Evidence for a neuropathic contribution to the development of spontaneous knee osteoarthrosis in a mouse model

Paul T Salo[1], Ruth A Seeratten[1], W Mark Erwin[2] and Robert C Bray[1]

[1]McCaig Centre for Joint Injury and Arthritis Research, Department of Surgery, University of Calgary and [2]Institute for Medical Science, University of Toronto. Correspondence: Paul T. Salo, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada. E-mail: salo@ucalgary.ca
Submitted 00-08-21. Accepted 01-06-15

ABSTRACT – Previous work has shown a progressive, age-related loss of knee joint innervation in the C57BL6Nia mouse. We did three experiments to describe further the loss and determine whether it might contribute to the development of knee osteoarthrosis in this model. Immunocytochemistry showed that the percentage of neurons expressing substance P and calcitonin gene-related peptide increased with age, indicating a relatively selective loss of mechanoreceptors. Histological examination of knee joints of mice at various ages showed that loss of joint innervation always preceded histological changes of cartilage degeneration. The mice usually developed a mild form of osteoarthrosis, but surgical ablation of joint innervation caused the development of severe patellofemoral osteoarthrosis.

The findings are consistent with the hypothesis that an age-related loss of joint innervation may contribute to the development of osteoarthrosis.

■

The cause of 'idiopathic' osteoarthrosis remains unknown, but abnormal loading of joint surfaces is possible in many patients (Radin 1983). It has been speculated that a loss or dysfunction of joint sensory innervation can result in abnormal joint loading in some patients (Cooke 1985, Marshall and Tatton 1990, O'Connor and Brandt 1993). The potential destructiveness of limb sensory denervation is exemplified clinically by the phenomenon of Charcot neuroarthropathy.

We have previously reported an age-related loss of knee joint sensory innervation in the C57BL6 mouse (Salo and Tatton 1993). Most neuronal loss occurred during the first third of the mouse's typical life span. Others had reported that a related strain of C57BL6 mouse developed knee arthritis with a prevalence dependent on age (Wilhelmi and Faust 1976, Pataki et al. 1980, Maier and Wilhelmi 1987, Wilhelmi and Maier 1987). The prevalence of knee arthrosis in this mouse increased substantially with age, in a manner that seemed to reflect a reciprocal relationship to the gradual loss of knee joint innervation that we observed.

To define more clearly the relationship between these two age-related phenomena, we made three experiments. First, we wished to confirm our impression that the mechanoreceptor neurons of large diameter were lost at a more rapid rate than the nociceptive neurons of smaller diameter. There are no selective histologic markers for mechanoreceptor neurons in the mouse, but a significant percentage of small diameter nociceptive neurons express the peptides substance P (SP) and/or calcitonin gene-related peptide (CGRP). We reasoned that a more rapid rate of loss of mechanoreceptor neurons would lead to relative increases in the percentage of joint afferents expressing SP and/or CGRP in older mice. Secondly, we aimed to correlate the loss of joint innervation with the prevalence and severity of degenerative change in the knee in a homogeneous group of mice. If there was a causal relationship, it would require that the loss of joint innervation precedes significant degeneration. Thirdly, we wished to determine whether early, near total ablation of joint afferent innervation would accelerate the development of spontaneous osteoarthrosis in the C57BL6 mouse strain.

If joint afferents mediate protective reflexes that can moderate loads damaging to joint surfaces, then an early, almost total loss of joint innervation should induce (or permit) an earlier and more severe arthritis in the denervated joint.

Methods

Neuropeptide expression in aging joint afferents

Male C57BL6/Nia mice were taken from the environmentally-isolated colony maintained by the U.S. National Institute for Aging. Mice were obtained at 3 ages: 8, 52 and 96 weeks (n 8 in each group), including most of the typical life span of this mouse strain. All animals were handled in accord with the guidelines of the Canadian Council on Animal Care and the local institution's animal care committee approved the experimental protocols.

