The effect of aging on the density of the sensory nerve fiber innervation of bone and acute skeletal pain

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Abstract

As humans age there is a decline in most sensory systems including vision, hearing, taste, smell, and tactile acuity. In contrast, the frequency and severity of musculoskeletal pain generally increases with age. To determine whether the density of sensory nerve fibers that transduce skeletal pain changes with age, calcitonin gene related peptide (CGRP) and neurofilament 200 kDa (NF200) sensory nerve fibers that innervate the femur were examined in the femurs of young (4-month-old), middle-aged (13-month-old) and old (36-month-old) male F344/BNF1 rats. Whereas the bone quality showed a significant age-related decline, the density of CGRP\textsuperscript{+} and NF200\textsuperscript{+} nerve fibers that innervate the bone remained remarkably unchanged as did the severity of acute skeletal fracture pain. Thus, while bone mass, quality, and strength undergo a significant decline with age, the density of sensory nerve fibers that transduce noxious stimuli remain largely intact. These data may in part explain why musculoskeletal pain increases with age.

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1. Introduction

Chronic musculoskeletal pain caused by conditions such as osteoarthritis, fractures, and low back disorders becomes more prevalent with age (Felson et al., 1987; Muraki et al., 2009; Woolf and Pfleger, 2003). These chronic skeletal pains, as well as the more acute musculoskeletal pain that follows moderate to strenuous physical exercise also increases with age (Fell et al., 2008), and all contribute to a loss of mobility, functional status, and quality of life (Brooks, 2006; Dominick et al., 2004; Woolf and Pfleger, 2003). Importantly, as the life expectancy in the developing and developed world is markedly increasing (Lutz et al., 2008), the toll that musculoskeletal pain will exact on individuals and society is also predicted to increase (Brooks, 2006; Woolf and Pfleger, 2003).

A major reason why musculoskeletal pain is such a medical and social burden is that our understanding of the factors that generate and maintain musculoskeletal pain is limited and even less is known about the mechanisms driving skeletal pain in older individuals. Currently, what we do know is that bones in older individuals (>50 years old) are more fragile than bones in younger individuals and less capable of sustaining damage before failure (Burr and Turner, 1999; Burstein et al., 1976; Currey et al., 1996). Equally important is that the ability of bone to absorb energy is significantly reduced with age, so that injury or fracture is much more likely to occur in an old bone than in a young bone upon impact loads of significant magnitude (Burr and Turner, 1999; Zioupos and Currey, 1998).
While there is an exhaustive literature on how bone deteriorates and becomes more fragile with age (Burr and Turner, 1999; Burstein et al., 1976; Currey et al., 1996; Ziopoulos and Currey, 1998), we know remarkably little about how the sensory nerve fibers that innervate the skeleton change with age. Currently, there are 3 major hypotheses as to why musculoskeletal pain increases with age. First, with age, the quantity and quality of bone decreases so that significant use and/or loads are more likely to lead to injury of bone tissue. Second, it has been hypothesized that musculoskeletal pain increases with age because the descending inhibition of pain in the spinal cord decreases with age (Edwards et al., 2003; Washington et al., 2000). Lastly, it has also been postulated that the brain’s perception of pain is different in the aged versus the young (Cole et al., 2010). An additional untested hypothesis is that unlike many other sensory systems, the density of the musculoskeletal pain sensory system does not undergo significant decline with age. Thus, while the bone itself declines in terms of mass, quality, and strength with age, if the sensory nerve fibers that innervate the bone remain largely intact, one would expect ever-increasing sensitization and activation of these nerve fibers as the bone is loaded and/or undergoes significant stress. Thus, the hypothesis that will be tested here is that while there is an age-related decline in bone progenitor cells, bone quality, bone mass, and fracture healing, there will not be a significant decline in the density of the sensory nerve fibers innervating the bone nor a decline in the severity of acute fracture-induced pain.

2. Methods

2.1. Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the VA Medical Center (Minneapolis, MN, USA) and the University of Arizona (Tucson, AZ, USA) and were in accordance with the National Institutes of Health guidelines for care and use of laboratory animals. All efforts were made to minimize the suffering and number of animals used.

