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Prolonging disuse in aged mice amplifies cortical but not trabecular bones' response to mechanical loading

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Abstract

Objective: Short-term neurectomy-induced disuse (SN) has been shown to restore load responses in aged mice. We examined whether this restoration was further enhanced in both cortical and trabecular bone by simply extending the SN. **Methods:** Following load:strain calibration, tibiae in female C57BL/J6 mice at 8, 14 and 20 weeks and 18 months (n=8/group) were loaded and bone changes measured. Effects of long-term SN examined in twenty-six 18 months-old mice, neurectomised for 5 or 100 days with/without subsequent loading. Cortical and trabecular responses were measured histomorphometrically or by micro-computed tomography. **Results:** Loading increased new cortical bone formation, elevating cross-sectional area in 8, 14 and 20 week-old ($p < 0.05$), but not 18 month-old aged mice. Histomorphometry showed that short-term SN reinstated load-responses in aged mice, with significant 33% and 117% increases in bone accrual at 47% and 37%, but not 27% of tibia length. Cortical responses to loading was heightened and widespread, now evident at all locations, following prolonged SN (108, 167 and 98% at 47, 37 and 27% of tibial length, respectively). In contrast, loading failed to modify trabecular bone mass or architecture. **Conclusions:** Mechanoadaptation become deficient with ageing and prolonging disuse amplifies this response in cortical but not trabecular bone.

Keywords: Mechanical Loading, Osteoporosis, Sciatic Neurectomy, Bone, Ageing

Introduction

Ageing is closely linked to vulnerability of the skeleton to osteoporosis and frailty. The mechanisms accounting for this age-related decline in mass that underpin bone's loss of functional competence have, however, yet to be fully elucidated. Age-related decline in bone mass affects both trabecular and cortical compartments of the appendicular skeleton and may reflect changes in gonadal status, nutrition or adaptation to mechanical loading, leading to decreased bone strength and consequently increased morbidity and mortality in the elderly[1].

It is well established that bones' adaptive response to mechanical loading is a primary determinant of bone mass and architecture by increasing bone thickness and mineral density in humans as well as in animal models. While it is clear that this adaptation is readily achieved in the young, load-bearing associated with habitual use appears insufficient however to maintain bone mass and architecture in aged individuals[2]. Previous studies have reported that adaptation to loading occurs primarily in the juvenile skeleton and that it progressively diminishes in skeletally mature animals with ageing[2-4]. It is possible, therefore, that a restricted capacity for adaptation to mechanical demands underpins the failure to retain bone mass during ageing.

A previous study investigating the role of the sympathetic nervous system in bone's anabolic response to loading, revealed that the imposition of 5 or ~100 days of sciatic neurectomy (SN)-induced hind-limb disuse in young, growing and skeletally mature female mice, amplifies load-induced increases in tibial cortical bone area and new bone formation[5]. This study found that SN indeed enhanced strain-related bone accrual even when applied loads had been

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calibrated to engender identical peak strain magnitudes. This suggests that habitual limb use, which is lacking following SN, may act to restrict bone's sensitivity to peak mechanical strain[6,7]. These data raise many questions, including: can the sensitivity of the mechano-adaptive response be adjusted by the habitual loading environment, is any such adjustment subject to exaggeration with prolongation and, a critical issue for age-related decline in bone mass, can strain-induced osteogenic responses be reinstated to potentially correct cortical and trabecular bone deficiencies in the aged skeleton? This is an exciting possibility, since approaches that disclose any enhancement of the aged skeleton's sensitivity to loading may effectively reveal new combination strategies for the treatment of age-related, as well as short and long term disuse-induced osteoporotic bone loss.

A more recent study found that reduction in capacity for load-induced osteogenesis in aged animals is also reversible by application of a short 4 day-long period of SN. It was suggested that short-term disuse was sufficient to eliminate any age-related differences to external mechanical stimuli at least in cortical bone[7]. Disuse osteoporosis in humans, however, often occurs following very extended periods of bed rest, spinal cord injury/nerve damage[8], spaceflight[9] and hemiplegia after stroke[10] leading to bone loss, increased risk of fracture and consequently life-long immobility. It remains to be seen whether longer-term disuse, induced for instance by SN, in animal models could also eliminate age-related differences to external mechanical stimuli in both cortical and trabecular bone compartments.

