Published in final edited form as:

Bone. 2010 April ; 46(4): 985–992. doi:10.1016/j.bone.2009.12.031.

Psychotropic drugs have contrasting skeletal effects that are independent of their effects on physical activity levels

Stuart J. Warden^{a,b,*}, Sean M. Hassett^a, Julie L. Bond^a, Johanna Rydberg^a, Jamie D. Grogg^a, Erin L. Hilles^a, Elizabeth D. Bogenschutz^a, Heather D. Smith^a, Robyn K. Fuchs^{a,b}, M. Michael Blizotes^{c,d}, and Charles H. Turner^e

^aDepartment of Physical Therapy, School of Health and Rehabilitation Sciences, Indiana University, Indianapolis, IN 46202, USA

^bDepartment of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

^cPortland Veteran Affairs Medical Center, Portland, OR 97201, USA

^dOregon Health and Science University, Portland, Oregon 97239, USA

^eDepartment of Biomedical Engineering, Purdue School of Engineering and Technology, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202

Abstract

Popular psychotropic drugs, like the antidepressant selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs), and the mood stabilizer lithium, may have skeletal effects. In particular, preclinical observations suggest a direct negative effect of SSRIs on the skeleton. A potential caveat in studies of the skeletal effects of psychotropic drugs is the hypoactive (skeletal unloading) phenotype they induce. The aim of this study was to investigate the contribution of physical inactivity to the skeletal effects of psychotropic drugs by studying bone changes in cage control and tail suspended mice treated with either vehicle, SSRI, TCA or lithium. Tail suspension was used to control for drug differences on physical activity levels by normalizing skeletal loading between groups. The psychotropic drugs were found to have contrasting skeletal effects which were independent of drug effects on animal physical activity levels. The latter was evident by an absence of statistical interactions between the activity and drug groups. Pharmacological inhibition of the 5-hydroxytryptamine (5-HT) transporter (5-HTT) using a SSRI reduced *in vivo* gains in lower extremity BMD, and negatively altered *ex vivo* measures of femoral and spinal bone density, architecture and mechanical properties. These effects were mediated by a decrease in bone formation without a change in bone resorption suggesting that the SSRI had anti-anabolic skeletal effects. In contrast, glycogen synthase kinase-3[beta] (GSK-3[beta]) inhibition using lithium had anabolic effects improving *in vivo* gains in BMD via an increase in bone formation, while TCA-mediated inhibition of the norepinephrine transporter had minimal skeletal effect. The observed negative skeletal effect of 5-HTT inhibition, combined with recent findings of direct and indirect effects of 5-HT on bone formation, are of interest given the frequent prescription of SSRIs for the treatment of depression

© 2009 Elsevier Inc. All rights reserved.

*Corresponding author: Stuart J. Warden, PT, PhD, FACSM, Department of Physical Therapy, School of Health and Rehabilitation Sciences, Indiana University, 1140 W. Michigan Street, CF-326, Indianapolis, IN 46202, USA. Phone: +1-317-278-8401; Fax: +1-317-278-1876; stwarden@iupui.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and other affective disorders. Likewise, the anabolic effect of GSK-3[beta] inhibition using lithium reconfirms the importance of Wnt/beta-catenin signaling in the skeleton and its targeting by recent drug discovery efforts. In conclusion, the current study demonstrates that different psychotropic drugs with differing underlying mechanisms of action have contrasting skeletal effects and that these effects do not result indirectly via the generation of animal physical inactivity.

Keywords

antidepressant; depression; fluoxetine; osteoporosis; prozac

INTRODUCTION

Psychotropic drugs act within the central nervous system to alter brain function resulting in temporary changes in perception, mood, consciousness and behavior. Popular psychotropic drugs include the antidepressant selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs), and the mood stabilizer lithium. These agents have proven clinical utility in treating affective disorders such as anxiety, depression and mania [1-3]; however, questions have recently been raised regarding their potential skeletal effects [4].

