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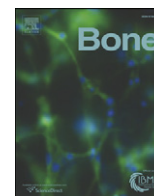Artigos e Materiais de Revistas Científicas - FMRP/RCM

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Trabecular bone loss after administration of the second-generation antipsychotic risperidone is independent of weight gain

Original Full length Article

Trabecular bone loss after administration of the second-generation antipsychotic risperidone is independent of weight gain

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ABSTRACT

Second generation antipsychotics (SGAs) have been linked to metabolic and bone disorders in clinical studies, but the mechanisms of these side effects remain unclear. Additionally, no studies have examined whether SGAs cause bone loss in mice. Using in vivo and in vitro modeling we examined the effects of risperidone, the most commonly prescribed SGA, on bone in C57BL6/J (B6) mice. Mice were treated with risperidone orally by food supplementation at a dose of 1.25 mg/kg daily for 5 and 8 weeks, starting at 3.5 weeks of age. Risperidone reduced trabecular BV/TV, trabecular number and percent cortical area. Trabecular histomorphometry demonstrated increased resorption parameters, with no change in osteoblast number or function. Risperidone also altered adipose tissue distribution such that white adipose tissue mass was reduced and liver had significantly higher lipid infiltration. Next, in order to tightly control risperidone exposure, we administered risperidone by chronic subcutaneous infusion with osmotic minipumps (0.5 mg/kg daily for 4 weeks) in 7 week old female B6 mice. Similar trabecular and cortical bone differences were observed compared to the orally treated groups (reduced trabecular BV/TV, and connectivity density, and reduced percent cortical area) with no change in body mass, percent body fat, glucose tolerance or insulin sensitivity. Unlike in orally treated mice, risperidone infusion reduced bone formation parameters (serum P1NP, MAR and BFR/BV). Resorption parameters were elevated, but this increase did not reach statistical significance. To determine if risperidone could directly affect bone cells, primary bone marrow cells were cultured with osteoclast or osteoblast differentiation media. Risperidone was added to culture medium in clinically relevant doses of 0, 2.5 or 25 ng/ml. The number of osteoclasts was significantly increased by addition in vitro of risperidone while osteoblast differentiation was not altered. These studies indicate that risperidone treatment can have negative skeletal consequences by direct activation of osteoclast activity and by indirect non-cell autonomous mechanisms. Our findings further support the tenet that the negative side effects of SGAs on bone mass should be considered when weighing potential risks and benefits, especially in children and adolescents who have not yet reached peak bone mass.

This article is part of a Special Issue entitled: Interactions Between Bone, Adipose Tissue and Metabolism.

Introduction

Second-generation antipsychotics (SGAs) are used to treat major psychiatric disorders in part due to their lower incidence of extrapyramidal side effects compared to first-generation antipsychotics (FGAs) [1]. Risperidone is one such SGA that is currently indicated for use in adolescents (as young as 10) and adults with schizophrenia and bipolar disorders, although it is widely used for attention deficit disorders. Risperidone is also approved for use in children as young as 5 years old for treatment of irritability associated with autism. Even though SGAs are highly prescribed, they have been linked to metabolic disorders including obesity, hyperglycemia and dyslipidemia [2–4]. The mechanism of metabolic changes associated with antipsychotics is unknown, but it is clear that a single dose (intravenous or intracerebroventricular) of certain SGAs (olanzapine, clozapine, and to a lesser degree risperidone) can dramatically reduce insulin sensitivity in the liver and this effect continues after multiple doses [5]. Additionally, SGA-linked obesity and type 2 diabetes mellitus are more prevalent in children and adolescents [6–8].

The relationship among energy metabolism, insulin signaling and bone remodeling is becoming more apparent, so it is likely that drugs that alter energy metabolism will ultimately affect bone mineral density [9]. Indeed, clinical studies demonstrate reduced bone mineral density and increased fracture risk in patients treated with risperidone and other SGAs, although these studies are confounded by indication (since the underlying disorders treated with antipsychotic medication can be associated with reduced bone mineral density in some cohorts) [10–16]. Risperidone can induce hyperprolactinemia by dopamine receptor blockade and this can lead to hypothalamic hypogonadism, which has been suggested as a possible mechanism of bone loss [11,12,15,17]. However, a recent study demonstrated that less than half of the patients using risperidone developed hyperprolactinemia [11]. In addition, the increase in prolactin serum levels due to risperidone treatment is often a temporary event [18]. Moreover, unlike FGAs that bind mainly dopamine (D2) receptors, SGAs bind to multiple targets, including serotonin (5-HT), histamine, and D2 receptors, and therefore can affect additional organ systems that indirectly impact skeletal remodeling.

Despite some evidence that SGAs have a deleterious effect on bone, rodent studies involving SGA administration have focused on prolactin and the major metabolic consequences including weight gain, fat redistribution and glucose intolerance. However, no studies have examined whether risperidone is capable of causing direct deleterious effects on bone, nor have any studies been reported in mice. Here, we examined the effects of risperidone on bone and adipose tissue metabolism, and found significant trabecular and cortical bone loss independent of weight gain and overt metabolic dysfunction using two methods of risperidone administration (orally in food and by subcutaneous infusion). Reduced trabecular bone mass in mice fed a diet containing risperidone was due to increased resorption, an effect that can be recapitulated by direct risperidone administration to primary bone marrow derived osteoclasts *in vitro*. On the other hand, chronic infusion of risperidone suppressed bone formation, and showed a trend toward increased resorption, indicating that the mode of delivery and age of mice are important variables. Despite these differences, we have clearly established a negative role for risperidone on bone metabolism through both direct and indirect mechanisms.

Materials and methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Maine Medical Center Research Institute. The animals were maintained on 12 h light/12 h dark cycles. The mice had free access to water and food.

Mice — oral risperidone administration

Male C57BL/6J (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 3 weeks of age. Mice were fed normal laboratory chow until 3.5 weeks of age, when they were switched to wet mash supplemented with risperidone such that the dose delivered was 1.25 mg/kg/day or control wet mash. Food intake was monitored twice per week to ensure correct dosing. Mice were maintained on risperidone or control diet for five (n = 8 control, 8 risperidone) or 8 weeks (n = 8 control, 8 risperidone), at which time mice were euthanized and tissues were collected after an overnight fast. Livers were stained for lipid deposition with Oil Red O.