The 25- and 36-month-old, naive male Fischer 344/Brown Norway F1 (F344/BNF1) rats were a generous donation from Dr. Priscia Honore (Abbott Laboratories, Abbott Park, IL, USA). Young (4- and 5-month-old) and middle-aged (13-month-old) F344/BNF1 rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). The F344/BNF1 rat is recommended for age-related studies by the National Institutes on Aging because this hybrid rat lives longer and has a lower rate of pathological conditions than inbred rats (Walker et al., 2006). The rats were housed in conventional facilities with a 12-hour light/dark cycle and given food and water ad libitum.

2.2. Bromodeoxyuridine administration

To visualize proliferating cells in the peristeme under normal conditions, rats were placed under isoflurane anesthesia and implanted subcutaneously with 2 miniosmotic pumps (model 1003D, Alzet, Cupertino, CA, USA) containing ~100 μL of 200 mg/mL bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA) solution. Rats were sacrificed 7 days post pump implantation and processed for immunohistochemistry (see below).

Because the number of cells labeled with BrdU following parenteral injections may be limited by the very short bioavailability of BrdU (half-life of 8 minutes; Taupin, 2007), studies designed to identify changes in the dividing cells due to age or to other factors have adopted and validated the use of continuous administration of BrdU by using miniosmotic pumps. This type of administration spans several days and allows obtaining consistent and reproducible intensity of BrdU staining (Baldauf and Reynmann, 2005; Cao et al., 2007; Soames et al., 1994; Tatematsu et al., 1989). Additionally, a previous study performed in bone showed sustained administration of BrdU via osmotic minipump provided a more substantial signal to noise ratio and reproducible immunostaining of BrdU in the periosteal cells as compared with single and multiple injections (Barou et al., 1997). Preliminary studies in our laboratory confirmed these results. In light of these findings, we administered BrdU by using miniosmotic pumps.

2.3. Euthanasia and microcomputed tomography (μCT) analysis

Naive rats were euthanized with CO2 and perfused intracardially as previously described (Jimenez-Andrade et al., 2009). The femurs were removed, postfixed for 4 hours in perfusion fixative, and placed in phosphate-buffered saline (PBS) solution at 4 °C. To characterize the age-related changes in mineralized bone microarchitecture, femurs were analyzed with an eXplore Locus SP microcomputed tomography (μCT) system (GE Healthcare, London, Ontario, Canada). This cone beam μCT scanner used a 2300 × 2300 charged couple device detector with current and voltage set at 80 μA and 80 KVP, respectively. Specimens were scanned in 900 views through 360° with a 2100 ms integration time. Scans were then reconstructed at 16 μm3 resolution using Reconstruction Utility software v.1.0 (GE Healthcare, London, Ontario, Canada).

To visualize overall differences in trabecular and cortical bone, μCT images of preprocessed femurs were rendered using the MicroView analysis software ABA 2.2 (GE Healthcare, London, Ontario, Canada) and assembled using Adobe Photoshop CS and Adobe Illustrator CS4.
2.4. Processing of femoral periostea for immunohistochemistry

Evaluation of age-related changes in sensory innervation was performed in whole-mount preparations and frozen sections of the periostea. The advantage of frozen sections is that one can visualize and quantify the innervation, cellular, and vascular density in each layer of the periostea (cambium and fibrous) separately. The advantage of whole mount preparations is that they allow the observer to get a “bird’s-eye” view of the mesh-like network of nerves in the periostea.

Periostea from the left diaphyseal shaft was removed as a whole mount and processed for immunohistochemistry according to a previously published protocol (Jimenez-Andrade et al., 2009). Briefly, periostea was obtained from the distal end growth plate region immediately below the third trochanter, as all of the experimental fractures were located in this anatomical region. Preparations were incubated overnight at room temperature (RT) with primary antibodies. Unmyelinated primary afferent sensory nerve fibers and some myelinated nerve fibers were labeled with polyclonal rabbit anti-rat calcitonin gene related peptide (CGRP; 1:12,000 dilution; Sigma, St. Louis, MO, USA; Lawson et al., 1993). Myelinated primary afferent sensory nerve fibers were immunostained for 200 kDa neurofilament H (NF200) (polyclonal chicken anti-mouse NF200; 1:1000; Chemicon, Temecula, CA, USA) (Lawson and Waddell, 1991). Preparations were then incubated for 3 hours at RT with secondary antibodies conjugated to fluorescent markers (Cy3, 1:600; Cy2, 1:200; Jackson ImmunoResearch, West Grove, PA, USA). Preparations were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:30,000; Invitrogen, Carlsbad, CA, USA) for 5 minutes. Finally, tissue was dehydrated through an alcohol gradient (70%, 80%, 90%, and 100%), cleared in xylene, mounted on gelatin-coated slides (attached muscle layer in contact with the slide) and coverslipped with di-n-butylphthalate-polystyrene-xylene (Sigma, St. Louis, MO, USA).