All bone types exhibit some highly conserved factors in their development, remodelling and repair. It is also apparent that the response to many *in vivo* challenges, including those engendered by external mechanical stimuli are however not always identical in cortical and trabecular bone types[11]. It is clear that despite constituting a relatively small fraction of total bone volume, organisational alignment in trabecular bone produces robust load-bearing and also provides much of the total bone surface area for remodelling. Herein, we have examined the hypothesis that restoration of load-induced osteogenesis by short-term SN can be further enhanced in both cortical and trabecular bone types by simply extending the period of disuse.

Materials and methods

Animals

Female C57BL/J6 mice (Charles River Company, UK) were randomized and housed in polypropylene cages in groups of 4, subjected to 12 h light/dark cycle with room temperature at 21±2°C and fed *ad libitum* with maintenance diet (Special Diet Services, Witham UK). All procedures complied with the Animals (Scientific Procedures) Act 1986 and local ethics committee.

Ex vivo load:strain calibration

For all ages, strain gauge recordings were used to establish the relationship between the applied axial load and the resultant bone strain by first undertaking a suitable calibration procedure as described previously[12]. Briefly, trimmed single element gauges (EA-06-015LA-120, Measurements Group Inc., Basingstoke, UK) were attached to medial and lateral surfaces of the tibial diaphyseal mid-shaft, proximal to the junction with the fibula, in longitudinal alignment. Gauges were connected to a strain conditioner/amplifier system and recordings captured and analysed using Workshop 96 software (Dartec Ltd., Stourbridge, UK). Hind limbs with strain gauges attached (n>6 at each age) were subsequently positioned in the axial loading apparatus, and load magnitudes required to engender peak surface strains of between 500 and 3000 με were determined *ex vivo*.

In vivo loading

The apparatus and protocol for dynamically loading the mouse tibia/fibula have been reported previously[12]. Briefly, under isoflurane-induced anaesthesia the right tibia of each mouse was held in the padded cups, and dynamic (40 cycles/day, 2 Hz, with 10 s rest periods between cycles) axial loads generating 2000me were applied through the knee on alternate days for two weeks; 6 loading episodes in total. From the strain gauge data (see "*Ex vivo* load: strain calibration"), different peak loads for young and aged mice were calculated (13N at 8, 14, 20 weeks, and 12N to tibiae of mice aged >18 months of age or 10N and 7N to similarly aged mice following either 5 or 100 days of SN, respectively). The loaded mice were able to move around in the cage and gained access to food and water without difficulties and no adverse effect was observed. Three days after the final loading episode, mice were sacrificed by cervical dislocation; right and left (left acting as contra-lateral control) tibiae were dissected and processed for bone histomorphometry analysis as previously described[12].

Assessment of the cortical response to mechanical loading

To assess bone formation, all animals received an intraperitoneal injection of calcein (7 mg/kg, Sigma Chemical Company, MO, USA) on the first and last days of loading and transverse sections of the tibia analysed by histomorphometry as described previously[12,13]. Briefly, left (control) and right (loaded) tibiae were dissected, fixed in buffered formal saline (BFS), washed and dehydrated through graded concentrations of alcohol. Tibiae were infiltrated and embedded using a flat polymethylmethacrylate (PMMA, British Drug House) bed technique. After polymerization at 42°C, the PMMA blocks were trimmed and cut with an annular diamond saw into 500 μm thick, serial planar parallel segments along the shaft length. Fluorochrome labels were observed using a laser scanning confocal microscope (Carl Zeiss Ltd., Herts., UK). Measurements at fixed sites were made from the outside edges of periosteal and inside edges of endosteal labels. The