Numerous studies have demonstrated associations between psychotropic drug use and osteoporotic fracture risk. In particular, osteoporotic fracture rates are increased two-fold with SSRIs [5], marginally increased with TCAs [6-9], and possibly decreased with lithium [6, 10]. The increased fracture risk associated with SSRIs may be attributable to confounding by indication, with depression being an independent risk factor for low bone mass and fracture risk [11]. However, this does not account for the higher rate of fractures among SSRI users compared to TCA users, and the observation that SSRIs users have increased bone loss and less bone mineral density (BMD) than TCA users despite both agents being prescribed for similar indications [6-9,12,13]. Similarly, an elevated risk of falling among SSRI users does not appear to explain their higher fracture risk as fall risk appears to be equivalent in users of TCAs [14,15], and the elevated fall risk with SSRI use declines over time which contrasts the increase in fracture risk with treatment duration with these agents [6,8,9].

It is possible that the negative skeletal effects of SSRIs, compared to TCAs and lithium, result from direct effects of serotonin (5-hydroxytryptamine [5-HT]) on the skeleton. SSRIs antagonize the 5-HT transporter (5-HTT) to inhibit uptake of 5-HT from the extracellular space and prolong 5-HT receptor activation. Recent evidence has identified that all of the major bone cell types (osteoblasts, osteoclasts and osteocytes) possess a functional 5-HTT that is highly specific for 5-HT uptake [16-19]. In addition, functional receptors for 5-HT have been identified in all the major bone cell types, and stimulation of these receptors influences bone cell activities [16-21]. These findings indicate that bone cells possess functional pathways for both responding to and regulating the uptake of 5-HT.

Animal studies support the potential for direct skeletal effects of SSRIs [22]. Mice with a null mutation in the gene encoding for the 5-HTT displayed a consistent skeletal phenotype of reduced mass, altered architecture, and inferior mechanical properties [23], whereas administration of a clinically popular SSRI (fluoxetine hydrochloride) to growing and adult mice resulted in reduced bone mineral accrual and accelerated bone loss following ovariectomy, respectively [23,24]. In contrast, a TCA (desipramine hydrochloride) had minimal skeletal effect compared to a SSRI (fluoxetine hydrochloride) in a preclinical animal study [25], and lithium appeared to be anabolic in another animal study [26].

Preclinical observations suggest a direct negative effect of SSRIs on the skeleton; however, a potential caveat in these studies is the hypoactive (skeletal unloading) phenotype that SSRIs induce in animals [22]. It currently remains unclear whether the preclinical skeletal effects of SSRIs, in contrast to other psychotropic drugs, are simply due to effects on animal physical activity levels. The aim of this study was to investigate the role physical activity on the skeletal effects of psychotropic drugs by studying the skeletal effects of an SSRI, TCA and lithium on bone changes in cage control and tail suspended mice. Tail suspension was used to control for drug differences on animal physical activity levels by normalizing skeletal loading between groups. If the negative skeletal phenotype associated with SSRIs in mice is due to the hypoactive phenotype these agents induce, this would be rescued compared to control animals when intervention is coupled with tail suspension.

MATERIALS AND METHODS

Animals

Eighty virgin female Swiss-Webster mice were purchased at 4 weeks of age (Taconic Farms, Inc., Hudson, NY) and acclimatized for 1 week. Animals were maintained under standardized conditions with *ad libitum* access to standard mouse chow and water. Procedures were performed with approval from the Institutional Animal Care and Use Committee of Indiana University.

Activity groups

Animals were randomly divided into two *activity* groups: 1) cage control (CON group) and 2) tail suspended (UNLOAD group). The latter group was tail suspended for 4 weeks. Half of a metal paper clip was made into a U-shape and its open end attached to the sides of the mouse tail with superglue. The rounded end of the paperclip was attached to a swivel and hung from an overhead wire. The height of the wire was adjusted to maintain the mice at approximately 25°-30° of head down tilt so that the hindlimbs but not forelimbs were elevated above the cage floor. The swivel allowed animal pivoting and slid freely on the wire to permit side-to-side movements. The floor of the cage was lined with a textured tape to allow the animals to grip and move via their forelimbs, and was sparsely lined with wood chips to absorb excretions. Suspended animals were floor fed.