In order to determine the age-related changes in cellular density, cellular proliferation, vascularization, and innervation of each layer from the periostea, cross-sectional/frozen section analysis of the right femurs from naive rats with the periostea attached to the bone were processed as follows. Right femurs were postfixed and decalcified. Demineralization was performed by placing bones in 10% ethylenediaminetetraacetic acid (EDTA) solution at 4 °C. EDTA solution was changed once per week for 3 months. For histology experiments, demineralized bones were embedded in paraffin. Seven-μm thick sections were cut in the coronal plane and stained with hematoxylin and eosin (H&E). For immunohistochemical experiments, decalcified bones were cryoprotected in 30% sucrose at 4 °C for at least 48 hours and serially sectioned on the coronal plane at a thickness of 30 μm. Bone sections used for immunohistochemistry were mounted on gelatinized slides. Sections were incubated overnight at RT with primary antibodies. Proliferating cells were immunostained for the BrdU antibody (1:500, US Biological, Swampscott, MA, USA). Blood vessels were immunostained for the platelet endothelial cell adhesion molecule RECA (monoclonal mouse anti-rat CD31, 1:500, BD Pharmingen, San Diego, CA, USA). Unmyelinated and myelinated primary afferent sensory nerve fibers were labeled with antibodies against CGRP and NF200, respectively, as previously described. Further immunohistochemical steps were performed as previously described.

2.5. Image acquisition and quantification of age-related cellular changes in the periostea

All analyses were performed within the distal metaphysis as the periostea is thickest within this region of the bone. For evaluation in whole mount and frozen sections, the area evaluated was within a 1.5-mm-long region, which started 2 mm above the distal femoral growth plate.

For whole mount preparations, 2 random images per rat at 400× magnification (320 μm × 320 μm) were obtained of the entire visible periostea using DAPI as a reference. Images were acquired using an Olympus Fluoview FV1000 laser scanning confocal imaging system (Olympus, America Inc, Melville, NY, USA software v. 5.0). The z-series of each field of view were then used to generate a single topographic image (2-dimensional projection).

For frozen sections, 3 random images per rat at 400× magnification (320 μm × 320 μm) were obtained for quantification purposes. Under the DAPI channel, the thickness of the fibrous and cambium layers was determined with FV1000 software. As all periosteal quantifications were performed in frozen sections, the area of each periosteal layer was calculated by multiplying the length of the section of periostea by the thickness of the fibrous or cambium layer. This area was subsequently multiplied by the thickness of the cryostat cut section (30 μm) and recorded as the volume of each individual periosteal layer.

In order to quantify the number of proliferating cells in the periostea, the number of BrdU+ nuclei and DAPI+ nuclei in each layer of the metaphyseal periostea was determined. Inclusion criteria for BrdU+ nuclei were similar to those reported by Horner et al. (2000). In particular, BrdU immunostaining needed to be located in the nucleus as determined by counterstaining sections with DAPI and BrdU+ nuclei needed to exhibit uniform staining throughout the nucleus. Results are presented as percent of DAPI nuclei in each periosteal layer that also exhibited BrdU immunoreactivity.

Density of blood vessels was determined by quantifying the total volume of blood vessels (Nielsen et al., 2008) in each layer of the periostea from z-series images of each field of view using Imaris Pro Software v. 6.0 (Bitplane AG,
Zurich, Switzerland). Data from at least 3 slices per animal were recorded and averaged.

Quantification of CGRP$^+$ and NF200$^+$ sensory nerve fibers in rat frozen sections was performed by determining the total length of nerve fibers in each periosteal layer (Xie et al., 2007). The confocal images were viewed on a high-resolution monitor and the length of nerve fibers was determined using Image Pro Plus v. 6.0 image analysis software.