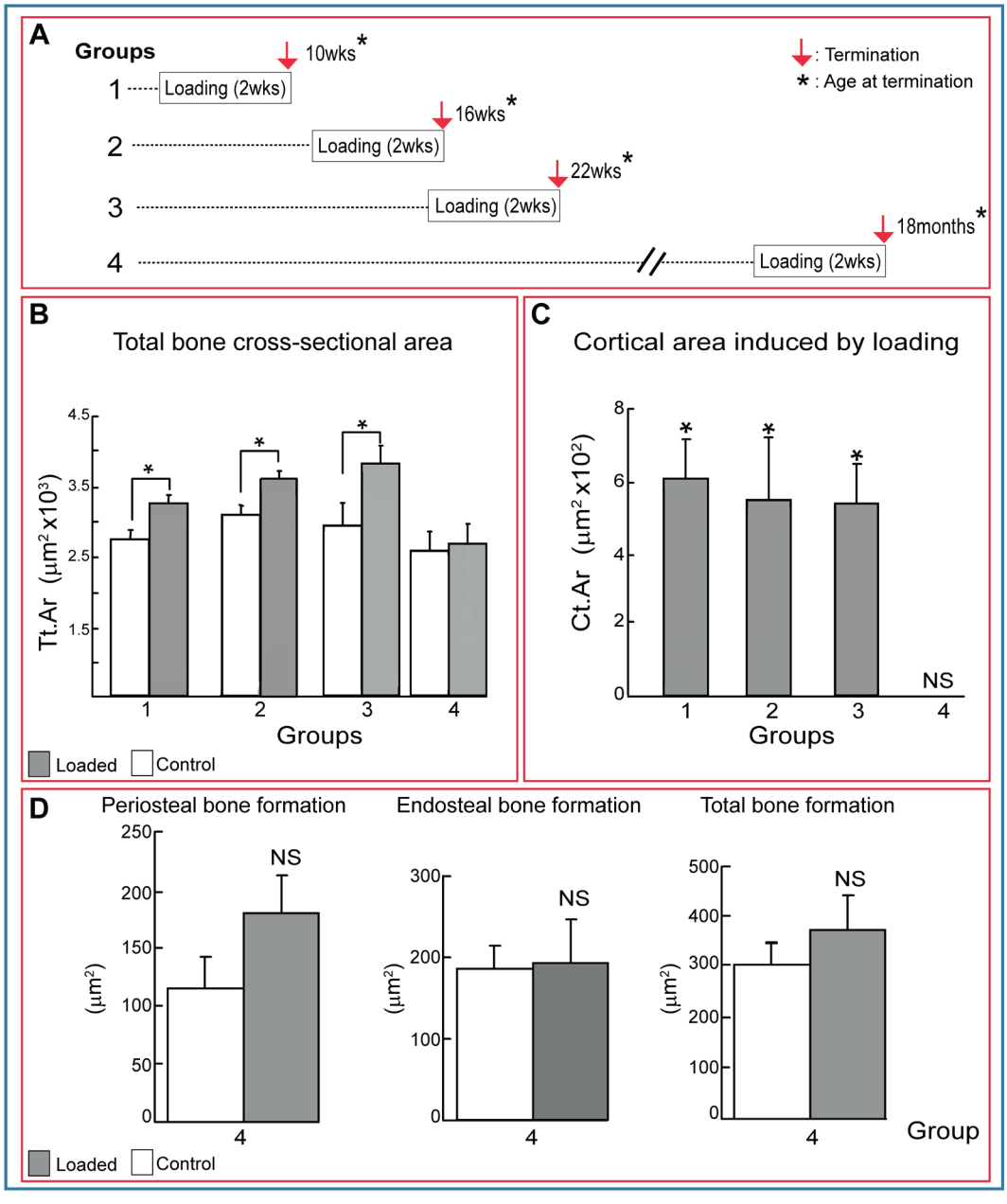

Figure 1. Adaptation to loading at various ages and lack of response in aged mice. A: Diagrammatic representation of loading protocol used to evaluate loading response in mice at 8, 14 and 20 weeks as well as 18 months of age. Calcein labels were administrated on the 1st and last days of loading during the 2 weeks loading period. **B:** Increases in total bone cross sectional area (Tt.Ar) in tibiae in mice at three ages at 2000με for 2-weeks, showing increases in cortical bone area (Ct.Ar) (μm^2) in response to first loading episode in three ages (Groups 1, 2 and 3) and not aged group (4). **C:** Increases in cortical bone area (μm^2) induced by loading (increases vs. non-loaded contralateral control). **D:** Response, in aged mice, to mechanical loading (2000με) for 2-weeks; periosteal bone formation (μm^2); endosteal bone formation (μm^2), and total bone formation (μm^2). Data shown are mean±SEM (n=8/group). "NS" denotes non-significance and *p<0.05.

area between the outer limit of the periosteal and endosteal internal label (μm^2) represents new bone formation.

Sciatic neurectomy (SN) and loading

To examine the response of bones in aged mice to defined load-engendered mechanical strain with and without a brief (5d) or prolonged (100d) period of prior disuse, imposed by SN, 26 female C57Bl/6J mice aged 18 months old were randomly divided into one of four groups, each of which was 18 month of age after SN. In the first group, the right leg was SN operated 5 days prior to sacrifice (group 5: n=8, 5d SN); in the second group the right leg was subjected to SN for

five days prior to the application of 2 weeks of axial loading (group 6: n=8, 5d SN+Load), in the third group, the right leg was SN operated 100 days prior to sacrifice (group 7: n=5, 100d SN); in the fourth group, the right leg was subjected to SN for 100 days prior to the application of axial loading (group 8: n=5, 100d SN+Load). The effect of SN disuse on the response to loading was studied after appropriate calibration as described previously (see "*ex vivo* load:strain calibration").