Mice — risperidone infusion

Female C57BL6/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age. At 7 weeks of age, mice were implanted with Alzet Osmotic Minipumps (Durect Corporation, Cupertino, CA) containing either 0.5 mg/kg/day risperidone (Sigma,

St. Louis, MO) or vehicle in the subcutaneous region dorsal to the right hip. Concentration of risperidone was based on the average weight of C57BL/6 female mice from 7 to 11 weeks of age. After 4 weeks of risperidone or vehicle infusion mice were euthanized and tissues collected after an overnight fast.

Dual-energy X-ray absorptiometry (DXA)

Control and risperidone treated mice were measured at the time of harvest for lean muscle mass, fat, and bone mineral density using the PIXImus dual-energy X-ray densitometer (GE-Lunar, Madison, WI, USA). The PIXImus was calibrated daily with a mouse phantom provided by the manufacturer. Mice were placed ventral side down with each limb and tail positioned away from the body. Full body scans were obtained, and X-ray absorptiometry data were gathered and processed with the manufacturer's supplied software (Lunar PIXImus 2, version 2.1). The head was specifically excluded from all analyses due to concentrated mineral in skull and teeth.

Micro-computed tomography (µCT)

Micro-architecture of the distal trabecular bone and midshaft cortical bone of the femur were analyzed by µCT (resolution 10 µm, VivaCT-40, Scanco Medical AG, Bassersdorf, Switzerland). Bones were scanned at energy level of 55 kVp, and intensity of 145 µA. The VivaCT-40 is calibrated weekly using a phantom provided by Scanco. Trabecular bone volume fraction and micro-architecture were evaluated in the secondary spongiosa, starting proximately at 0.6 mm proximal to the distal femoral growth plate, and extending proximally 1.5 mm. Approximately 230 consecutive slices were made at 10.5 µm interval at the distal end of the growth plate and extending in a proximal direction, and 180 contiguous slices were selected for analysis. Measurements included bone volume/total volume (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th.) and trabecular separation (Tb.Sp.). Scans for the cortical region were measured at the mid-point of each femur, with an isotropic pixel size of 21 µm and slice thickness of 21 µm, and used to calculate the average bone area (BA), total cross-sectional area (TA), bone area/total area (BA/TA), and cortical thickness (Ct.Th.). For mid-shaft analysis, the cortical shell was contoured by user-defined threshold and iterated across the 50 slices. All scans were analyzed using a manufacturer software (Scanco, version 4.05).

Histology and quantitative histomorphometry

Qualitative histologic analysis and quantitative static and dynamic histomorphometry were performed as described previously [19]. To examine bone-formation rates, calcein label (20 mg/kg) and demeclocycline label (20 mg/kg) were injected intraperitoneally at 9 and 2 days prior to euthanization, respectively. Immediately after euthanization, tibias for histomorphometry were placed in 70% ethanol and maintained in the dark at 4 °C. Histomorphometric measurements were performed on the secondary spongiosa of the proximal tibia metaphysis using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA, USA). For dynamic histomorphometry, mineralizing surface per bone surface (MS/BS, %) and mineral apposition rate (MAR, µm/day) were measured in unstained sections under ultraviolet (UV) light and used to calculate bone-formation rate with a surface referent (BFR/BS, $\mu m^3/\mu m^2$/year), volume referent (BFR/BV, %/year) and tissue referent (BFR/TV, %/year). Static measurements included BV/TV (%), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, /mm), trabecular separation (Tb.Sp, µm), eroded surface per bone surface (ES/BS, %), osteoid surface per bone surface (OS/BS, %), osteoid volume per tissue volume (OV/TV, %), osteoid thickness (O.Th, µm), osteoblast surface per bone surface (Ob.S/BS, %), osteoclast surface per bone surface (Oc.S/BS, %), osteoblast number
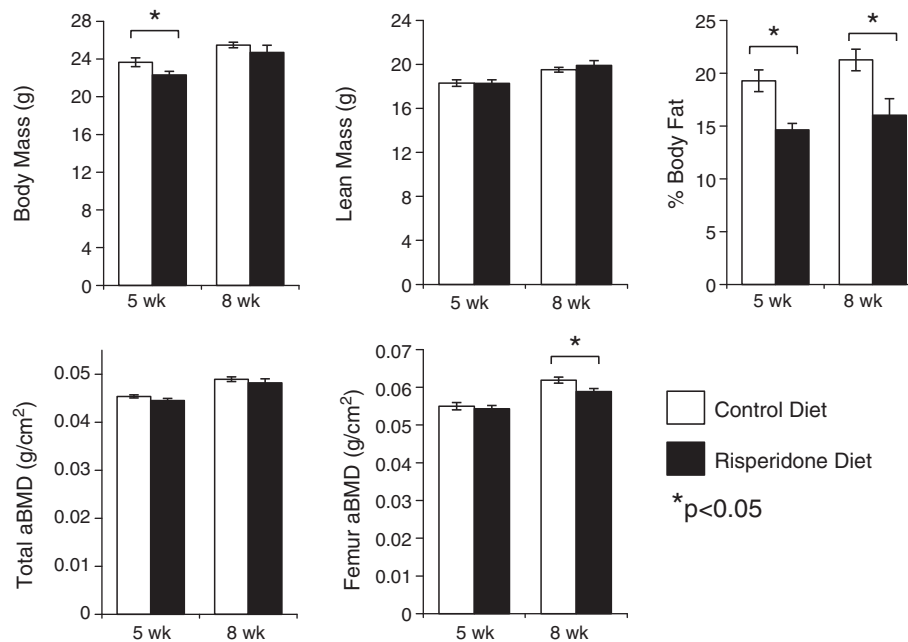
Fig. 1. Reduced body fat and femur areal bone mineral density after risperidone administration. Risperidone was administered to male B6 mice orally in the food for five or 8 weeks, starting at 3.5 weeks of age. Lean mass, fat mass, total and femur areal BMD were measured by DXA. Bars represent mean ± standard error of untreated (white) and risperidone treated mice (black). n = 8. *p<0.05 by Student's t-test.

per bone perimeter (N.Ob/B.Pm, /mm), osteoblast number per tissue area (N.Ob/T.Ar, /mm^2), osteoclast number per bone perimeter (N.Oc/B.Pm, /mm), osteoclast number per tissue area (N.Oc/T.Ar, /mm^2), and adipocytes per total area (/mm^2). Terminology and units followed the recommendations of the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [20].