Technique of labeling joint afferents. Under general inhalational anesthesia (O2/N2O/halothane), the knee joint was stabilized in a positioning device and a 27 gauge needle was inserted into the space between the patella and femur. The joint was injected with 2 μL of 2% Fluoro-Gold (FG) followed by 6 μL of 0.1 M phosphate buffer (to precipitate the FG in the joint space), containing 0.1% Fast Green (to detect extravasation) according to our previously described technique (Salo and Tatton 1993). Older mice had only one knee injected because of their limited tolerance of general anesthesia (for the numbers of mice used and joints injected, see descriptions of individual experiments below).

Dorsal root ganglion histology. 7 days after the FG labeling procedure, the mice were deeply anesthetized with intraperitoneal sodium pentobarbital and perfused for 1 minute via the left ventricle with ice-cold heparinized normal saline (4 units/mL) followed by ice-cold fresh 4% paraformaldehyde in 0.1 M phosphate buffer for 15 minutes. The spine was then immediately removed to a dissecting board. Complete laminectomies were performed from T13 to S1, the levels known to contain the entire population of knee joint afferents in the mouse. The pair of dorsal root ganglia at each level was removed and postfixed overnight in fresh ice cold 4% paraformaldehyde contained in 0.1 M phosphate buffer. On the following day, the ganglia were washed in buffer and placed in 20% sucrose overnight. The next day, they were blotted dry, embedded in Tissue-Tek and frozen by immersion in liquid nitrogen.

Specimens were stored at –20 °C until sectioning. Blocks were mounted in a Reichert-Jung cryostat and serial 6 μm transverse frozen sections collected on gelatin-coated slides. Sections were counterstained in aqueous 1% toluidine blue, dehydrated and coverslipped with Eukitt.

Immunohistochemistry. After fixation and embedding, as described above, 10 μm serial frozen sections were cut from the L2 and L3 DRGs harvested from 8 mice prelabeled by intraarticular FG injection. We used only the L2 and L3 ganglia for immunohistochemistry because we had found that, on average, over 80% of the joint afferents were located there (Salo and Tatton 1993).

The detailed immunohistochemistry protocol for visualization of the neuropeptides has been reported elsewhere (Salo and Theriault 1997). Briefly, after washing and blocking with normal goat serum, primary incubation was performed overnight in humidity chambers at 4 °C. Anti-SP (Peninsula) was used at a titer of 1/5000 in 2% normal goat serum and anti-CGRP (Genosys) at a titer of 1/3000 in 2% normal goat serum. Specific binding was visualized by incubating for 1 h with biotinylated goat antirabbit IgG followed by Texas red avidin D (Vector). The slides were rinsed in phosphate-buffered saline and coverslipped with Mowiol. Slides were stored at 4 °C in the dark for no more than 1 week before viewing. Every 4th section was examined using a Leitz DMRB fluorescence microscope, alternating filters appropriate for FG (excitation: 355–425 nm, long-pass filter: 460 nm) or Texas red (excitation: 515–560 nm, long-pass filter: 580 nm). We examined every 4th section to reduce the risk of double-counting a labeled cell. In each section we first counted the total number of FG-labeled profiles and then the number of FG-labeled profiles that also contained Texas red immunofluorescence.

Correlation between loss of joint innervation and osteoarthrosis

21 male C57BL6/Nnia mice were obtained at each of three ages, 7 at 26, 8 at 52 and 6 at 96 weeks.

Fluoro-Gold labeling of joint afferents was done as described above. In 8 mice, only 1 knee was injected with Fluoro-Gold. In 13 mice, both knees were injected. 1 week after FG labeling, mice were sacrificed for counting of labeled knee joint afferents in the lumbar dorsal root ganglia and histologic grading of degenerative change in the corresponding knee.

Counting of FG-labeled joint afferents. Every section through every ganglion (ranging from 80 to 160 sections per ganglion) was examined alternately under brightfield and U.V. epifluorescence with appropriate filters for FG (excitation: 355–425 nm, long-pass filter: 460 nm) using a Leitz DMRB microscope with fluorescence illuminator. Comparison of each section with the previous and following sections in the series allowed the labeled cellular profiles to be followed ensuring that each labeled cell was counted only once (serial reconstruction method of Coggeshall et al. 1990). The total number of FG-positive somata was counted for each ganglion.