SN was accomplished as previously described[12,14]. Briefly, under isoflurane-induced anaesthesia, an incision was made caudal to the right hip joint and the biceps femoris muscle elevated to expose the nerve and SN was achieved by resecting a 3-4 mm segment of the sciatic nerve posterior to the hip joint. Sham surgery was performed on the right limb of control group animals. The neurectomised and sham-operated mice were able to move around in the cage and gained access to food and water without difficulties. Mice were killed, and right and left (used as contralateral control) tibias were dissected and processed for bone histomorphometry.

Assessment of the trabecular response to mechanical loading following SN

Tibiae were scanned (by Maiko Matsuura, Ludwig-Maximillian University, Munich, Germany) using a micro-computed tomography system (μCT20; Scanco Medical AG, Basserdorf, Switzerland) to evaluate trabecular architecture as previously described[12]. High resolution scans with an isotropic voxel size of 9 μm and an integration time of 200 ms were acquired. The bone tissue was segmented from non-bone tissue by applying a Gaussian filter and a fixed threshold procedure. Trabecular bone volume (BV/TV), number (Tb.N; /mm), thickness (Tb.Th; mm), trabecular separation (Tb.Sp) and structural measure index (SMI) were evaluated.

Statistical analysis

Cortical and trabecular bone analyses were performed using student's t-test to compare between right (loaded) and left contra-lateral (non-loaded, control) tibiae in individual mice. Where appropriate comparison was made between right tibiae exposes to either 5 or 100 days of SN, with or without subsequent loading. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) and results are expressed as the mean±standard error of the mean (SEM). $P<0.05$ was considered to be significant.

Results

Loading-induced adaptation is achieved in tibia of young, growing and mature, but is absent in aged female mice

We initially examined whether a 2-week long period of episodic dynamic tibial loading was capable of provoking changes in cortical bone structure in a range of age groups (Figure 1A). In keeping with previous findings[12] we found that loading, produced significant new cortical bone formation, with increases in total bone cross-sectional area (Tt.Ar) observed in female mice aged 10, 16 and 22 week at termination of the study (Groups 1, 2 and 3 respectively; $p<0.05$, Figure 1A/B). Our data indicate that tibial loading promotes new bone formation at multiple stages of skeletal maturity into adulthood.

Confirmation that these osteogenic responses to loading were evident in young, growing and skeletally mature mouse tibiae, allowed us to examine whether their diminution was apparent in the ageing female tibia. Prior to *in vivo* tibial loading in ageing mice, *ex vivo* calibration was performed to ensure that loading could be controlled to engender identical peak strain magnitudes to those applied to tibiae in younger mice. Following calibration, we found that identical load-induced peak strain levels (2000με) did not induce any increases in new bone formation in tibiae of 18 month-old mice (at 37% of length, group 4; Figure 1B-D), showing that load-induced new bone formation was absent in aged mice. Indeed, periosteal, endosteal and total new bone formation in the cortex at 37% of the tibial length in 18-month old mice was unmodified by application of identical peak strain magnitudes (Figure 1D).

Extended periods of SN-induced disuse enhance load-induced cortical bone formation in aged mice

It is well established that SN results in disuse-related bone loss[15,16]. To examine whether SN could similarly increase sensitivity of aged bone to identical peak strain magnitudes, it was first necessary to generate load: strain calibration curves for aged tibiae that had been subjected previously to SN either for a short or extended period (5 or 100 days respectively; Figure 2B). This showed that applied loads of 10N and 7N were sufficient to generate 2000me after 5 or 100 days of SN, respectively. Two groups of mice were then subjected to SN for either 5 or 100 days (groups 5 and 7; Figure 2A) and two subjected to SN for 5 or 100 days prior to application of tibial loading for two weeks (groups 6 and 8). Histomorphometric analysis showed, in agreement with a previous study[7], that short-term SN can reinstate a response to loading in the tibia of aged mice (Figures 2C-D). We found that this restoration of load response was, however, location-dependent with significant 33% and 117% increases in new bone formation at sites 47% and 37% along the tibial proximo-distal length which were absent at 27% of tibia length. Our data revealed that the cortical bone response to loading in aged mice was heightened and affected more locations following prolonged SN, with elevated magnitudes of load-induced new bone formation evident at all three locations examined (108, 167 and 98% at 47, 37 and 27% of the tibial length, respectively; Figures 2C-D). These data indicate that prolonged periods of SN resulted in more robust increases in load-induced cortical bone formation in aged mice.

