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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) and old (36 month) male F344/BNF1 rats. Whereas the bone quality showed a significant age-related decline, the density of CGRP⁺ and NF200⁺ nerve fibers that innervate the bone remained remarkably unchanged as well as 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.

Keywords

periosteum; orthopedic; elderly; fracture healing; analgesia

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).

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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 yrs 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 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; Zioupos and Currey, 1998), we know remarkably little about how sensory nerve fibers that innervate the skeleton change with age. Currently, there are three major hypotheses as to why musculoskeletal pain increases with age. First, with age the quantity and quality of bone decrease 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) and the University of Arizona (Tucson, AZ) 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, naïve male Fischer 344/Brown Norway F1 (F344/BNF1) rats were a generous donation from Dr. Prisca 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 periosteum under normal conditions, rats were placed under isoflurane anesthesia and implanted subcutaneously with two mini-osmotic pumps (model # 1003D, Alzet, Cupertino, CA) containing ~100 µl of 200 mg/ml

bromodeoxyuridine (BrdU, Sigma, St. Louis, MO) solution. Rats were sacrificed 7 days post-pump implantation and processed for immunohistochemistry (see below).

Since the number of cells labeled with BrdU following parenteral injections may be limited by the very short bioavailability of BrdU (half-life of 8 min (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 mini-osmotic pumps. This type of administration spans several days and allows obtaining consistent and reproducible intensity of BrdU staining (Baldauf and Reymann, 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/ noise ratio and reproducible immunostaining of BrdU in the periosteal cells as compared to 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 mini-osmotic pumps.

2.3 Euthanasia and micro-computed tomography (μ CT) analysis

Naïve rats were euthanized with CO₂ and perfused intracardially as previously described (Jimenez-Andrade et al., 2009). The femurs were removed, post-fixed for 4 hours in perfusion fixative and placed in PBS solution at 4°C. In order to characterize the age-related changes in mineralized bone micro-architecture, femurs were analyzed with an eXplore Locus SP micro-computed tomography (μ CT) system (GE Healthcare London, Ontario, Canada). This conebeam μ CT scanner used a 2300×2300 CCD detector with current and voltage set at 80 μ A and 80 KVp, respectively. Specimens were scanned in 900 views through 360° with a 2100 ms integration time. Scans were then reconstructed at 16- μ m³ resolution using Reconstruction Utility software (GE Healthcare).

To visualize overall differences in trabecular and cortical bone, μ CT images of pre-processed femurs were rendered using the MicroView analysis software (GE Healthcare) and assembled using Adobe Photoshop CS and Adobe Illustrator CS4.

2.4 Processing of femoral periosteum for immunohistochemistry

Evaluation of age-related changes in sensory innervation was performed in whole-mount preparations and frozen sections of the periosteum. The advantage of frozen sections is that one can visualize and quantify the innervation, cellular and vascular density in each layer of the periosteum (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 periosteum.

Periosteum 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, periosteum 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, 1:1,000, Chemicon, Temecula, CA, USA) (Lawson and Waddell, 1991). Preparations were then incubated for three 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 DAPI (4'-6-diamidino-2-phenylindole, 1:30000, Invitrogen, Carlsbad, CA, USA) for 5 min. 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 periosteum, cross sectional/frozen section analysis of the right femurs from naïve rats with the periosteum attached to the bone were processed as follows. Right femurs were post-fixed and decalcified. Briefly, demineralization was performed by placing bones in 10% 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 30µm. 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). Blood vessels were immunostained for the platelet endothelial cell adhesion molecule RECA (monoclonal mouse anti-rat CD31, 1:500, BD Pharmingen, San Diego, CA). 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 periosteum

All analyses were performed within the distal metaphysis as the periosteum 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, two random images per rat (400× magnification, 320µm×320µm) were obtained of the entire visible periosteum 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 (two-dimensional projection).