Drug groups

Animals in each activity group were randomly divided into four drug groups: 1) vehicle treated [VEH group]; 2) fluoxetine hydrochloride (20 mg/kg) treated [SSRI group]; 3) desipramine hydrochloride (20 mg/kg) treated [TCA group], and; 4) lithium chloride (200 mg/kg) treated [LITH group]. Fluoxetine hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO) and desipramine hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO) were chosen as they have strong selectivity for the 5-HTT and norepinephrine transporter, respectively [27]. In addition, they have previously been shown to have anti-anxiolytic [28,29] and skeletal effects [23-25] in mice at the doses administered. The fluoxetine dose of 20 mg/kg/d in mice results in serum fluoxetine and norfluoxetine (active metabolite of fluoxetine) concentrations equivalent to those observed with the maximum (40-80 mg/d) recommended fluoxetine dose used to treat depression in humans [29-31]. Thus, the chosen dose produces clinically comparable serum fluoxetine and norfluoxetine concentrations. Lithium chloride (Sigma-Aldrich, Inc., St. Louis, MO) was chosen as it is a common agent for the treatment of both depression and mania, is a known inhibitor of glycogen synthase kinase-3 β (GSK-3 β) [32], and has previously been shown to have skeletal effects at the dose administered [26]. All drugs were mixed in a vehicle of ultra pure water (NANOpure Diamond, Barnstead International, Dubuque, IA) and administered via intraperitoneal injection (10 ml/kg) at the same time daily for 4 weeks.

Physical activity levels

Physical activity levels were assessed during the third week of intervention in the CON group. Each animal was treated with their respective drug in their home cage and 30 min later placed in a novel, non-illuminated test environment (VersaMax Animal Activity Monitoring System; AccuScan Instruments, Inc., Columbus, OH) for a 30 min measurement period. The total distance traveled by each mouse was recorded from the number of laser beam breaks during the test period.

In vivo skeletal assessments

In vivo skeletal assessments were performed under anesthesia at baseline and following 4 weeks intervention. Dual energy X-ray absorptiometry (DXA; PIXImus II, Lunar Corp., Madison, WI) and peripheral quantitative computed tomography (pQCT; Norland Medical Systems Stratec XCT Research SA+, Stratec Electronics, Pforzheim, Germany) were used to measure areal BMD (aBMD) within the rear half of the animals (pelvis, femur, tibia and feet [the tail vertebrae were excluded below the level of ischial tuberosities]) and localized tibial volumetric BMD (vBMD), respectively. pQCT involved taking transverse midshaft and proximal tibial scans as sites representative of predominantly cortical and trabecular bone, respectively. The proximal tibial scan was 15% of tibial bone length from its proximal end. All pQCT scans were performed using a 70 μm voxel size, and the bone edge was detected using contour mode 1 with a threshold of 400 mg/cm^3 . Cortical bone parameters were recorded from midshaft scans and total (cortical and trabecular) bone parameters from proximal scans. Proximal scans were not separated into cortical and trabecular compartments due to partial-volume limitations associated with assessing trabecular sites in mice.

Euthanasia and tissue handling

Animals were euthanized by exsanguination under anesthesia following 4 weeks intervention (animal age = 9 weeks). Bloods were collected from the CON group for assessment of circulating 5-HT levels (see below), and the lumbar spine (L1-L5) and femurs removed from both activity groups for further analyses. The lumbar spine and right femur were stored in 70% ethanol at 4°C for later assessment of *ex vivo* BMD, architecture and mechanical properties, while the left femur was fixed in 10% neutral buffered formalin for 72 hours before being processed for histomorphometry.

Ex vivo bone mineral density and architecture

DXA was performed as described above to measure *ex vivo* spinal (L1-L4) and femoral aBMD. Femoral midshaft geometry and fifth lumbar (L5) vertebrae trabecular architecture were assessed using micro-computed tomography. For femoral midshaft geometry, a single transverse midshaft slice was acquired using a 7 μm voxel size on a μCT -40 machine (Scanco Medical AG, Auenring, Switzerland). The slice image was imported into Scion Image for Windows (Beta 4.0.2; Scion Corporation, Frederick, MD) wherein cortical area (Ct.Ar) was determined. For L5 vertebrae trabecular architecture, a 250 μm thick cross-sectional region with 12 μm voxel size resolution was acquired distal to the mid-point of the pedicles on a SkyScan 1172 high-resolution μCT (SkyScan, Kontich, Belgium). The area for analysis was outlined within the trabecular compartment, excluding the cortical and subcortical bone, and bone volume fraction (BV/TV), trabecular number, trabecular thickness, and trabecular separation acquired.