Magnetic resonance imaging

Axial and coronal images were acquired in a 7.0 T Bruker PharmaScan magnet. The animals were anesthetized with inhaled isoflurane 1%, 0.4 l/min. The infused volume was adjusted, occasionally, depending on animal respiration frequency. The acquisition method used for body fat was: TR 600 ms, TE 87 ms, FOV 40×40×10 mm, matrix size 256×256×32, slice thickness 10 mm, 1 slice, no fat suppression, 4 average, with total scan time of about 11 min. For single pulse spectroscopy: TR 1000 ms, no fat suppression, 400 averages, with total scan time of about 7 min. Spectroscopy was carried out by using the following parameters: TR = 2500 ms, TE = 20 ms, voxel = 0.75×7×0.75 mm, no fat suppression, 400 averages, with total scan time about 17 min.

Serum measurements

Serum adiponectin was measured with the Mouse Adiponectin Assay Kit (Meso Scale Discovery, Gaithersburg, MD). Serum prolactin was measured with the Mouse/Rat Prolactin Kit (Calbiotech, Spring Valley, CA). Serum P1NP and CTx were measured with the Rat/Mouse P1NP EIA and the RatLaps EIA, respectively (Immunodiagnostic Systems, Scottsdale, AZ).

Insulin and glucose tolerance

For the insulin tolerance test (ITT), mice were fed *ad libitum* and injected I.P. with insulin at a dose of 1 U/kg. Glucose levels were then measured at 0, 20, 40, 60, 100, 120 and 150 min post injection. For the glucose tolerance test (GTT), mice were placed in a clean cage with

water and fasted overnight (16 h). A 1 g/kg dose of glucose was administered I.P. and blood glucose levels were measured at 0, 20, 40, 60, 80, 100, 120, 150 and 180 min post injection. Glucose levels were measured using the OneTouch Ultra Glucometer (LifeScan, Inc., Milpitas, California, USA) per manufacturer's instructions.

Mass spectrometry

Risperidone and the active metabolite 9-OH risperidone levels were measured by mass spectrometry in serum as previously shown [21].

Osteoclast culture

Whole bone marrow was isolated from 8 week old female C57BL6/J mice and plated in 96-well plates at a density of 2×10^6 cells/well and cultured in the presence of RANKL and M-CSF as previously described
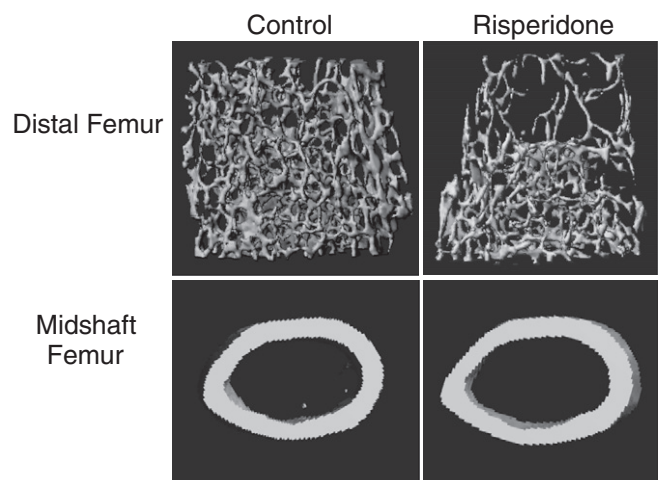

Fig. 2. Reduced trabecular and cortical bone mass in the femur after 8 weeks of risperidone administration. Shown are representative three-dimensional μCT images from control and risperidone treated mice.

Table 1
Male femur µCT after five and 8 weeks of risperidone treatment.

	Five week treatment		Eight week treatment	
	Control (n = 8)	Risperidone (n = 8)	Control (n = 8)	Risperidone (n = 8)
Trabecular				
BV/TV (%)	8.1 ± 0.6	7.7 ± 0.8	9.5 ± 0.4	7.3 ± 0.6[**]
Tb.N (1/mm)	4.5 ± 0.1	4.3 ± 0.2	4.6 ± 0.1	4.2 ± 0.1[*]
Tb.Th (mm)	0.0379 ± 0.0010	0.0378 ± 0.0012	0.0401 ± 0.0007	0.0383 ± 0.0005
Tb.Sp (mm)	0.219 ± 0.007	0.233 ± 0.011	0.207 ± 0.004	0.232 ± 0.007[*]
Conn.D (1/mm³)	100 ± 12	92 ± 15	130 ± 12	82 ± 12[*]
Cortical				
Tt.Ar (mm²)	1.96 ± 0.03	1.84 ± 0.02[**]	1.95 ± 0.02	1.98 ± 0.04
Ma.Ar (mm²)	1.12 ± 0.02	1.07 ± 0.01	1.05 ± 0.01	1.09 ± 0.02
Ct.Ar (mm²)	0.84 ± 0.02	0.77 ± 0.01[**]	0.90 ± 0.01	0.89 ± 0.02
Ct.Ar/Tt.Ar (%)	42.9 ± 0.3	41.7 ± 0.5[*]	46.1 ± 0.3	44.9 ± 0.4[*]
Ct.Th (mm)	0.191 ± 0.003	0.180 ± 0.002[**]	0.204 ± 0.002	0.201 ± 0.002

Abbreviations: BV/TV, bone volume/total volume; Tb., trabecular; N, number; Th, thickness; Sp, separation; Conn.D, connectivity density; Tt., total; Ar, area; Ma, medullary; Ct., cortical.

[**] p<0.01 compared to age-matched control by two-tailed Student's t-test.

[*] p<0.05 compared to age-matched control by two-tailed Student's t-test.

