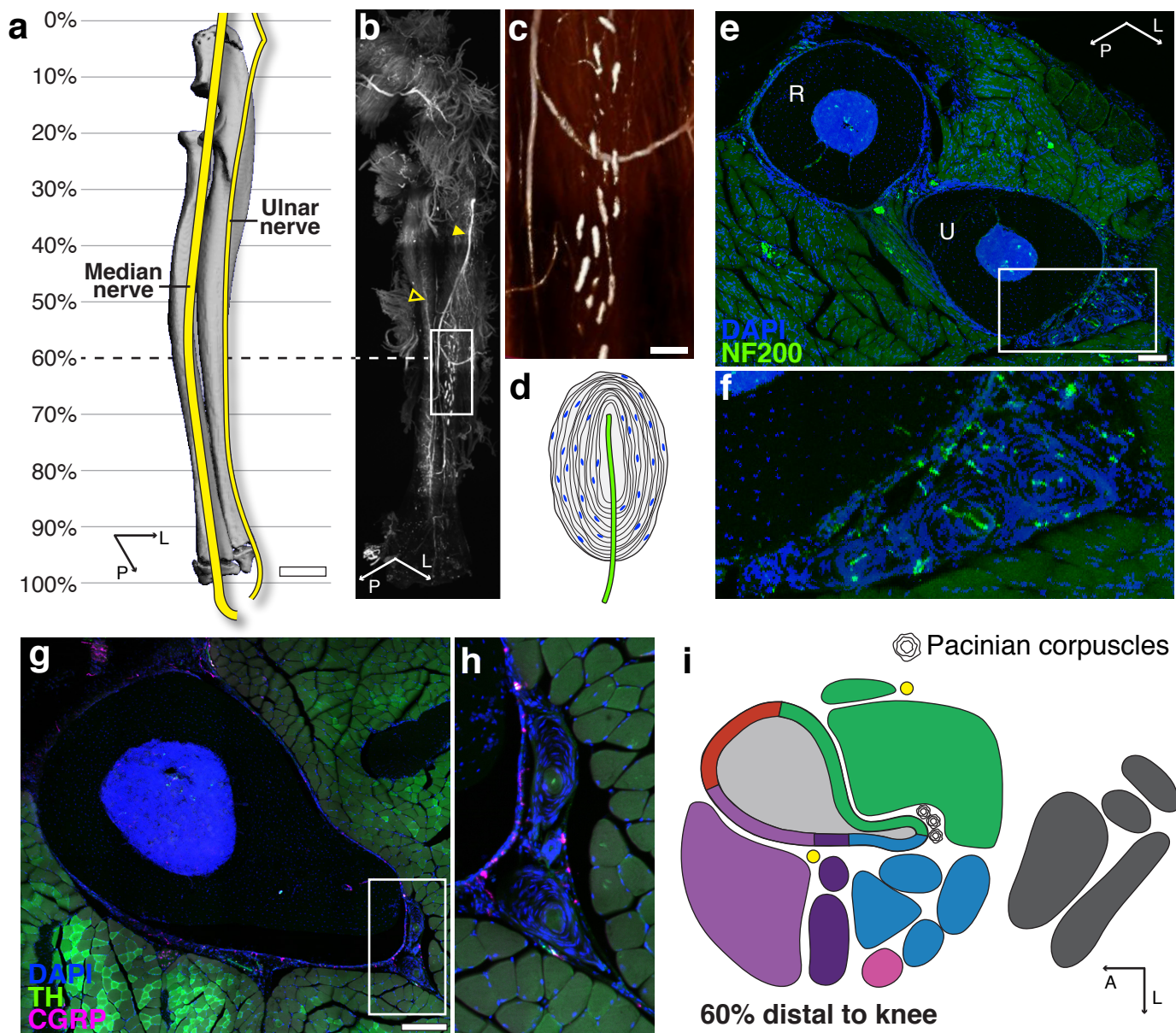
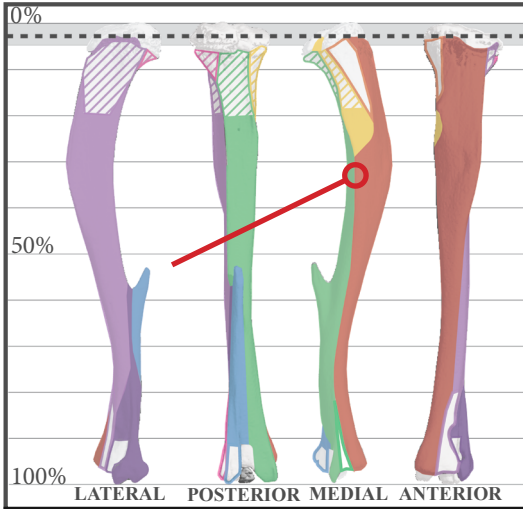