Mechanical properties

Mechanical properties of the femoral midshaft and L5 vertebrae were determined following overnight rehydration of the specimens in saline. Femurs were positioned cranial side up across the lower supports of a miniature materials testing machine (Vitrodyne V1000; Liveco, Inc.,

Burlington, VT). The supports had a span width of 10.0 mm, and the bones were fixed with ~0.1 N static preload before being loaded to failure in three-point bending using a crosshead speed of 0.2 mm/sec. During testing, force and displacement measurements were collected every 0.01 sec from which ultimate force was derived. For L5 vertebrae mechanical properties, the end plates of the vertebral bodies were removed via parallel cuts on a diamond wafering saw (Isomet; Buehler, Lake Bluff, IL). After removing the neural arch by clipping through the pedicles, the vertebral bodies were tested in axial compression at a cross-head speed of 0.01 mm/s. During testing, force and displacement measurements were collected every 0.02 sec from which ultimate force was derived.

Histomorphometry

Calcein injections (30 mg/kg body mass; Sigma Chemical Co., St. Louis, MO) were given 7 and 3 days prior to euthanasia to permit determination of bone formation rates. The left femur was embedded undecalcified in 99% methyl-methacrylate with 3% dibutyl phthalate (Sigma-Aldrich, St. Louis, MO). Transverse thick (40-50 μ m) sections were removed from the midshaft using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives), and mounted unstained to assess periosteal bone formation rates. Frontal plane thin (4 μ m) sections of the distal femur were taken using a microtome (Reichert-Jung 2050; Reichert-Jung, Heidelberg, Germany) and stained with tartrate-resistant acid phosphatase and counterstained with hematoxylin (Sigma-Aldrich, Kit #387A-1KT, St. Louis, MO) to allow identification of trabecular osteoclasts.

Sections were montaged using Image-Pro Plus (Version 6.3; Media Cybernetics, Inc., Bethesda, MD) on a Leica DMI6000 inverted microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and stored digitally. Dynamic parameters were measured from the unstained midshaft femur sections, and included single-label perimeter (sL.Pm), double-label area (dL.Ar) and perimeter (dL.Pm), and interlabel width (Ir.L.Wi). The following were derived from these primary data: mineralizing surface ($MS/BS = [1/2sL.Pm + dL.Pm]/B.Pm$), mineral apposition rate ($MAR = dL.Ar / (0.5dL.Pm) / 4 \text{ days}$), and bone formation rate ($BFR/BS = MAR \times MS/BS \times 3.65$). Bone resorption was determined from stained sections of the distal femur by counting the number of bone-adherent, multinucleate, tartrate-resistant acid phosphatase positive cells (osteoclasts) within approximately 5 mm² of the secondary spongiosa and normalizing to bone surface (N.Oc/BS).

Circulating 5-HT levels

Serum and platelet-free plasma 5-HT levels were assessed in the CON group from blood collected at euthanasia. A shortened (15 mm) non-coated microhematocrit capillary tube was used to penetrate the retro-orbital plexus. The free end of the capillary tube was positioned over a microtainer blood collection tube coated with dipotassium salt EDTA (BD Microtainer Blood Collection Tube #365973; BD Diagnostics, Franklin Lakes, NJ). Dipotassium salt EDTA tubes were used to prevent blood clotting which results in platelet activation and the release of their 5-HT stores. A sample of 250-500 μ l of blood was collected to allow assessment of platelet-free plasma (circulating extracellular) 5-HT levels. The remaining blood was used to determine serum 5-HT levels and was collected in microtainer blood collection tubes containing spray-coated silica (BD Microtainer Blood Collection Tube #365956; BD Diagnostics, Franklin Lakes, NJ). These tubes promote clotting and platelet release of their sequestered 5-HT stores. Samples for platelet-free plasma and serum 5-HT levels were prepared and assessed using a standard 5-HT ELISA assay, as per the manufacturer's instructions (Immuno Biological Laboratories Inc., Minneapolis, MN). All samples were analyzed in duplicate and read on a microplate reader (VersaMax microplate reader; Molecular Devices, Sunnyvale, CA). 5-HT levels in the SSRI, TCA and LITH groups were expressed relative to those in the VEH group.