[22]. Five replicates were performed per experiment. Risperidone (0, 2.5 or 25 ng/ml) was added with each media change at days 0, 3 and 6. Osteoclasts were harvested at day 7 by fixation with 2.5% glutaraldehyde and stained for TRAP (Sigma Kit, St. Louis, MO). TRAP positive osteoclasts with three or more nuclei were counted.

Statistical analyses

All data is presented as mean ± standard error. Statistical significance was determined using a two-tailed Student's t-test (alpha = 0.05) with Microsoft Excel (Microsoft, Redmond, WA).

Results

Oral risperidone administration

Risperidone was administered to male 3.5 week old B6 mice at a dose of 1.25 mg/kg per day in the food to examine its effects on bone metabolism. Risperidone fed mice had significantly lower body weights and lower body fat compared to controls after 5 weeks of treatment (Fig. 1). After 8 weeks, the lower body fat remained statistically significant but there was no significant difference in body mass. Although there was no detectable difference in total aBMD by DXA at either time point, risperidone-fed mice had significantly lower femur aBMD after 8 weeks of treatment compared to untreated controls.

After 8 weeks of risperidone administration, distal femur trabecular bone volume fraction (BV/TV) was 23% lower (p<0.01) than that of untreated control mice (Fig. 2, Table 1). At this time point, trabecular number and connectivity density were significantly lower while trabecular separation was significantly higher than control. By the fifth week of treatment, risperidone treated mice had reduced femur midshaft cortical thickness, which can be attributed to a reduction in periosteal circumference compared to controls (Table 1). After 8 weeks, however, the difference in periosteal circumference was no longer evident, although risperidone-treated mice continued to have significantly lower percent Ct.Ar/Tt.Ar (Table 1).

Table 2
Male static and dynamic tibia histomorphometry after five and 8 weeks of risperidone treatment.

	Five week treatment			Eight week treatment		
	Control	Risperidone	p value	Control	Risperidone	p value
	(n = 6–8)	(n = 8)		(n = 7–8)	(n = 8)	
BV/TV (%)	8.8 ± 0.4	7.3 ± 0.5[*]	0.03	9.9 ± 1.1	6.9 ± 0.7[*]	0.03
Tb.Th (µm)	29.9 ± 1.2	27.2 ± 1.0	0.10	31.3 ± 1.2	28.5 ± 0.9	0.09
Tb.N (/mm)	2.95 ± 0.12	2.66 ± 0.12	0.11	3.12 ± 0.22	2.38 ± 0.19[*]	0.03
Tb.Sp (µm)	313 ± 15	353 ± 16	0.09	301 ± 24	415 ± 42[*]	0.03
MS/BS (%)	27.7 ± 4.2	23.2 ± 2.1	0.32	26.3 ± 2.5	27.6 ± 2.3	0.71
MAR (µm/day)	2.03 ± 0.09	2.11 ± 0.18	0.72	1.18 ± 0.08	1.02 ± 0.07	0.15
BFR/BS (µm³/µm²/year)	199 ± 24	180 ± 22	0.57	113 ± 14	103 ± 13	0.61
BFR/BV (%/year)	1301 ± 150	1273 ± 146	0.90	706 ± 61	741 ± 113	0.78
BFR/TV (%/year)	117 ± 17	92 ± 10	0.21	76 ± 14	49 ± 7	0.13
Ob.S/BS (%)	5.2 ± 1.1	6.5 ± 1.3	0.43	2.3 ± 0.6	3.0 ± 1.0	0.53
N.Ob/T.Ar (/mm²)	26.2 ± 4.9	28.9 ± 4.9	0.70	12.2 ± 3.2	11.2 ± 3.0	0.81
N.Ob/B.Pm (/mm)	4.6 ± 1.0	5.4 ± 0.8	0.57	2.1 ± 0.5	2.4 ± 0.7	0.78
OV/TV (%)	0.044 ± 0.011	0.047 ± 0.011	0.89	0.012 ± 0.004	0.015 ± 0.006	0.63
OS/BS (%)	3.2 ± 0.8	3.3 ± 0.7	0.89	0.8 ± 0.3	1.6 ± 0.6	0.24
O.Th (µm)	2.1 ± 0.3	2.5 ± 0.2	0.35	2.7 ± 0.4	1.6 ± 0.4	0.08
Oc.S/BS (%)	2.5 ± 0.6	5.7 ± 0.9[*]	0.01	1.5 ± 0.3	2.9 ± 0.5[*]	0.02
N.Oc/T.Ar (/mm²)	6.4 ± 1.6	11.8 ± 1.2[*]	0.02	3.3 ± 0.6	5.2 ± 0.9	0.11
N.Oc/B.Pm (/mm)	1.1 ± 0.3	2.3 ± 0.3[*]	0.01	0.6 ± 0.1	1.1 ± 0.1[*]	0.01
ES/BS (%)	0.98 ± 0.20	2.56 ± 0.38[*]	<0.01	0.53 ± 0.14	0.93 ± 0.27	0.21

Abbreviations: BV, bone volume; TV, tissue volume; Tb., trabecular; N, number; Th, thickness; Sp, separation; MS, mineralizing surface; BS, bone surface; MAR, mineral apposition rate; BFR, bone formation rate; Ob.S, osteoblast surface; N.Ob, number of osteoblasts; T.Ar, tissue area; B.Pm, bone perimeter; OV, osteoid volume; OS, osteoid surface; O.Th, osteoid thickness; Oc.S, osteoclast surface; N.Oc, number of osteoclasts; ES, eroded surface.

[*] p<0.05 by two-tailed Student's t-test.

Static and dynamic histomorphometry was performed on trabecular bone of the proximal tibia in order to determine how risperidone altered remodeling. Consistent with femur µCT results, after 8 weeks of treatment, trabecular BV/TV and trabecular number were significantly lower in the risperidone treated group compared to untreated controls (Table 2). Additionally, the reduction in BV/TV was also statistically significant in the tibia by 5 weeks of treatment, which was not apparent in the femur µCT. Neither treatment period significantly altered measurements of mineralization, bone formation, or osteoblast numbers. However, risperidone treated mice did have significantly elevated bone resorption parameters, at both five and 8 weeks of treatment, including percent of bone surface in contact with osteoclasts and number of osteoclasts per unit bone surface. Erosion surface was also 2.5-fold higher in mice treated with risperidone for 5 weeks compared to control mice. This difference was not statistically detectable at the eight-week time point. Bone marrow adipocyte number was not significantly different after five or 8 weeks of oral risperidone administration vs control treated animals (not shown).