For frozen sections, three random images per rat at 400× magnification (320µm×320µm) 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 periosteum by the thickness of the fibrous or cambium layer. This area was subsequently multiplied by the thickness of the cryostat cut section (30µm) and recorded as the volume of each individual periosteal layer.

In order to quantify the number of proliferating cells in the periosteum, the number of BrdU⁺ nuclei and DAPI⁺ nuclei in each layer of the metaphyseal periosteum was determined. Inclusion criteria for BrdU⁺ nuclei were similar to those reported by Horner et al (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 % 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 periosteum from z-series images of each field of view using Imaris Pro Software v.5.7 (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 non-visible 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 two 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 to the right hind limb (intact hind limb) using an incapitance 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 five 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: $[\text{weight on fractured hind limb} / (\text{weight on fractured hind limb} + \text{weight on intact hind limb})] \times 100$.

Our experimental protocol consisted of four 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) of fractured femurs were obtained immediately post-fracture and at all behavior 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 Image J software (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 one 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). For data obtained from periosteal tissue, a Friedman non-parametric 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 two-group comparisons. To determine differences between groups in the behavior and radiographic results, a one-way ANOVA followed by Mann-Whitney two-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 μ CT 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 μ CT 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 three age groups (Fig 2D–F, 4A). In contrast, the thickness of the cambium layer decreased from $51.21 \pm 3.41 \mu\text{m}$ in 4 month-old rats to $25.53 \pm 1.46 \mu\text{m}$ in 13 month-old rats ($p < 0.005$, Figs 2A–2B, 4A). This thickness further decreased to $21.59 \pm 1.54 \mu\text{m}$ in 36 month-old rats (Figs 2A–C, 4A). These observations are consistent with published data, which have shown that the thickness of 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 (Fig 2D–I, 4B), and this was not significantly different between young and middle-aged rats (Fig 4B). However, in the 36 month-old rats, cell density was significantly reduced compared to 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 periosteal 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 three 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 had a greater density of blood vessels compared to the fibrous layer (Fig 2G–I, 4D). No significant changes in the density of blood vessels were found in the fibrous layer in the three age groups. In contrast, there was a decrease in the density of blood vessels in the cambium layer for middle-aged rats compared to young rats (Fig 4D). There were no further changes in vascularization in the cambium layer of old rats (Fig 4D).

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 envelops 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 three age groups, the vast majority of CGRP⁺ and NF200⁺ fibers were present in the fibrous layer of the periosteum (Fig 4A–F). As the fibrous layer does not significantly change in thickness with age, these results are not confounded 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 of periosteum of young male rats, was not significantly different when compared to 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 to rats with the pin alone (Fig 5A–C). There were no significant differences in the magnitude of the pain-related behaviors between young and old rats with a fracture (Fig 5A–C). Both young and old rats with a pin 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 compared to 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 two-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 (Ostbye et al., 2004; Shane Anderson and Loeser, 2010). 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 innervation of the bone is clearly correlated with the severity of bone pain following injury (Jimenez-Andrade et al., 2009; Wong et al., 2008), so that a reduction (>70%) in the density of CGRP⁺ sensory nerve fibers innervating the femur resulted in a 50% reduction in fracture pain related behaviors (Jimenez-Andrade et al., 2009).