Extended period of disuse blunt SN-related enhancement of load-induced trabecular bone response in aged mice

We also examined whether tibial trabecular bone load responses in aged mice were similarly enhanced by

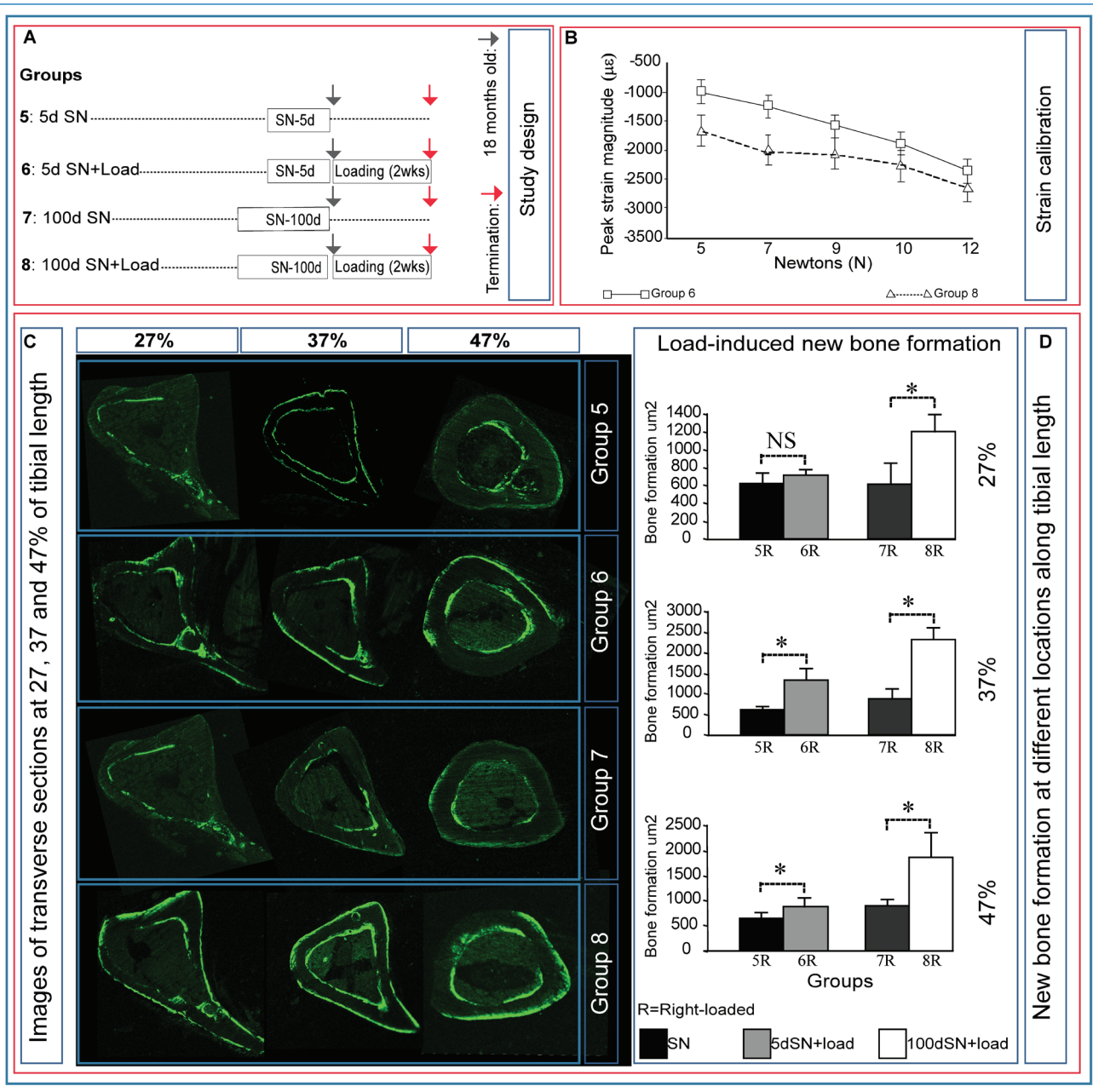

Figure 2. Neurectomy rescues osteogenic response to loading in aged mice. A: Diagrammatic representation of experimental protocols used to evaluate response to loading in mice at 18 months of age following either SN-induced disuse for 5 days (n=8) with or without load (groups 5 and 6 respectively) and SN-induced disuse for 100 days (n=5) with or without load (groups 7 and 8 respectively). **B:** *Ex vivo* calibration of load: strain relationship in tibiae from limbs of 18 month-old female mice after either 5 or 100 days of neurectomy (n=4/group). **C:** Representative confocal images of transverse sections at 27, 37 and 47% of tibial length in all groups. **D:** Total new bone formation in all groups between right SN control limbs and right SN limbs that were subjected to two weeks of axial loading (2000με). Data are mean±SEM (*p<0.05).

prolonging SN-related disuse. Our initial analyses comparing left (control) and right (SN) tibiae showed, as expected[15,16], that SN produced significant reduction in trabecular BV/TV and raised SMI within 5 days of SN, and that these changes were greater with extended periods of disuse; 100 days of SN additionally significantly reduced trabecular number/thickness and increased separation (groups 5 and 7, Table 1; all p<0.05). Analogous to data from cortical bone, we find that short-term SN before load application (group 6) was sufficient to protect against the reduction in BV/TV and raised SMI induced by SN alone (group 5), conserving levels to those evident in control (left) tibiae. Short-term SN also promoted

Table 1. Short SN preceding tibial loading does not restore load-induced trabecular bone response and exaggerates bone loss in long-term SN followed by loading. Mean value of morphometric parameters obtained by micro-computed tomography representing trabecular mass and architecture of all groups. Group sizes were n=8 for groups 5 and 6 and n=5 for groups 7 and 8. Two-sample t-test was used to compare means between left control and right treated tibiae. Data are mean±SEM.

		5 days Sciatic Neurectomy		100 days Sciatic Neurectomy	