In order to further investigate adipose tissue changes observed by DXA, we examined abdominal and subcutaneous fat by MRI. Representative MR images demonstrate that both subcutaneous and visceral fats were reduced after 5 weeks of risperidone treatment (Fig. 3A). This reduction in mass of normal white adipose tissue

depots was coupled with an increase in liver adiposity as measured by Oil Red O staining and by weight measurements (Fig. 3B).

Given the potential variability due to food supplementation, we measured risperidone and 9-OH risperidone levels in the serum of mice at each time point. Although risperidone levels were low to undetectable by mass spectrometry (likely due to the overnight fast and therefore no new drug exposure), the more stable 9-OH risperidone was detectable at levels of 7.9 ± 3.5 ng/ml (range 1.6–24.1 ng/ml) for the five week treatment and 6.0 ± 0.8 ng/ml (range 4.6–9.3 ng/ml) for the eight week treatment, which are within the range obtained in human patients [23–25].

Subcutaneous risperidone infusion

Due to variability in the amount of risperidone delivered per mouse by food supplementation, we administered risperidone (0.5 mg/kg per day) to 7-week old C57BL/6 J mice via subcutaneous osmotic minipumps for 28 days. In the literature, female mice are more likely to have a weight-gain side effect of SGA administration; therefore we used female mice instead of males to try to exclude weight loss as a confounding factor in the bone effects we observed with male mice. At two and 4 weeks after pump implantation, serum risperidone concentration was 5.9 ± 0.7 ng/ml (range 5.0–7.9 ng/ml) and 1.3 ± 0.3 ng/ml (range 0.8–2.3 ng/ml), respectively. The concentration of 9-OH risperidone was 3.8 ± 0.5 ng/ml (range 2.8–5.7 ng/ml) at 2 weeks and 2.4 ± 0.3 ng/ml (range 1.5–3.1 ng/ml) at 4 weeks, which was considerably less variable than levels measured during oral administration.

Mice did not have significantly altered body mass, fat mass or lean mass after infusion of risperidone (Fig. 4A). Additionally, insulin tolerance (Fig. 4B) and glucose tolerance (Fig. 4C) were not different between treatment groups. Similar to oral administration, whole body aBMD was not different between groups. Despite the shortened treatment duration and lower dose, femur aBMD was lower in mice treated with risperidone compared to control (Fig. 4D). Both distal femur trabecular BV/TV and connectivity density were significantly reduced (44% and 64%, respectively) (Table 3). Cortical thickness, cortical area and percent of the total area were also lower in the femur of risperidone treated mice compared to control.

Serum P1NP, a marker of bone formation, was lower in risperidone treated mice while there was no difference in CTx, a marker of resorption (Fig. 4E). Consistent with these data, histomorphometric analysis of the tibia in 7 week old mice treated with subcutaneous risperidone infusion revealed significantly suppressed MAR (decreased 18%) and BFR/BV (decreased 34%). In addition, osteoblast number was markedly reduced (p = 0.05) and there was a 53% increase in osteoclast number although it did not reach statistical significance (p = 0.12) (Table 4).

Direct effect of risperidone on osteoclast differentiation

In order to determine whether the effects of risperidone on resorption parameters could be due to direct interaction with osteoclasts, we cultured primary osteoclasts from bone marrow in the presence or absence of risperidone. Addition of both 2.5 and 25 ng/ml risperidone with each media change increased TRAP positive osteoclast number in a dose dependent manner (Fig. 5). On the other hand, when bone marrow stromal cells were exposed to risperidone *in vitro*, there were no differences in osteoblast proliferation or differentiation compared to untreated cells (data not shown).

Discussion

In this paper we have clearly established that risperidone has a negative impact on bone mass in mice and that this effect may be due to both direct and indirect mechanisms. Using both male and female

A MR-Abdominal Adipose
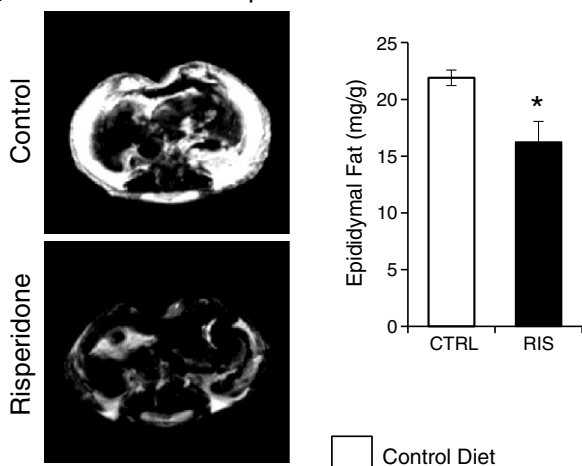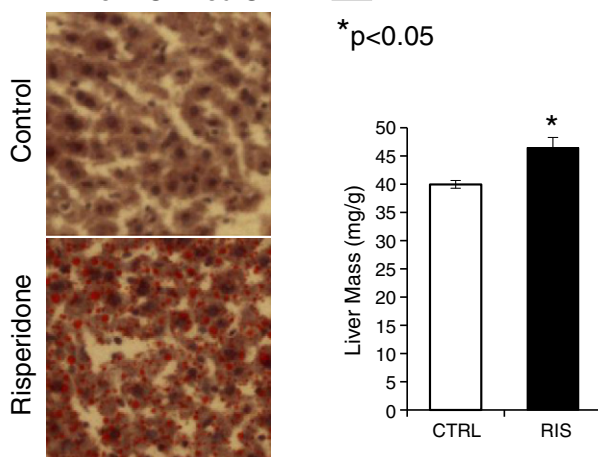