Joint histology. Mice were perfused with 4% paraformaldehyde and knees removed en bloc by transecting the limbs at mid-femur and mid-tibia. The joints were postfixed overnight in 4% paraformaldehyde and then placed in 10% formic acid decalcifying solution for 4 weeks, making changes in the solution every 3 days. The joints were trimmed and the tissue blocks dehydrated, infiltrated and paraffin-embedded. Serial 8 µm sagittal sections were cut through the entire joint, so that the medial, lateral and patellofemoral compartments could all be examined. Sections were collected alternately onto slides to create two sets of alternate sections from each joint. 5 sections were mounted on each slide. An average of 125 sections was obtained from each joint.

One set of alternate sections was stained with hematoxylin and eosin; the other set was stained with hematoxylin, fast green and safranine-O (Mankin et al. 1971).

Osteoarthrosis grading and scoring. The entire set of serial sagittal sections was reviewed for each joint. Histological lesions were identified and graded according to the simplified criteria of Maier and Wilhelmi (1987) (Figure 1). The number of lesions and grade of each were recorded for each joint. We then assigned an osteoarthrosis score

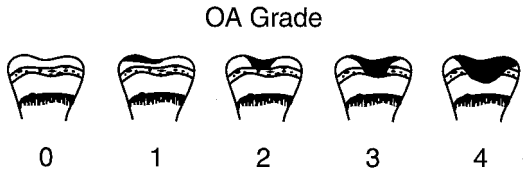

Figure 1. OA grading (adapted from Maier and Wilhelmi 1987).
Grade 0: joint surface intact.
Grade 1: superficial fissuring and fibrillation.
Grade 2: small "punched out" defects, no deeper than the tidemark.
Grade 3: shallow defects extending into calcified cartilage, subchondral sclerosis.
Grade 4: deep defects extending into bone, eburnation, prominent osteophytes.

to each joint by totaling the number of cartilage lesions observed, with each lesion graded from 1–4 by the Maier and Wilhelmi's method for classifying severity. Thus for each knee joint we determined both the number of joint afferents and an osteoarthrosis score.

Effect of joint denervation on prevalence and severity of osteoarthrosis

Ablation of joint afferents. 12 male, 8-week-old C57BL6Nnia mice were used. Under general anesthesia, using sterile technique and with the aid of an operating microscope, the left L3 dorsal root ganglion was resected via a posterior approach. Mice returned to normal cage activity within 1 day of surgery. We detected no defects in mouse ambulation, grooming or feeding behavior. The mice were housed four to a cage, in an environmentally-isolated colony, for the next 14 months.

Joint histology. 14 months after the denervation procedure, all mice were killed and both knees removed and processed, as described above, for histological assessment of the joint surfaces. For each mouse, the contralateral knee served as a control.

Statistics

In the first experiment, we determined the average number of neurons expressing each of two different neuropeptides, at three ages. The number of neurons expressing a given neuropeptide within a definable population of neurons would be expected to be normally distributed about a mean. We therefore used a one-way analysis of variance to determine the significance of differences seen between the three age groups.

In the second experiment, we determined the number of joint afferents and the osteoarthrosis score for knee joints from the three age groups. The number of joint afferents (DRG neurons) and severity of osteoarthrosis (OA score), plotted on histograms, were not normally distributed. We therefore used Kruskal-Wallis one-way non- parametric analysis of variance (ANOVA) and Spearman's rho non- parametric correlation analyses. All analyses were done using Systat v.10 for Windows (SPSS 2000).

In the third experiment, we studied denervated and non-denervated knees from the same animal. All of the denervated knees differed markedly in appearance from the non-denervated knees. The appearance of the latter were also compared to the appearance of knee joints of similar age from the second experiment (external controls). However, no statistical analysis was done.