2.6. Fracture protocol

To measure pain-related behaviors following femoral fracture, we used 5- and 26-month-old F344/BNF1 male rats. The 36-month-old F344/BNF1 rats were not used for bone fracture analysis due to surgical procedure survival concerns. To provide mechanical stabilization of the fracture site during and following fracture, a stainless steel pin was inserted into the medullary cavity of the femur as previously described (Freeman et al., 2008). A closed mid-diaphyseal fracture of the left femur was produced 2 weeks post pin placement in anesthetized rats (5% isoflurane) as originally described by Bonnarens and Einhorn (Bonnarens and Einhorn, 1984). Exclusion criteria included fractures located in the metaphysis, dislodged pins and nonvisible fracture following impact (Gerstenfeld et al., 2007). Following recovery from anesthesia, rats were allowed unrestricted movement and weight-bearing of the fractured limb.

2.7. Pain-related behaviors

Pain-related behaviors were evaluated before fracture (day 0) and at day 1, 3, 7, 10, 14, 17, and 21 following fracture to assess ongoing (spontaneous) fracture pain-related behaviors (guarding and flinching) as previously described (Freeman et al., 2008). Briefly, the number of hind limb flinches and time spent guarding over a 2-minute observation period were recorded as measures of ongoing pain (Santy and Mackintosh, 2001). Fracture-induced pain was also assessed by differences in weight distribution of the left hind limb (fractured or pin hind limb) as compared with the right hind limb (intact hind limb) using an incapacitation meter as previously described (Freeman et al., 2008). Weight-bearing was used as an endpoint in this study as this has been widely used in humans to evaluate bone healing following fracture (Corrales et al., 2008). Briefly, the mean force applied during 3 seconds by each hind limb was measured in 5 trials. Weight-bearing of the affected left hind limb was calculated as percentage of total weight bearing on both hind limbs by the following equation: (weight on fractured hind limb / [weight on fractured hind limb + weight on intact hind limb]) × 100.

Our experimental protocol consisted of 4 different groups: pin 5-month-old (n = 3), pin 26-month-old (n = 3), pin + fracture 5-month-old (n = 7), and pin + fracture 26-month-old (n = 5).

2.8. Radiographic analysis

Digital radiograph images (MX20 DC12, Faxitron XRay Corporation, Wheeling, IL, USA) of fractured femurs were obtained immediately post-fracture and at all behavior test time points (days 7, 10, 14, 17, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 92, 98, and 105). The area of the callus was determined by a blinded investigator using ImageJ software v. 1.41B (NIH). A complete bone union was considered as bridging of both cortical walls (value of 2), while a partial union was defined as bridging of 1 cortical wall of the femur (value of 1) (Jimenez-Andrade et al., 2007).

2.9. Statistical analyses

Statistical analysis was performed using SPSS v. 11 statistics package (SPSS, Chicago, IL, USA). For data obtained from periosteal tissue, a Friedman nonparametric analysis of variance (ANOVA) was used to compare the 3 groups (4 month, 13 month, and 36 month) for each condition type at each measurement time point. Significant ANOVA results were followed by Mann-Whitney 2-group comparisons. To determine differences between groups in the behavior and radiographic results, a 1-way ANOVA followed by Mann-Whitney 2-group comparisons was performed. Results were considered statistically significant at p < 0.05. In all cases, the investigator was blind to the experimental status of each animal.

3. Results

3.1. Age-related changes in the microarchitecture of the bone

The μCT analysis of femurs from young (4-month-old), middle-aged (13-month-old), and old (36-month-old) male rats revealed an age-related loss of cortical and trabecular bone. In particular, a cross-sectional view of the mid-diaphysis of a young, middle-aged, and old femur revealed a gradual reduction in cortical thickness with age (Fig. 1A–C). Additionally, high resolution μCT images of the distal metaphysis showed an age-related reduction in trabecular number and increased trabecular separation (Fig. 1D–F).