B Liver - Oil Red O

Fig. 3. Reduced white adipose tissue and increased liver adiposity after 5 weeks of risperidone treatment. (A) Representative abdominal MR images and epididymal fat mass. (B) Liver Oil Red O staining (red stain indicates presence of lipids) and liver mass. Bars represent mean ± standard error of untreated (white) and risperidone treated mice (black). n = 8. *p < 0.05 by Student's t-test.
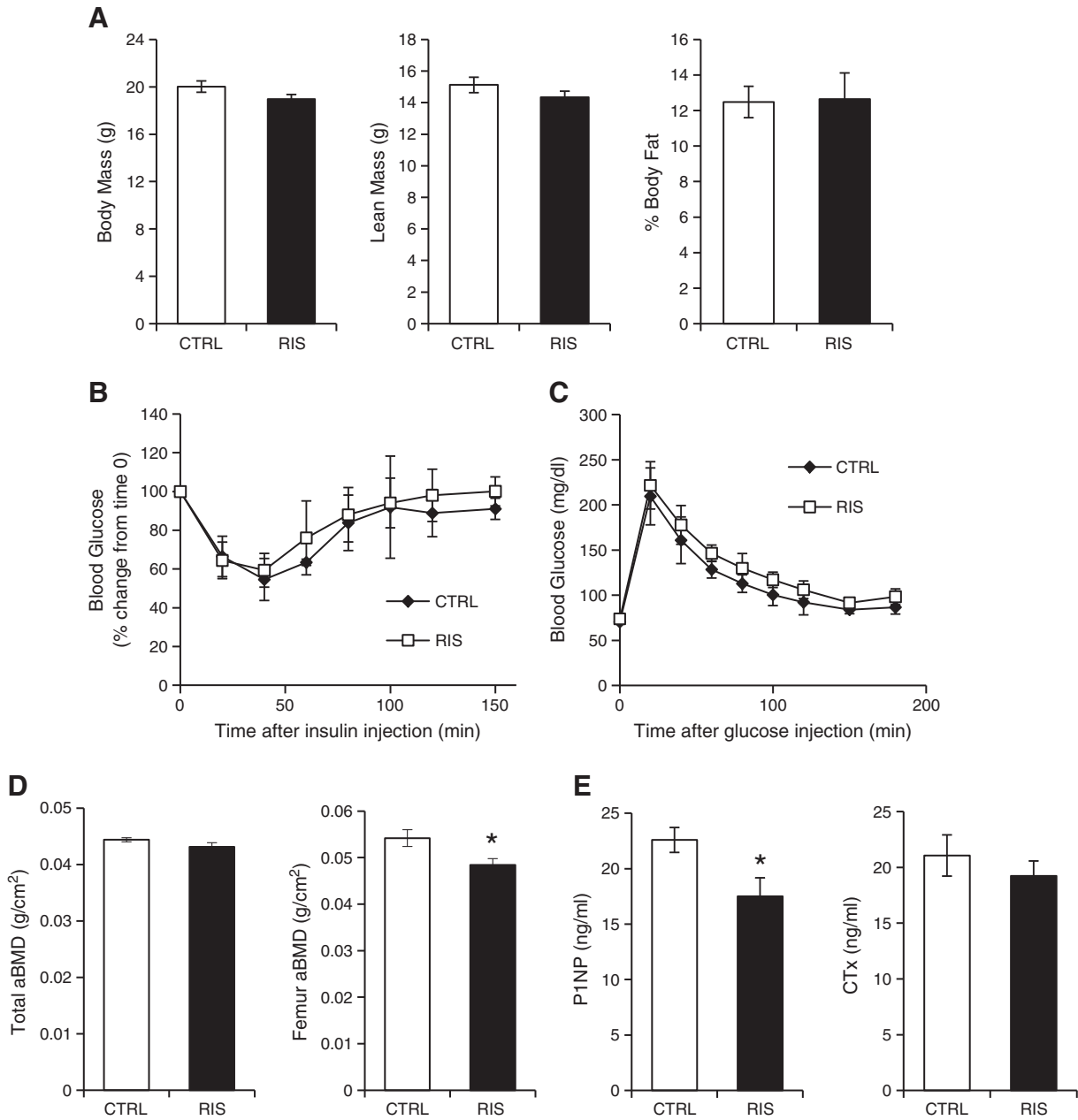
Fig. 4. Subcutaneous infusion of risperidone did not alter total body, lean or fat mass, but did reduce femur areal BMD and serum P1NP. Risperidone was administered to female B6 mice by subcutaneously implanted osmotic minipump for 4 weeks, starting at 8 weeks of age. (A) Lean mass and fat mass were measured by DXA. (B) Insulin tolerance test. (C) Glucose tolerance test. (D) Total and femur aBMD were measured by DXA. (E) Serum P1NP and CTx were measured after an overnight fast. Bars represent mean ± standard error of untreated (white) and risperidone treated mice (black). n = 5. *p < 0.05 by Student's t-test.

mice, and both oral administration and subcutaneous infusion, we found that risperidone reduces both trabecular and cortical bone mass, and that this effect is likely due to imbalanced bone remodeling (Table 2). Interestingly, both modes of administration of risperidone reduced bone mass but histomorphometric analyses revealed there were different mechanisms of action for risperidone. For example, oral risperidone treatment was associated with a primary increase in bone resorption with little change in bone formation, whereas subcutaneous infusion of risperidone resulted in a reduction in P1NP (Fig. 4B) and suppression of both bone formation and mineral apposition rates. These data suggest there may be both cell autonomous (i.e. on osteoclasts) and non-cell autonomous effects (i.e. on osteoblasts) since *in vitro* studies could not demonstrate significant changes in the pace of osteoblast differentiation in response to risperidone.

However, it should be noted that only 7-week-old female mice were administered subcutaneous risperidone whereas only 3.5-week-old male mice were treated with the oral form. At 7 weeks of age, female B6 mice are near their peak in trabecular number and trabecular BV/TV in the distal femur [26]. Thereafter, trabecular BV/TV in the distal femur declines due to enhanced resorption relative to formation. Risperidone treatment accelerated trabecular bone loss in B6 females by further suppressing bone formation. Male B6 mice gain trabecular BV/TV and number up to 8 weeks of age, thus during the first treatment period (3.5 to 8.5 weeks of age) normal modeling favors bone formation, whereas during the second period (8.5 to 11.5 weeks of age) modeling tends to favor resorption [26]. Therefore, from 3.5 to 8.5 weeks of age, trabecular bone mass in the risperidone treated animals is likely unable to reach its peak due to elevated

Table 3
Female tibia μCT after 4 weeks of risperidone infusion.