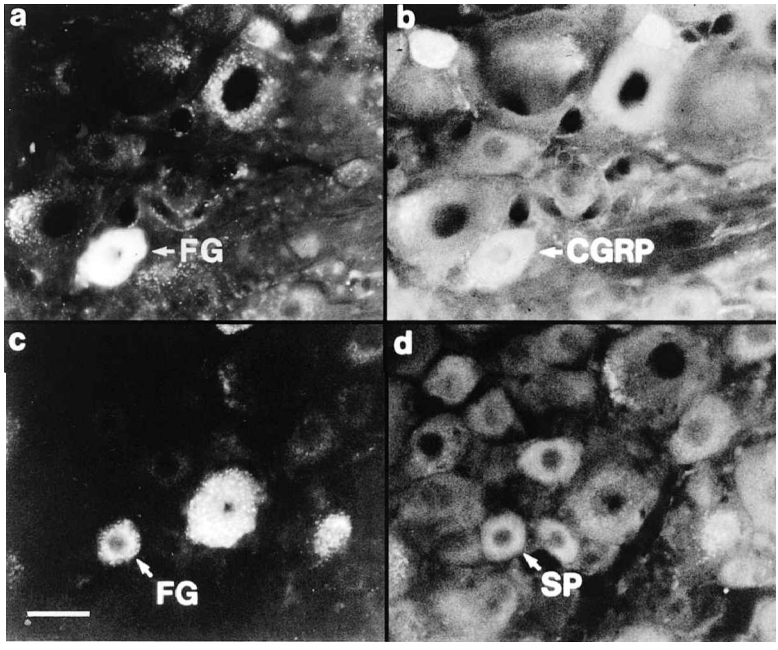

Figure 2. Composite photomicrograph of two sections of mouse DRG stained for CGRP (above) or SP (below). On the left, UV epifluorescence for Fluoro-Gold, on the right, same section viewed with UV epifluorescence for CGRP (above) or SP (below). Scale bar = 25 microns.

Results

Neuropeptide expression in aging joint afferents

Fluoro-Gold was found to be fully compatible with fluorescent immunohistochemistry in this model (Figure 2). At 8 weeks of age, 11 (1.8)% (mean (SD)) of FG-labeled joint afferents expressed SP-like immunoreactivity and 22 (8.6)% expressed CGRP-like immunoreactivity. By 52 weeks, these percentages increased to 15 (3.3)% and 31 (5.0)%, respectively. At 96 weeks, the corresponding percentages were 16 (2.5)% and 33 (9.2)%. The increases in the proportions of neurons expressing neuropeptide immunoreactivity from 8 weeks to 96 weeks of age were significant for SP and CGRP, using a one-way analysis of variance ($p = 0.02$).

Correlation between loss of joint innervation and osteoarthrosis

The types of cartilage lesions observed are illustrated in Figure 3. Most of the mice showed relatively mild cartilage degeneration, consisting of surface ulceration or fibrillation. No mice had grade 3 or 4 lesions.

The data comparing the number of joint afferents with the osteoarthrosis score are shown in Figure 4. Below an arbitrary threshold of 100 afferents (40% of the average number at 8 weeks of age), the prevalence of degenerative change seemed to be increased.

The Kruskal-Wallis ANOVA analysis demonstrated a statistically signficant relationship between the OA score and number of joint afferents in the three age groups ($p = 0.001$). This level of statistical significance indicates a high correlation between osteoarthrosis and the number of joint afferents. Spearman's rho correlation revealed that the OA score and number of joint afferents had a strong inverse correlation of –0.75 ($p = 0.001$).

However, in this group of mice with spontaneous osteoarthrosis, higher OA scores usually reflected