3.2. Age-related changes in the morphology, cellular density, cellular proliferation, vascularization, and sensory innervation of the periosteum

As illustrated in H&E sections and frozen sections counterstained with DAPI (Fig. 2A–C), the thickness of the fibrous layer of the periosteum did not change significantly in the 3 age groups (Fig. 4A). In contrast, the thickness of the cambium layer decreased from 51.21 ± 3.41 μm in 4-month-old rats to 25.53 ± 1.46 μm in 13-month-old rats (p < 0.005, Figs. 2A and B, 4A). This thickness further decreased to 21.59 ± 1.54 μm in 36-month-old rats (Figs. 2A–C, 4A). These observations are consistent with pub-
the majority of BrdU fracture (Allen et al., 2004). We determined periosteal cell that are vital for bone healing in response to trauma and layer, the percent of DAPI
was twice as high in the cambium layer than in the fibrous layer (Figs. 2D–I, 4B), and this was not significantly dif-
cantly reported (O’Driscoll et al., 2001; Tonna, 1961),
cel density was significantly different compared with young and middle-aged rats (Fig. 4B).

The periosteum contains mesenchymal progenitor cells that are vital for bone healing in response to trauma and fracture (Allen et al., 2004). We determined peristeal cell proliferation by evaluating the uptake of BrdU. As previously reported (O’Driscoll et al., 2001; Tonna, 1961), the majority of BrdU+ proliferating cells were located in the cambium layer across all 3 age groups (Fig. 2D–F). The number of proliferating cells in the cambium layer decreased significantly with age (Fig. 4C). In the cambium layer, the percent of DAPI+ nuclei that express BrdU in the middle-aged group decreased to 1.16% from a value of 9.1% in young rats, and further decreased in the old group to 0.47% (Fig. 4C).

We evaluated the density of RECA blood vessels in each layer of the periosteum at different ages. The cambium layer dramatically decreases with age (O’Driscoll et al., 2001).

Cell density, as evaluated by number of DAPI+ nuclei, was twice as high in the cambium layer than in the fibrous layer (Figs. 2D–I, 4B), and this was not significantly dif-
cantly between young and middle-aged rats (Fig. 4B). However, in the 36-month-old rats, cell density was significa-
cantly reduced compared with young and middle-aged rats (Fig. 4B).

3.3. Age-related changes in the sensory innervation of the periosteum

Confocal micrographs of whole mount metaphyseal periosteum preparations revealed that CGRP+ and NF200+ nerve fibers have a linear and bifurcating pattern. These sensory fibers form a mesh-like network that envelopes the naïve, unfractured bone (Fig. 3A–F). Sensory fibers in the periosteum appear as single nerve fibers or nerve bundles. As frozen sections through the bone provide a coherent anatomical view of nerve fibers in each periosteal layer, we obtained the length of CGRP+ and NF200+ nerve fibers in each layer of the periosteum using this approach. Across all 3 age groups, the vast majority of CGRP+ and NF200+ fibers were present in the fibrous layer of the periosteum (Fig. 4E–F). As the fibrous layer does not significantly change in thickness with age, these results are not con-
ffounded by an age-related reduction in layer thickness, such as that which occurs in the cambium layer. The density of CGRP+ nerve fibers, expressed as length of nerve fibers/mm3, in the periosteum of young male rats, was not significa-
cantly different when compared with that in middle-aged male rats and old rats (Fig. 4E). Likewise, there were no significant differences in the density of NF200+ fibers in the periosteum between young versus middle-aged versus old male rats (Fig. 4F).

3.4. Age-related pain behaviors

All pin + fracture rats exhibited a greater time spent guarding, an increased number of flinches, and a marked reduction in weight-bearing as compared with rats with the pin alone (Fig. 5A–C). There were no significant differences in the magnitude of the pain-related behav-
ors between young and old rats with a fracture (Fig. 5A–C). Both young and old rats with a pin only showed minimal number of flinches, time spent guarding, and hind limb weight-bearing which was not significantly different from that observed in naïve rats (baseline values, data not shown).

3.5. Age-related changes in fracture-induced bone healing

Quantitative analysis of the bone healing process was performed by determining the callus area, defined as the total radio-opaque area within the outermost boundary of the fracture callus. Callus was first evident at day 7 post fracture in both groups. With the exception of day 10 post fracture, callus area was not significantly different from day 7 to day 35 post fracture (data not shown). The callus area was significantly greater in the old group when
Fig. 2. Age-related changes in the periosteum of the rat. (A–C) Seven-μm thick longitudinal cross-sections of the distal metaphysis of the femur stained with hematoxylin and eosin (H&E) display the composition and appearance of the layers of the periosteum in young (A), middle-aged (B), and old (C) male F344/BNF1 rats. Note that the cambium layer of the periosteum in middle-aged and old animals is thinner compared with young animals. In contrast, minimal changes in the thickness of the fibrous layer are observed with age. (D–F) Confocal photomicrographs from sections (30 μm) of young (D), middle-aged
compared with the young group from day 42 to day 105 post fracture, suggesting a delay in the reabsorption of the callus in the 26-month-old rats versus 5-month-old rats (data not shown). There was a significant age-related delay in bridging, as bones from young animals showed union and partial union at day 77, whereas bones from old animals still did not display union at day 105 post fracture.