		Group 5 (5d-SN)	Group 6 (5d-SN+Load)	Group 7 (100d-SN)	Group 8 (100d-SN+Load)
BV/TV	Left control	0.10±0.008	0.10±0.009	0.12±0.025	0.09±0.010
	Right treated	0.06±0.011	0.10±0.006	0.04±0.010	0.03±0.002
	T-test left vs. right	0.0002	NS	0.0182	0.0008
	T-test right vs. right		0.0150		NS
SMI	Left control	2.36±0.158	2.46±0.046	2.40±0.209	2.71±0.167
	Right treated	2.84±0.175	2.46±0.106	3.40±0.264	3.44±0.117
	T-test left vs. right	0.0019	NS	0.0055	0.0007
	T-test right vs. right		NS		NS
Tb.N	Left control	3.25±0.150	3.11±0.140	3.76±0.428	3.41±0.099
	Right treated	3.14±0.136	3.11±0.139	2.90±0.215	2.83±0.065
	T-test left vs. right	NS	NS	0.0299	0.0005
	T-test right vs. right		NS		NS
Tb.Th	Left control	0.06±0.001	0.07±0.001	0.06±0.002	0.06±0.002
	Right treated	0.05±0.001	0.07±0.001	0.05±0.003	0.05±0.002
	T-test left vs. right	NS	0.0252	0.0424	NS
	T-test right vs. right		0.0000		NS
Tb.Sp	Left control	0.32±0.017	0.32±0.014	0.27±0.031	0.30±0.009
	Right treated	0.32±0.015	0.32±0.018	0.35±0.030	0.36±0.008
	T-test left vs. right	NS	NS	0.0090	0.0004
	T-test right vs. right		NS		NS

significant load-induced trabecular thickening (group 6) to levels greater than in both SN alone and contralateral control tibiae. In contrast to cortical bone, where prolonged SN amplifies the load response, we found that load application failed to elicit any modification in trabecular bone mass or architecture beyond that provoked by solely SN-related disuse (group 8; Table 1), indicating that longer-term SN-related rescue of the load response is not replicated in trabecular bone.

Discussion

We have tested whether a capacity to adapt to mechanical load is reinstated in the tibia by short, and further enhanced by long-term neurectomy-induced hind-limb unloading in aged mice. We find that: i) in agreement with previous studies, load responses become defective in aged mice and are indeed restored by short-term SN-related disuse; ii) prolonged functional SN disuse further augments load responses in cortical bone but, iii) blunts any rescue in the trabecular compartment elicited by short-term disuse. These findings indicate that lengthening the period of disuse more effectively eliminates age-related differences to external mechanical stimuli in cortical bone but abolishes the beneficial effects of short-term SN-related disuse in the trabecular compartment.