To examine the possibility that unlike bone itself, sensory nerve fibers that innervate bone and transduce noxious stimuli do not undergo a significant decline with age, immunohistochemistry and confocal microscopy were employed to quantify the density of sensory nerve fibers in young, middle-aged, and old rats. It was found that neither of the two 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. This data partially agrees 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 animal. 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 suggests 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 weeks 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 is that if fracture healing time takes longer in old versus young rats, why is the time course of fracture pain behaviors in the older animals not significantly longer than 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 mechano-sensitive 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 be expected 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 to other organs, bone is unique in being a remarkable reservoir of growth factors (e.g. transforming growth factor beta (TGF-beta), bone morphogenetic proteins (BMPs), 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. Non-steroidal anti-inflammatory drugs are effective in attenuating some musculoskeletal pain states (Balano, 1996; Greenfield, 2006); but these therapies frequently may have significant gastrointestinal, cardiovascular, renal and hepatic toxicity with extended use (Lanas et al., 2010; Weinblatt, 1991). For severe musculoskeletal pain, opiates can be used (Mahowald et al., 2005; Mamaril et al., 2007) but opiate-induced side effects such as sedation, cognitive impairment, clouding of mental status and loss of balance tend to be more severe in the elderly (Davis and Srivastava, 2003; Feinberg, 2000). Thus, a major challenge is to develop new analgesics that more effectively relieve skeletal pain without the unwanted side effects. Understanding the changing interaction(s) that occur between the bone and sensory nerve fibers as vertebrates age has the potential to more clearly define the mechanisms and

therapeutic targets for alleviating age-related musculoskeletal pain, and if effective would significantly contribute to healthy aging.

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Figure 1.

Micro-computed tomography (μ CT) images illustrating age-related decline in the cortical and trabecular bone of the rat femur. Longitudinal μ CT image of young (4 months-old) (A), middle-aged (13 months-old) (B), and old (36 months-old) (C) male F344/BNF1 rat femur. Note that the thickness of the diaphyseal cortical bone decreases with age. High power μ CT images (D, E, F) taken from the center of the intramedullary space of the distal metaphysis show a progressive age-related reduction in trabecular number, and trabecular thickness, and an increased trabecular separation.

Figure 2.

Age-related changes in the periosteum of the rat. (A–C) 7 μ m-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 to young animals. In contrast, minimal changes in the thickness of the fibrous layer are observed with age. (D–F) Confocal photomicrographs from sections (30 μ m) of young (D), middle-aged (E) and old (F) bones immunostained with a proliferation marker antibody (BrdU, black) and counterstained with a nuclear cell marker (DAPI, gray). Note that in the young femur; most of the

proliferating cells (BrdU⁺ nuclei) are located in the cambium layer. In contrast, there were very few to zero BrdU⁺ nuclei in the periosteum of middle-aged and old rats. (G–I) Representative confocal images of the bone sections stained with an antibody that labels endothelial cells forming blood vessels (RECA, black) show that the number of blood vessels decreases progressively with age in the inner cambium layer of the periosteum.

Figure 3.

Age-related preservation of the density of sensory nerve fibers innervating the rat femoral periosteum. Representative confocal images of whole mount preparations of the periosteum stained for calcitonin gene-related peptide (CGRP) and 200 kDa neurofilament H (NF200) sensory nerve fibers obtained from the femurs of 4 (A, D), 13 (B, E) and 36 (C, F) month-old F344/BNF1 rats. CGRP is a neuropeptide found mainly in unmyelinated nerve fibers (C-fibers) and some thinly myelinated (A-delta) sensory nerve fibers (A–C), whereas NF200 is expressed by myelinated (A-delta and A-beta) primary afferent sensory nerve fibers (D–F). Note that the density and net-like arrangement of the sensory nerve fibers in the periosteum is maintained in the three age groups. Confocal images (100µm z-series) were projected from 100 optical sections acquired at 1.0µm intervals. Scale bar A–D: 50 µm.

Figure 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 three age groups. Each bar represents the mean $\pm$ SEM. * $p < 0.05$.

Figure 5.

The severity of fracture-induced pain behaviors is similar in the young and old rat. Following closed fracture of the femur, young (5 month-old) and old (26 month-old) F344/BNF1 rats exhibited dramatically increased time spent spontaneously guarding (A), a greater number of spontaneous flinches (B), and reduced weight bearing of the fractured limb (C) as compared to animals that received intramedullary pin placement alone. Note that the magnitude and duration of the pain-related behaviors following fracture were not influenced by age.