Statistics

Two-way factorial analyses of variance (ANOVAs) were used to compare interventions, with activity (CON vs. UNLOAD) and drug (VEH vs. SSRI vs. TCA vs. LITH) being the independent variables. Main effects were explored in the event of a non-significant interaction, with Fisher's protected least-significant difference (PLSD) used for post-hoc analyses of significant drug main effects. To control for body weight influences on bone properties, body weight was used as a covariate in architecture and mechanical property comparisons. Body weight was not used as a covariate in analyses of bone density or histomorphometric measures as these are normalized for bone size. For univariate measures (distance traveled and circulating 5-HT levels), one-way ANOVAs were performed with post-hoc analyses performed as described above. All analyses were performed with the Statistical Package for Social Sciences (SPSS 15.0.1; SPSS Inc., Chicago, IL) software, and were two-tailed with a level of significance set at 0.05.

RESULTS

Animal characterization

There were no baseline differences in body mass (all $p = 0.90$); however, both activity and drug intervention had independent main effects on final body mass (all $p < 0.05$; Fig. 1A). UNLOAD gained 20.5% less body mass than CON during the study period ($p < 0.001$), while SSRI had 7.5% and 9.7% less mass at the end of the study than VEH ($p = 0.04$) and LITH (all $p < 0.01$), respectively. Drug intervention had a significant effect on activity levels ($p < 0.01$), with LITH traveling 47%, 56% and 58% of the total distance of VEH, SSRI and TCA, respectively (all $p < 0.03$; Fig. 1B). SSRI and TCA did not influence distance traveled compared to VEH (all $p = 0.20$ -0.85).

Skeletal effects of activity and drug intervention combined, and the independent effects of activity

There were no interactions between activity and drug intervention for any skeletal measure (all $p = 0.21$ to 0.90), indicating that activity and drug effects were independent. Activity modification induced changes consistent with the known skeletal effects of unloading. UNLOAD had smaller gains in *in vivo* aBMD and vBMD (all $p < 0.05$; Fig. 2), and inferior *ex vivo* aBMD, bone architecture and bone mechanical properties than CON (all $p < 0.05$, Fig. 3).

Effect of drug intervention on *in vivo* bone measures

Drug intervention had significant effects on the percent change in hindlimb aBMD ($p = 0.03$; Fig. 2B) and proximal tibial vBMD ($p < 0.001$; Fig. 2D), but not tibial length ($p = 0.20$; Fig. 2A) or midshaft tibial vBMD ($p = 0.57$; Fig. 2C). SSRI suppressed proximal tibial vBMD gain by 9.0% (95% confidence interval [95%CI]: 3.3% to 14.8%) compared to VEH ($p < 0.01$; Fig. 2D). In contrast, LITH gained 10.9% (95%CI: 3.4% to 18.4%) more hindlimb aBMD ($p < 0.01$; Fig. 2B) and 6.7% (95%CI: 0.8% to 12.5%) more proximal tibial vBMD ($p = 0.03$; Fig. 2D) than VEH. There were no differences between TCA and VEH for change in either hindlimb aBMD ($p = 0.50$; Fig. 2B) or proximal tibial vBMD ($p = 0.15$; Fig. 2D).

Effect of drug intervention on *ex vivo* bone measures

There was a drug intervention main effect on femoral and spine aBMD, with SSRI having 8.3%-9.5% lower femoral aBMD than all other drug groups and 10.1%-10.5% lower spine aBMD than VEH and LITH (all $p < 0.05$; Fig. 3A,B). Midshaft femur Ct.Ar and L5 vertebrae BV/TV were also lower in SSRI compared to both VEH and LITH (all $p < 0.05$; Fig. 3C-E). There were no effects of TCA or LITH compared to VEH on femoral aBMD and Ct.Ar or spine

aBMD and BV/TV (all $p = 0.11$ to 0.91 ; Fig. 3A-E). There were significant drug intervention effects on femoral midshaft and L5 vertebrae ultimate force (all $p \leq 0.03$; Fig. 3F,G). SSRI did not differ from VEH at the femoral midshaft, but could resist 12.1%-14.9% less force than both TCA and LITH at this site (all $p < 0.05$; Fig. 3F). At the L5 vertebrae, SSRI had 18.5% and 23.2% lower ultimate force than VEH and LITH, respectively (all $p < 0.05$; Fig. 3G). TCA was 9.9% stronger and 18.6% weaker than VEH at the femur midshaft and L5 vertebrae, respectively, and 23.3% weaker than LITH at the L5 vertebrae (all $p < 0.05$; Fig. 3F,G).