4. Discussion

In the present study, we show that the density of CGRP* and NF200* sensory nerve fibers in the femoral periosteum was not significantly different between the young (4 month), middle-aged (13 month) and old (36 month) rats. Furthermore, the severity of acute fracture-induced pain-related behaviors did not change in young versus old animals. In contrast, progenitor cell proliferation, vascularization of the periosteum, thickness of the cambium layer (required for fracture healing), and fracture healing itself all dramatically declined with age.

In general, as humans age there is a loss of sensitivity in nearly all sensory modalities including vision, hearing, taste, smell, and tactile acuity (Helzner et al., 2005; Schiffman, 1997; Weale, 1986). For vision, this loss is attributed to a decrease in the accommodative ability of the eye, and a loss of photoreceptors, resulting in a reduction in sensitivity to low levels of illumination and visual acuity (Weale, 1986). Deterioration of hearing also occurs with age such that the prevalence of hearing loss is 0.5% at birth, 5% in children, and 30% to 83% in individuals aged 65 and older, depending upon the definition used (Helzner et al., 2005). The spatial acuity of touch, like that of vision and hearing, tends to decline in nearly all individuals (Stevens and Cruz, 1996). Thus, when the acuity threshold is assessed repeatedly in the index finger as measured by: the ability to discriminate tactile gaps (by a refined version of 2-point threshold), orientation of lines (across vs. along the finger), and length of lines (all of which relate to prominent discriminatory features of Braille) all decline, and have been shown earlier to decrease, on average, by about 1% per
annum over the adult life span from about 20 to 80 years (Stevens and Cruz, 1996). Although there are clearly differences among the elderly subjects, all tested consistently worse than the least acute young adult controls (Stevens and Cruz, 1996).

In contrast to most sensory systems, the musculoskeletal pain sensory system appears to increase with age, at least in terms of the frequency it is perceived and its severity (Brooks, 2006; Dominick et al., 2004; Woolf and Pfleger, 2003). Previous studies that have examined age-related increase in musculoskeletal pain have largely focused on decreases in the strength, mass, and quality of the skeleton and muscle (Shane Anderson and Loeser, 2010; Ostbye et al., 2004). However, another reason as to why the incidence and duration of musculoskeletal pain increases with age may be that while bone and muscle integrity clearly decline, the nociceptors that innervate bone remain largely intact. Previously, it has been shown that the density of CGRP⁺ and NF200⁺ nerve fibers in the distal metaphyseal periosteum is not significantly different between the 3 age groups. Each bar represents the mean ± standard error of the mean (SEM) *p < 0.05.

![Fig. 4. Age-related changes in the thickness, cellular density, cellular proliferation, vascularization, and sensory innervation of the rat periosteum. Periosteal layer thickness (A), cellular density (B), cellular proliferation (C), vascularization (D), and sensory innervation (E, F) were analyzed in 4-, 13-, and 36-month-old rats. Note that while the number of proliferating cells (BrDU⁺) and density of blood vessels (RECA⁺) decreases with age, the density of CGRP⁺ and NF200⁺ nerve fibers in the distal metaphyseal periosteum is not significantly different between the 3 age groups. Each bar represents the mean ± standard error of the mean (SEM) *p < 0.05.](image-url)
of the 2 major populations of sensory nerve fibers that innervate the bone (one which expresses CGRP and one which expresses NF200) (Jimenez-Andrade et al., 2010) show significant age-related decline when comparing the total density of nerve fibers in young (4 month), middle-aged (13 month), or old (36 month) male rats. It is interesting to note that the majority of CGRP+ and NF200+ nerve fibers are located in the outer fibrous layer of the periosteum, which unlike the inner cambium layer of the periosteum, does not undergo significant age-related reduction in layer thickness (Nakahara et al., 1991; O’Driscoll et al., 2001; Tonna, 1974). Thus, the preservation of the total density of sensory nerve fibers observed in the present study is not due to an age-related reduction in layer thickness as there is not a significant reduction in thickness of the fibrous layer of the periosteum with age. These data partially agree with previous observations which have been made in human vertebrae which suggested that aged, degenerating vertebrae actually show an increase in the number of CGRP+ sensory nerve fibers due to hypothesized sprouting (Peng et al., 2005) and that in equine sesamoid bone there is an increase in the number of sensory fibers in 10-year-old horses versus 54-day-old foals (Cornelissen et al., 1998).