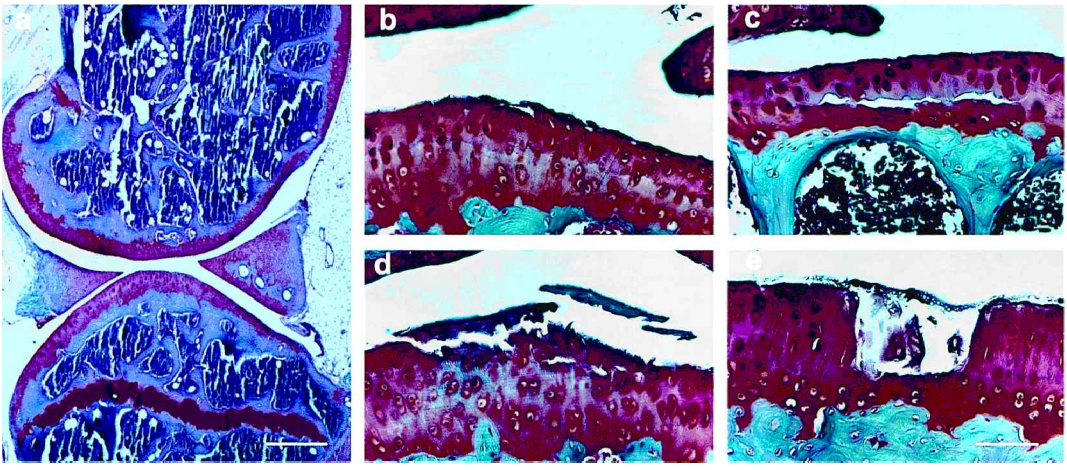

Figure 3. Composite photomicrograph illustrating typical cartilage lesions in knee joints of mice at various ages.
a. Low power view of a complete sagittal section of a normal joint (scale bar = 350 microns).
b. surface fibrillation.
c. delamination at the tidemark.
d. erosion down to tidemark.
e. "punched-out" lesion (scale bar = 100 microns).

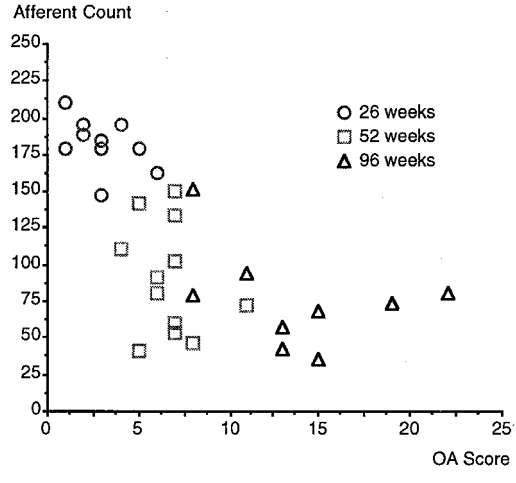

Figure 4. Scatterplot of the number of joint afferents versus OA score for each joint. Each point is the result from one knee joint. The differently-shaped symbols indicate the age of the mouse at killing.

a larger number of lesions in the joint, not a greater severity of individual lesions.

Effect of joint denervation on prevalence and severity of osteoarthrosis

1 mouse died under anesthesia at the time of the denervation procedure, so 11 mice were available for review 14 months after denervation. All knee joints ipsilateral to the L3 dorsal root ganglionectomy had severe (grade 4) patellofemoral arthrosis with ablation of the cartilage surface, subchondral sclerosis, marginal osteophytes and eburnation (Figure 5). 1 mouse also developed similar grade 4 degenerative changes in the medial joint compartment. These changes were far more severe than any of the spontaneous changes seen in aging mice.

None of the knee joints contralateral to the L3 dorsal root ganglionectomy developed patellofemoral cartilage degeneration. These joints seeemed to have the same appearance as those from 52-week-old mice described in the second experiment, showing mild (grade 1–2) tibio-femoral degeneration and sparing of the patellofemoral joint. None of the aging mice in the second experiment developed spontaneous patellofemoral arthrosis.

Discussion

Neuropeptide expression in aging joint afferents

Age-related loss of joint innervation has not been reported in any other species. The finding that the percentage of neuropeptide-expressing joint afferent neurons increased with age is additional evidence that the spontaneous, age-related loss of joint afferents is relatively selective for mechanoreceptors. In our previous study, we showed that most