	Osmotic minipumps		p value
	Saline (n=5)	Risperidone (n=5)	
Trabecular			
BV/TV (%)	4.3 ± 0.6	2.4 ± 0.3*	0.02
Tb.N (1/mm)	3.1 ± 0.2	2.9 ± 0.1	0.07
Tb.Th (mm)	0.036 ± 0.002	0.033 ± 0.001	0.15
Tb.Sp (mm)	0.322 ± 0.017	0.359 ± 0.007	0.08
Conn.D (1/mm^3)	28 ± 7	10 ± 2*	0.03
Cortical			
Tt.Ar (mm^2)	1.73 ± 0.01	1.67 ± 0.03	0.14
Ma.Ar (mm^2)	0.98 ± 0.01	0.99 ± 0.02	0.56
Ct.Ar (mm^2)	0.75 ± 0.01	0.68 ± 0.02*	0.03
Ct.Ar/Tt.Ar (%)	43.2 ± 0.5	40.6 ± 0.5*	0.02
Ct.Th (mm)	0.182 ± 0.003	0.168 ± 0.004*	0.02

Abbreviations: BV/TV, bone volume fraction; Tb., trabecular; N, number; Th, thickness; Sp, separation; Conn.D, connectivity density; Tt., total; Ar, area; Ma, medullary; Ct., cortical.
* p<0.05 by two-tailed Student's t-test.

resorption compared to B6 controls. During the remainder of the treatment time (i.e. 8.5 to 11.5 weeks), formation and resorption begin to decrease, but resorption in the risperidone treated mice does not decrease to the level of the control mice. This effect, coupled to the inability of risperidone administered mice to reach peak trabecular bone mass, contributes to the greater % trabecular BV/TV reduction observed in the mice with longer risperidone exposure. Thus, gender differences, age differences, levels of risperidone achieved, as well as the direct and indirect effects of the drug on bone cells contribute to the skeletal response to this drug. Notwithstanding these confounding variables, it is clear that bone loss occurs with risperidone treatment of mice, independent of weight gain.

At a first glance, hyperprolactinemia would be another mechanism of bone loss secondary to risperidone, which is a dopamine receptor antagonist [11,12]. However, there is controversy about risperidone-induced hyperprolactinemia and we were unable to show an elevation in serum prolactin with either type of risperidone administration.

Table 4
Female static and dynamic histomorphometry after 4 weeks of risperidone infusion.

	Osmotic minipumps		p value
	Saline (n=4–5)	Risperidone (n=4–5)	
BV/TV (%)	5.1 ± 1.1	3.7 ± 0.9	0.34
Tb.Th (μm)	32.7 ± 2.5	30.4 ± 1.9	0.49
Tb.N (/mm)	1.50 ± 0.26	1.15 ± 0.24	0.34
Tb.Sp (μm)	775 ± 217	1230 ± 494	0.42
MS/BS (%)	30.4 ± 3.1	27.4 ± 2.8	0.49
MAR (μm/day)	2.96 ± 0.04	2.42 ± 0.10*	<0.01
BFR/BS (μm^3/μm^2/year)	329 ± 32	241 ± 25	0.08
BFR/BV (%/year)	2181 ± 241	1436 ± 167*	0.04
BFR/TV (%/year)	95 ± 20	58 ± 18	0.22
Ob.S/BS (%)	6.6 ± 1.5	2.0 ± 1.4	0.05
N.Ob/T.Ar (/mm^2)	15.5 ± 5.2	4.8 ± 3.1	0.12
N.Ob/B.Pm (/mm)	5.4 ± 1.4	1.5 ± 1.0	0.05
OV/TV (%)	0.003 ± 0.003	0.002 ± 0.001	0.82
OS/BS (%)	0.26 ± 0.26	0.46 ± 0.29	0.62
O.Th (μm)	0.69 ± 0.69	0.75 ± 0.46	0.95
Oc.S/BS (%)	2.9 ± 0.7	4.4 ± 0.9	0.19
N.Oc/T.Ar (/mm^2)	3.1 ± 0.8	4.2 ± 1.1	0.43
N.Oc/B.Pm (/mm)	1.1 ± 0.2	1.7 ± 0.2	0.12
ES/BS (%)	1.49 ± 0.42	1.86 ± 0.39	0.53
N.Ad/T.Ar (/mm^2)	7.9 ± 1.5	32.5 ± 10.0	0.07

Abbreviations: BV, bone volume; TV, tissue volume; Tb., trabecular; N, number; Th, thickness; Sp, separation; MS, mineralizing surface; BS, bone surface; MAR, mineral apposition rate; BFR, bone formation rate; Ob.S, osteoblast surface; N.Ob, number of osteoblasts; T.Ar, tissue area; B.Pm, bone perimeter; OV, osteoid volume; OS, osteoid surface; O.Th, osteoid thickness; Oc.S, osteoclast surface; N.Oc, number of osteoclasts; ES, eroded surface; N.Ad, number of adipocytes.
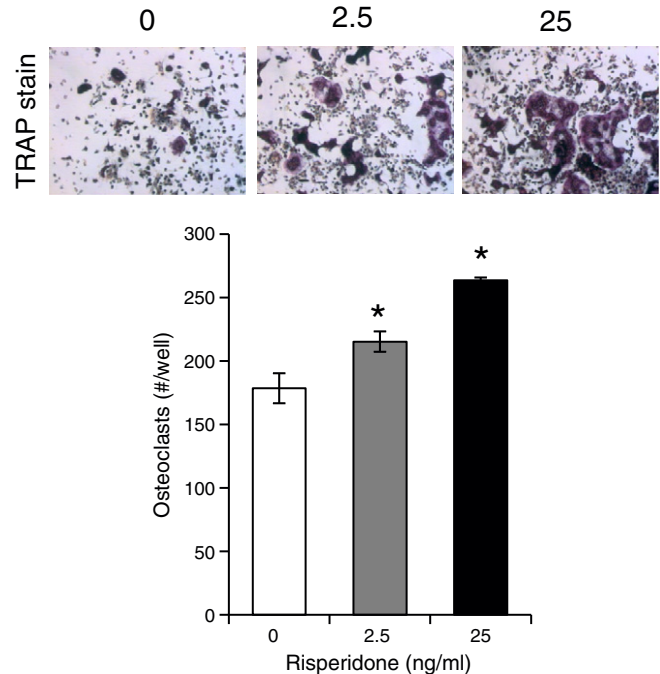* p<0.05 by two-tailed Student's t-test.