While bone quality (Burr and Turner, 1999; Currey et al., 1996; Zioupos and Currey, 1998) and repair (Nilsson and Edwards, 1969; Parker et al., 2007) appeared to undergo a marked decline in age, what is clear in the present study is that the acute pain response of the animal to fracture as measured by time spent guarding, flinching, and the ability to bear weight following fracture appeared to be fully preserved when comparing the young and the old animals. This contrasts with most other sensory systems, which undergo a marked decline with age (Felder and Schrott-Fischer, 1995; Helzner et al., 2005; Schiffman, 1997; Stevens and Cruz, 1996; Weale, 1986). Interestingly, human psychophysical studies that compared young versus old human volunteers also suggested that pain due to deep pressure applied to the finger (presumably also stimulating the underlying bone) increases with age, whereas responses to non-noxious (warmth, cold, vibration) (Lautenbacher et al., 2005) or noxious stimulation of the superficial skin either remains the same or decreases with age (Kenshalo, 1986; Lin et al., 2005). It should be stressed that while the present data suggest that there is not a significant decline in the density of the sensory innervation of bone with age, it will also be equally important to examine how the phenotype of sensory nerve fibers changes with age. Thus, previous studies in normal 6-8 week old vs. 2-year-old mice showed that older mice not only showed a reduced thermal sensitivity in the skin but that this reduction was also accompanied by a reduced expression of Nav1.8 sodium channel and the capsaicin receptor (TRPV1) in the dorsal root ganglia and peripheral nerve (Wang et al., 2006). Understanding how the density and phenotype of sensory nerve fibers, as well as the structures they are innervating, change with age may...
shed light on why chronic pain generally increases in the elderly and how this pain might be better controlled.

One obvious question that arises from the results presented here is that if fracture is that if fracture healing time takes longer in old versus young rats, why is the time course of fracture pain behavior in the older animals not significantly longer than that in the young animals? Previous studies in humans and rodents have shown that fracture pain declines with stabilization of the bone, either by fixation or casting of the bone in humans (Bone et al., 2004; Santy and Mackintosh, 2001) or due to formation of a callus in the rat or mouse (Freeman et al., 2008; Jimenez-Andrade et al., 2007). In light of these results it is hypothesized that, at least following acute fracture of the femur, a significant component of fracture pain is mediated by physical distortion of the mechanosensitive sensory nerve fibers that richly innervate the periosteum (Jimenez-Andrade et al., 2007). In the present study, as the rate of callus formation which stabilizes the fractured bone appears to be similar if not greater in the old versus young, one would expect that the pain behaviors would diminish at a similar rate.

What still remains to be defined is why the sensory innervation of bone is maintained with age when so many other sensory systems undergo a marked decline. Compared with other organs, bone is unique in being a remarkable reservoir of growth factors (e.g. transforming growth factor beta, bone morphogenetic proteins, nerve growth factor, insulin-like growth factor-1) (Garcia et al., 2004; Solheim, 1998). One potential explanation as to why the sensory innervation of bone appears to be largely preserved with age is that as the bone deteriorates there is a release of trophic growth factors that are normally sequestered in the bone that support the survival of sensory nerve fibers that innervate the bone (Murakami et al., 2006).

A major problem with effectively treating age related musculoskeletal pain is that although there is a striking increase in this type of pain, there is a very limited repertoire of effective analgesics that attenuate this pain without significant unwanted side effects. Nonsteroidal anti-inflammatory drugs are effective in attenuating some musculoskeletal pain, and if effective, would significantly contribute to healthy aging.

Disclosure statement

All authors declare no conflicts of interest.

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