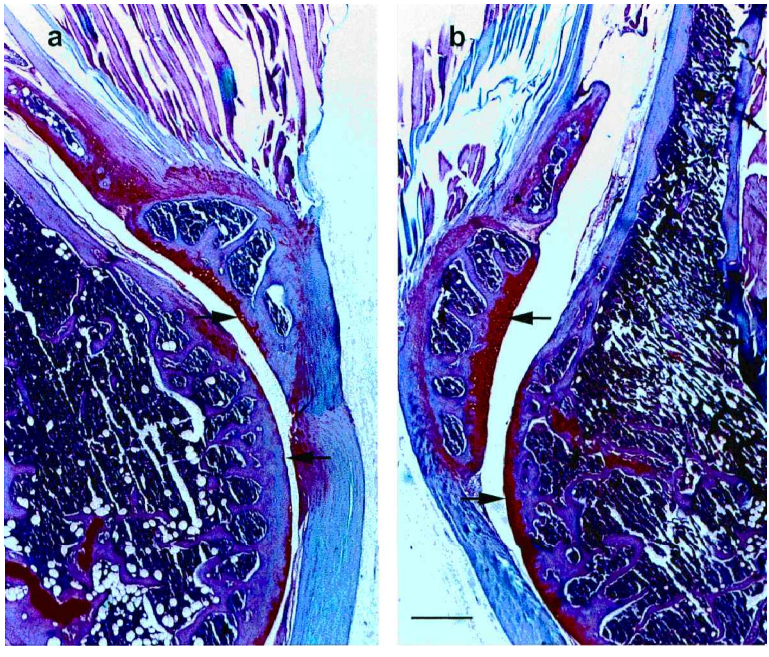

Figure 5. Composite photomicrograph showing left and right patellofemoral joints of a mouse 14 months after unilateral (left) L3 dorsal root ganglionectomy. On the left (a), the denervated joint, on the right (b), joint with intact innervation. Note the loss of cartilage matrix on both patellar and femoral articular surfaces, with eburnation (arrows). The red staining in the proximal patellar tendon of the denervated joint is an area of chondrometaplasia (scale bar = 200 microns).

of the spontaneous loss of joint innervation and of the relative increase in peptide-expressing neurons occurred in the first year of life.

Correlation between loss of joint innervation and osteoarthrosis

The apparent correlation between the loss of joint innervation and the development of spontaneous degenerative change should not be considered proof of a causal relationship. However, the fact that the loss of joint innervation always occurred before the appearance of significant degenerative change is consistent with the hypothesis that a loss of joint innervation contributes to or accelerates the degenerative process.

The spontaneous loss of knee joint innervation averaged about 60% by 96 weeks of age. We previously showed that the mouse knee joint is initially supplied by an average of about 240 neurons. It may be that some of these neurons mediate a larger contribution to protective neuromuscular reflexes than others. If all neurons of a certain type are equally predisposed to premature death, then loss of an individual neuron is probably a random event. The ability of surviving neurons to compensate for loss of other neurons is unknown. We were also unable to record or analyze differences in the activity levels of individual mice. Some of these factors may explain why there is not a closer correlation between the number of surviving joint afferents and the severity of joint degeneration.

Accelerated osteoarthrosis after surgical denervation

In our third experiment, L3 dorsal root ganglionectomy resulted in an immediate loss of 80% of the ipsilateral knee joint sensory neurons. This caused a degenerative change which affected every denervated knee.

It is unclear why the patellofemoral joint was preferentially affected. Perhaps, in a knee joint with intact ligamentous stabilizers, the patellofemoral contact forces are most disturbed by L3 ganglionectomy, hence the patellofemoral joint would be more likely to develop the degenerative changes normally mitigated by protective reflexes. Further

experiments may be able to address this indirectly by measuring quadriceps activity with quantitative EMG monitoring before and after ganglionectomy.

Previous reports of the effect of joint denervation on the subsequent occurrence of degenerative arthritis have been inconsistent. An early report suggested that denervation alone could induce cartilage degeneration in cats (Eloesser 1917). Another report with a rabbit model describes the rapid onset of cartilage degeneration after knee joint denervation (Finsterbush and Friedman 1975). However, a series of more recent experiments using a dog model produced contradictory results (O'Connor et al. 1985, 1992). These authors found that joint denervation alone, by dorsal root ganglionectomy or articular neurectomy, did not induce degenerative change in animals observed as long as 16 months after the procedure. If denervation was combined with anterior cruciate ligament transection, however, then an extremely rapid and destructive degeneration of the femoral condyle cartilage ensued. O'Connor and colleagues concluded that intact joint innervation gave protection only against OA in unstable joints.

The experiments in the present study, using the isogenic C57BL6Nnia mouse, do not refute the results reported by O'Connor and colleagues (O'Connor et al. 1985, 1992). In essence, surgical denervation accelerates cartilage degeneration. Our study differs from those of O'Connor et al. in that we did not damage the knee joints to induce instability. However, the fact that most C57BL6/NNia mice develop a mild spontaneous osteoarthrosis suggests that there may be an intrinsic abnormality of the cartilage matrix or joint biomechanics. Our results are consistent with the view that denervation of the joint induces a sufficient change in the biomechanics of the joint to compromise further the ability of the cartilage to maintain homeostasis, thereby accelerating degeneration. Nevertheless, since almost all C57BL6/NNia mice lose some knee innervation during their lifetime, our results do not exclude the possibility that spontaneous denervation is the only joint abnormality and the primary cause of the degenerative process in this model.