It is recognized that absence of osteogenic responses to mechanical stimuli may underpin the decline in aged bone's fracture resistance. Herein, we addressed whether a long-term absence of background activity, promoted by SN, may rescue this age-related blunting. Our studies agree with those showing that tibiae of aged animals show increased thresholds for the generation of an osteogenic response, and respond less exuberantly to loading than young adults[2,4,7,17]. There are alternative explanations for this lack of sensitivity to loading in aged bones that both our own and previous studies disclose. It is possible that responses may take much longer to develop or that many more loading bouts are required before an adaptive response is initiated. If the latter were indeed the case, it would oppose the prevailing current view that short, transient loading periods are sufficient to prompt the longer term adaptive changes in bone mass and architecture. Further studies are required for us to fully define the biological changes in bone responses to loading in the aged. Our results nevertheless disclose modifications that shift the dynamics or sensitivity (or both) of the loading response in aged mice and that these differ in cortical and trabecular bone.

Surgical SN is a widely used model of disuse-related bone loss, in which bone deficits are caused by early increases in

resorption and by depressed formation as a result of impaired osteoblast activity. It was therefore expected that both 5 and 100 days of SN would result in decreased tibial periosteal envelope size; consistent with decreased formation on the periosteal surface and less formation and elevated resorption on endocortical surfaces[18]. Our data, however, show that 100 but not 5 days of SN leads to a significant alteration in cortical bone mass in aged mice. They also indicate that trabecular bone exhibits modified sensitivity to disuse with reductions in mass elicited within 5 days that are reduced further by 100 of SN. This suggests that SN-disuse differentially impacts cortical and trabecular bone or, alternatively that they exhibit distinct dependence on bone innervation.

The enhanced response to external mechanical stimuli induced by short-term SN in cortical bone of aged mice agrees with a previous study[7]. Further heightening of this response by prolonged SN, with elevated magnitudes of load-induced new bone formation across more tibia locations is however novel. It is an original extension to our appreciation of the aged 'bone-phenotype', indicating that age-related blunting of osteogenic responses to load can be more fully rescued by prolonged disuse in 18-month-old limbs in which load-induced cortical bone formation is normally absent. The exact mechanism by which this enhanced osteogenic response to mechanical load is provoked by short and long-term SN remains undefined and further studies are needed to define their cellular basis.

One possibility is that restoration of the mechano-adaptive responses may be attributed to a 'repopulation' of such functionally isolated bone by cells engaged in disuse-related remodelling activity; this offers an explanation for the findings after only 5 days of disuse. It is, however, somewhat difficult to reconcile with our data demonstrating that prolonged disuse further enhances osteogenic cortical responses to loading, since it is likely that such active disuse-related remodelling will have lessened after the extended 100 day long period of disuse. Rather, these findings indicate that a more enduring disuse phase instead further sensitises cortical bone to reloading. It is vital to point out that calibration of load-strain relationships in the tibiae subjected to disuse, means that our data support a greater sensitivity to identical peak strains in bone that has experienced periods of disuse. This interpretation is in keeping with our earlier studies which showed SN-induced disuse only increased the extent of load-related new bone formation in tibiae of young mice[12].

Any possible explanation for the reinstating (at 5 days of SN) and augmentation (at 100 days of SN) of cortical bone responses to loading in aged mice, should however be viewed in the light of our findings in trabecular bone. These are distinguished on the basis that longer periods of SN disuse, effectively blunts the rescued load-response elicited by short-term disuse. It is tempting to speculate that short-term SN reinstates both cortical and trabecular responses to loading via acute changes in innervation, but that their differing responses to chronic disuse (long-term SN) underpins their divergent sensitivities to load. Continued decline in bone mass with prolonged disuse in only the trabecular compartment, is the most obvious distinction and suggests that sensitivity to disuse may override any load-sensitivity that bone in this compartment might otherwise exhibit.

In conclusion, we find that longer-term SN-related disuse more effectively eliminates age-related differences to external mechanical stimuli in cortical bone, but abolishes the beneficial effects of short-term SN-related disuse in trabecular bone. Our data point to the requirement for a combined approach for restoring mechanoadaptive response in bones of aged mice. Elucidation of the underpinning mechanisms may allow improved exercise regimens and targeted pharmacological interventions to limit bone frailty in ageing.