Fig. 5. Risperidone induces osteoclastogenesis *in vitro*. Whole bone marrow was cultured in the presence of M-CSF and RANKL to induce osteoclastogenesis. From day 0, wells were also treated with 0, 2.5 or 25 ng/ml risperidone with each media change. Cells were fixed on day 6 and stained for TRAP. Osteoclasts (positive for TRAP and containing 3 or more nuclei) were counted. Bars represent mean ± standard error. n = 3. *p<0.05 by Student's t-test.

Furthermore, female mice subjected to risperidone treatment did not show uterine atrophy that would be expected with hypogonadism (not shown). While there are data suggesting that high PRL levels are a long-term complication of risperidone treatment [27,28], others indicate that its occurrence is a transient side effect [18]. Therefore, an alternative mechanism likely exists for the profound alterations in bone turnover that we observed.

In addition to its actions as a dopaminergic antagonist, risperidone also has anti-serotonergic effects. Recently, serotonin was found to be a crucial intermediate molecule in the regulation of bone mass by leptin [29,30]. Leptin modulates bone metabolism indirectly through the hypothalamus by activating the sympathetic nervous system (SNS), thereby suppressing bone formation while stimulating bone resorption via skeletal beta adrenergic receptors on osteoblasts [31,32]. Leptin-deficient *ob/ob* mice show increased cancellous spine bone mass and overt obesity in spite of hypogonadism and elevated cortisol levels [33]. The pattern of bone changes in leptin-deficient mice may be reproduced by a chemical lesion of neurons in the region of the ventromedial hypothalamus [34]. However, another component of the central action of leptin emerged recently, when it was demonstrated that leptin receptor in hypothalamic ventromedial neurons is not essential to trigger leptin action [35]. Likely, leptin acts in other sites of the brain to regulate bone remodeling. Serotonergic neurons of the brainstem are a reasonable candidate to mediate this action, particularly since the consequences of leptin deficiency on bone turnover can be reversed by hampering serotonin production in the brainstem. Mice deficient in brain-derived production of serotonin (*Tph2*$^{-/-}$) exhibit the opposite phenotype of leptin-deficient mice. Notably, *Tph2*$^{-/-}$ mice have decreased bone mass associated to low bone formation and increased bone resorption, albeit they exhibit normal levels of leptin [36]. Thus, it is plausible that the sympathetic nervous system activity may be elevated during risperidone administration and this may contribute to the non-cell autonomous effects on osteoblasts observed with subcutaneous administration.

Ota et al. also demonstrated that rats treated with risperidone are resistant to weight gain [37,38]. These authors attributed this observation to increased energy expenditure as suggested by the enhanced gene expression of *Ucp1* in brown adipose tissue, and *Ucp3, β1 adrenergic receptor* and lipolytic enzymes in white adipose tissue. In accordance with the Ota results, our risperidone-treated mice did not show an obesity phenotype. Moreover, the present study shows that risperidone treatment was associated with increased fat deposition within the liver. This occurrence might reflect increased lipolysis in visceral adipose tissue, which promptly releases free fatty acids into the portal system, or may be due to a generalized redistribution of fat mass. However, we only demonstrated a non-significant increase in marrow adiposity in the subcutaneously infused mice ($p = 0.07$) and no change in marrow adiposity in the orally administered mice. Taken together, the evidence from histomorphometry, bone turnover markers and μCT in risperidone treated mice prevent us from excluding a non-cell autonomous effect on bone remodeling due to increased sympathetic nervous system activity.

In our study and other rodent SGA studies, risperidone treated mice did not gain weight as is often observed in human clinical trials. However, it is necessary to take into account that the circumstances of our experiments are different from those observed in clinical investigation. Different psychiatric disorders (i.e., anorexia nervosa, schizophrenia and depression) have diverse impacts on body weight and eating behavior, probably reflecting typical alteration on brain physiology [30,39,40]. Thus, it can be hypothesized that the impact of drugs, with actions on neuromodulators, or on eating behavior in individuals can be different from that observed in conditions of psychiatric disorders.

Despite clear evidence for a mechanism of risperidone affecting bone metabolism indirectly through the central nervous system, we did observe increased osteoclast differentiation with direct risperidone administration *in vitro* (Fig. 5). Osteoclasts express serotonin receptors and others that are potential targets for risperidone. Moreover, the *in vitro* changes in osteoclast differentiation are consistent with the *in vivo* histomorphometric data when mice are administered oral risperidone. However, as noted, we cannot exclude that there is a non-cell autonomous effect of risperidone on osteoblasts that stimulates RANKL and subsequently causes increased bone resorption. One possibility is that this is sympathetically mediated. Another possible mechanism is that risperidone activates PPARγ which can suppress bone formation and enhance osteoclastogenesis. Interestingly, in the mice treated by minipump, there was a four-fold increase in marrow adipocytes ($p = 0.07$) that was not seen with oral administration.

In conclusion, the present data show that risperidone impacts bone remodeling in mice by acting on resorption and formation in both a cell autonomous and non-cell autonomous manner. Consequently, we observed decreased bone mass and altered bone microarchitecture. In addition to reduced fat mass, risperidone therapy was associated with enlarged fatty liver. Thus, in addition to risperidone therapy creating conditions that could affect the central regulation of bone mass, the present study mandates that further clinical studies be focused on its direct effects on bone turnover, particularly in adolescents when peak bone mass is being acquired.

Acknowledgments

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