Figure 5

a

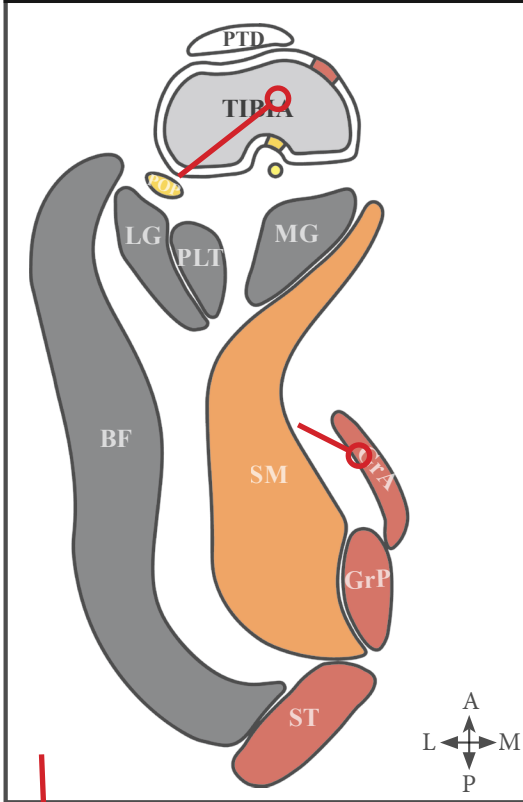
Section 1: 2% Site Proximal Epiphysis

e

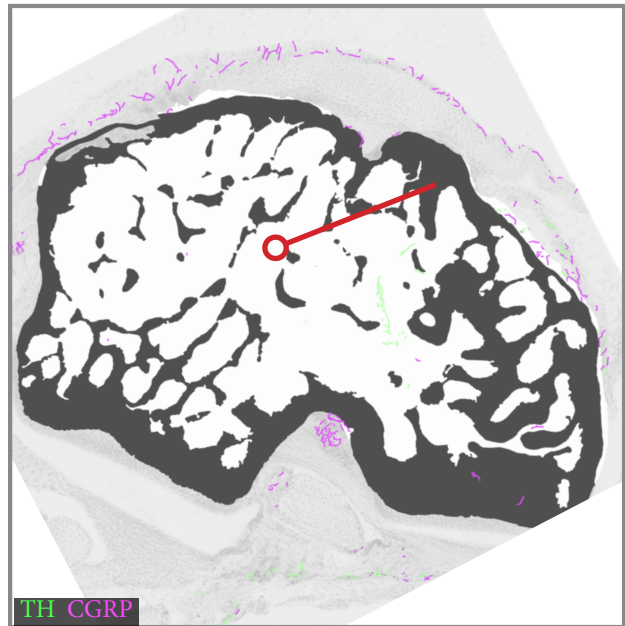


b

d






c



f

Color Legend

Pattern Legend

 Hover over icon for more information
 Hover over abbreviations for full definition
 Use arrows to access keys and icons