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References

1. Reeve J, Loveridge N. The fragile elderly hip: Mechanisms associated with age-related loss of strength and toughness. Bone 2014;61(100):138-48.
2. Birkhold AI, Razi H, Duda GN, Weinkamer R, Checa S, Willie BM. The influence of age on adaptive bone formation and bone resorption. Biomaterials 2014;35(34):9290-301.
3. Meakin LB, Galea GL, Sugiyama T, Lanyon LE, Price JS. Age-related impairment of bones' adaptive response to loading in mice Is associated with sex-related deficiencies in osteoblasts but no change in osteocytes. Journal of Bone and Mineral Research 2014;29(8):1859-71.
4. Razi H, Birkhold AI, Weinkamer R, Duda GN, Willie BM, Checa S. Aging leads to a dysregulation in mechanically driven bone formation and resorption. Journal of Bone and Mineral Research 2015;30(10):1864-73.
5. de Souza RL, Pitsillides AA, Lanyon LE, Skerry TM, Chenu C. Sympathetic nervous system does not mediate the load-induced cortical new bone formation. Journal of Bone and Mineral Research 2005;20(12):2159-68.
6. Holguin N, Brodt MD, Silva MJ. Activation of Wnt signaling by mechanical loading is impaired in the bone of old mice. Journal of Bone and Mineral Research 2016;31(12):2215-26.
7. Meakin LB, Delisser PJ, Galea GL, Lanyon LE, Price JS. Disuse rescues the age-impaired adaptive response to external loading in mice. Osteoporosis International 2015;26(11):2703-8.
8. Gaudio A, Pennisi P, Bratengeier C, Torrisi V, Lindner B, Mangiafico RA, et al. Increased sclerostin serum levels associated with bone formation and resorption markers in patients with immobilization-induced bone loss. The Journal of Clinical Endocrinology & Metabolism 2010;95(5):2248-53.
9. Smith SM, Heer MA, Shackelford LC, Sibonga JD, Ploutz-Snyder L, Zwart SR. Benefits for bone from resistance exercise and nutrition in long-duration spaceflight: evidence from biochemistry and densitometry. Journal of Bone and Mineral Research 2012;27(9):1896-906.

10. de Brito CMM, Garcia ACF, Takayama L, Fregni F, Battistella LR, Pereira RMR. Bone loss in chronic hemiplegia: a longitudinal cohort study. Journal of Clinical Densitometry 2013;16(2):160-7.
11. Willie BM, Birkhold AI, Razi H, Thiele T, Aido M, Kruck B, et al. Diminished response to *in vivo* mechanical loading in trabecular and not cortical bone in adulthood of female C57Bl/6 mice coincides with a reduction in deformation to load. Bone 2013;55(2):335-46.
12. de Souza RL, Matsuura M, Eckstein F, Rawlinson SCF, Lanyon LE, Pitsillides AA. Non-invasive axial loading of mouse tibiae increases cortical bone formation and modifies trabecular organization: A new model to study cortical and cancellous compartments in a single loaded element. Bone 2005;37(6):810-8.
13. Baron R, Vignery A, Neff L, Silverglate A, Santa Maria A. Processing of undecalcified bone specimens for bone histomorphometry. Bone histomorphometry: techniques and interpretation 1983;1:13-35.
14. Weinreb M, Rodan G, Thompson D. Osteopenia in the immobilized rat hind limb is associated with increased bone resorption and decreased bone formation. Bone 1989;10(3):187-94.
15. Baldock P, Sainsbury A, Couzens M, Enriquez R, Thomas G, Gardiner E, et al. Hypothalamic Y2 receptors regulate bone formation. J Clin Invest 2002;109:915-21.
16. Burt-Pichat B, Lafage-Proust MH, Duboeuf F, Laroche N, Itzstein C, Vico L, et al. Dramatic decrease of innervation density in bone after ovariectomy. Endocrinology 2005;146(1):503-10.
17. Holguin N, Brodt MD, Sanchez ME, Silva MJ. Aging diminishes lamellar and woven bone formation induced by tibial compression in adult C57BL/6. Bone 2014; 65:83-91.
18. Takahashi T, Tonami Y, Tachibana M, Nomura M, Shimada T, Aburada M, et al. Tetrandrine prevents bone loss in sciatic-neurectomized mice and inhibits receptor activator of nuclear factor κB ligand-induced osteoclast differentiation. Biological and Pharmaceutical Bulletin 2012;35(10):1765-74.