Effect of drug intervention on bone formation and resorption

Drug intervention had a significant effect on bone formation at the femoral midshaft, with SSRI suppressing formation by 37.8% compared to VEH, and LITH having 39.8%-77.6% greater formation than all other drug groups (all $p \leq 0.02$; Fig. 4A). There was no effect of drug intervention on the number of osteoclasts within the distal femur ($p = 0.71$; Fig. 4B).

Effect of drug intervention on circulating 5-HT levels

Serum and platelet-free plasma 5-HT levels were 31.2%-40.5% lower in SSRI than all other drug intervention groups (all $p < 0.001$; Fig. 5).

DISCUSSION

The results of this study suggest psychotropic drugs have contrasting effects on the growing mouse skeleton which are independent of drug effects on animal physical activity levels. Daily introduction of the clinically popular SSRI antidepressant fluoxetine hydrochloride reduced *in vivo* gains in lower extremity BMD, and negatively altered *ex vivo* measures of femoral and spinal bone density, architecture and mechanical properties. These effects were mediated by a decrease in bone formation without a concomitant change in bone resorption suggesting that the SSRI had anti-anabolic skeletal effects. In contrast, the mood stabilizer lithium had apparent anabolic effects improving *in vivo* gains in BMD via an increase in bone formation, and the TCA desipramine hydrochloride had minimal skeletal effects. The skeletal effects associated with each agent were independent of their effects on animal physical activity levels as there were no statistical interactions between the drug and activity groups. Also, the observed skeletal phenotypes did not result from negative effects of the psychotropic agents on body mass as the latter was used as a covariate in statistical comparisons and body weight corrected values were reported. These data indicate that SSRI had negative, lithium had positive and TCA had minimal skeletal effects that were not influenced by drug effects on physical activity levels or body mass.

The negative skeletal effect of SSRI in this study is consistent with our previous work demonstrating genetic inactivation or pharmacological inhibition of the 5-HTT in growing mice results in a consistent skeletal phenotype of reduced mass, altered architecture and inferior mechanical properties [23]. The current study adds to this body of work by demonstrating that the negative skeletal effects associated with pharmacological 5-HTT inhibition are independent of drug effects on animal physical activity levels. An established effect of inhibition of the 5-HTT in mice is heightened anxiety-like behavior, which manifests in a hypoactive locomotor behavioral phenotype [29,33]. Any reduction in cage activity may contribute to bone changes observed with 5-HTT inhibition due to relative skeletal unloading. While reduced physical activity levels were not statistically detected with SSRI intervention in the current study, we prospectively controlled for this potential caveat by normalizing skeletal loading across drug groups via the introduction of tail suspension. Tail suspension of mice is an established skeletal unloading model [34] and also an established model for assessing the antidepressant properties of pharmacological agents [35]. Antidepressant intervention in mice actually reduces the immobility associated with tail suspension [35], an effect that may potentiate the rescue of any

hypoactivity-induced skeletal phenotype induced by these agents in cage control animals. This was not the case in the current study as statistical interactions or trends towards interaction were not observed between the drug and activity groups for any skeletal measure. Also, physical inactivity does not explain why mice treated with the SSRI exhibited a consistent negative skeletal phenotype whereas animals treated with lithium had a positive skeletal phenotype despite the later displaying greater physical inactivity. Similarly, physical inactivity does not explain why TCA did not induce a skeletal phenotype despite it previously being shown to induce greater physical inactivity than SSRI [25]. Collectively, the current data suggest that altered skeletal loading is not responsible for the negative skeletal effects of SSRI.