A possible criticism of the earlier dog experiments is the relative duration of follow-up after denervation. O'Connor et al. waited 16 months from denervation until assessment, which was about 15% of a dog's typical life span (O'Connor et al. 1985). We waited 14 months between denervation and assessment, but this is about 60% of the natural life span of the mouse. Osteoarthritis is a disease of aging, and is rare during the first half of the normal human life span, so regardless of the etiology, the pathogenesis must be a long process. One wonders whether similar degenerative changes might have developed in the denervated knee joints of dogs if they had been followed for an equivalent percentage of their natural life span.

It is also possible that the effect of joint denervation is species-specific. Dogs may be less likely to develop degenerative arthritis than inbred mice. More experiments in other species may be justified to tackle this question.

Funding to support this research was received from the Orthopaedic Research and Education Foundation, the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

Coggeshall R E, La Forte R et al. Calibration of methods for determining numbers of dorsal root ganglion cells. J Neurosci Meth 1990; 35: 187-94.

Cooke T D V. Pathogenetic mechanisms in polyarticular osteoarthritis. Clin Rheum Dis 1985; 11 (2): 203-38.

Eloesser L. On the nature of neuropathic affections of the joints. Ann Surg 1917; 66: 201-7.

Finsterbush A, Friedman B. The effect of sensory denervation on rabbits' knee joints: a light and electron microscopic study. J Bone Joint Surg (Am) 1975; 57: 949-56.

Maier I, Wilhelmi G. Osteoarthrosis-like disease in mice: effects of anti-arthrotic and anti-rheumatic agents. In: Studies in osteoarthrosis: pathogenesis, intervention and assessment. (Ed. Lott D J et al.). John Wiley and Sons Chichester, New York, Brisbane, Toronto, Singapore 1987: 75-83.

Mankin H J, Dorfman H et al. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. J Bone Joint Surg (Am) 1971; 53 (3): 523-37.

Marshall K W, Tatton W G. Joint receptors modulate short and long latency muscle responses in the awake cat. Exp Brain Res 1990; 83: 137-50.

O'Connor B L, Brandt K D. Neurogenic factors in the etiopathogenesis of osteoarthritis. Rheum Dis Clin North Am 1993; 19 (3): 581-605.

O'Connor B L, Palmoski M J et al. Neurogenic acceleration of degenerative joint lesions. J Bone Joint Surg (Am) 1985; 67 (4): 562-72.

O'Connor B L, Visco D M et al. Neurogenic acceleration of osteoarthritis. J Bone Joint Surg (Am) 1992; 74 (3): 367-76.

Pataki A, Ruttner J R et al. Age-related histochemical and histological changes in the knee joint cartilage of C57BL mice and their significance to the pathogenesis of arthritis. I. Oxidative enzymes. Exp Cell Biol 1980; 48: 329-48.

Radin E L. The relationship between biological and mechanical factors in the etiology of osteoarthritis. J Rheumatol (Suppl 9) 1983; 10: 20-1.

Salo P T, Tatton W G. Age-related loss of knee joint afferents in mice. J Neurosci Res 1993; 35: 664-77.

Salo P T, Theriault E T. Number, distribution and neuropeptide content of rat knee joint afferents. J Anat 1997; 190: 515-22.

SPSS (2000). Systat Version 10 for Windows, www.spssscience.com.

Wilhelmi G, Faust R. Suitability of the C57 black mouse as an experimental animal for the study of skeletal changes due to ageing, with special reference to osteo-arthrosis and its response to tribenoside. Pharmacology 1976; 14 (4): 289-96.

Wilhelmi G, Maier R. Observations on the influence of weight-bearing stress and movement on the joints of mice predisposed to osteo-arthritis. Aktuel Rheumatol 1987; 12 (2): 161-7.