As effects on animal physical activity levels do not explain the skeletal effects of the agents investigated other mechanisms need to be considered, with agent-specific targeting of different underlying molecular pathways being a leading candidate. Fluoxetine and desipramine hydrochloride preferentially inhibit the 5-HTT and norepinephrine transporter, respectively, while lithium is an inhibitor of GSK-3 β . Antagonism of the 5-HTT using fluoxetine inhibits 5-HT uptake from the extracellular space prolonging its activation of 5-HT receptors. Yadav et al. [21] recently demonstrated that an increase in circulating 5-HT levels decreases bone formation by the activation of osteoblastic 5-HT_{1B} receptors and subsequent inhibition of osteoblast proliferation. While demonstrating that 5-HT has direct skeletal effects, this observation does not fully explain the skeletal effects of SSRI-mediated inhibition of the 5-HTT. Chronic administration of SSRIs results in substantially reduced circulating 5-HT levels [36], a finding confirmed in the current study. This reduction results from the fact that platelets typically transport >95% of circulating 5-HT and that their only means of obtaining 5-HT is via active uptake using the 5-HTT (platelets lack the rate-limiting enzyme tryptophan hydroxylase required for 5-HT synthesis). Applying the findings of Yadav et al. [21] to the SSRI scenario, the reduction in circulating 5-HT with chronic SSRI administration suggests these agents may increase osteoblast proliferation and bone formation. This was not the case in the current study, or in our previous studies [23,24] and those performed by independent groups [25,37]. One working hypothesis is that SSRIs impact the skeleton by directly inhibiting the 5-HTT located on bone cell membranes [38]. This may increase local 5-HT levels, in spite of decreased circulating 5-HT, by reducing its removal from the bone cell microenvironment enabling it to activate osteoblastic 5-HT_{1B} receptors and inhibit bone formation. This hypothesis requires investigation.

Indirect effects beyond drug-induced animal physical inactivity may also contribute to the skeletal phenotype observed with SSRI-mediated inhibition of the 5-HTT. In addition to demonstrating a direct skeletal effect of 5-HT, Yadav et al. [36] recently demonstrated that 5-HT within the central nervous system indirectly influences the skeleton. In particular, they observed that an increase in brainstem-derived 5-HT indirectly stimulated bone formation via its activation of hypothalamic 5-HT_{2C} receptors and subsequent inhibition of the bone suppressive influence of the sympathetic nervous system. While demonstrating that 5-HT has potential indirect skeletal effects, this observation appears at odds with the observed negative skeletal effects of 5-HTT inhibition. SSRI treatment increases extracellular 5-HT concentration within the central nervous system [40,41] which should increase bone formation, according to the observations of Yadav et al. [36]. This does not appear the case, highlighting the need for further investigation into the mechanism/s by which SSRI-mediated inhibition of the 5-HTT influences the skeleton.

The observed anabolic skeletal effect of lithium was not unexpected considering it is a potent inhibitor of GSK-3 β [32]. GSK-3 β functions as an intermediary in numerous intracellular signaling pathways, including the skeletally important Wnt/ β -catenin pathway which has become the target of recent drug discovery efforts. In Wnt/ β -catenin signaling, GSK-3 β phosphorylates β -catenin creating a signal for its rapid ubiquitin-dependent degradation by

proteosomes. When GSK-3 β is inhibited it enables β -catenin to remain stable (unphosphorylated) allowing it to accumulate in the cytoplasm to the point where translocation to the nucleus occurs and gene transcription is initiated. While previous work has established the skeletal benefits of lithium-mediated inhibition of GSK-3 β [26], the current study furthers the body of knowledge by demonstrating that lithium has anabolic effects despite inducing a significant hypoactive phenotype and maintains its anabolic effect even in the presence of more extreme skeletal unloading (tail suspension). These findings indicate lithium has potent anabolism independent of skeletal loading status and reconfirm the Wnt/ β -catenin pathway as a prime candidate for drug discovery efforts.

The current data need to be interpreted with caution in light of some study limitations. In particular, the psychotropic drugs investigated have minimal biochemical comparability as they target differing underlying molecular pathways. However, the goal of this study was not to compare drugs within the same biochemical family, but rather compare compounds within the same therapeutic family. The data indicate that drugs within the psychotropic family commonly prescribed for the treatment of affective and mood disorders have contrasting skeletal effects. These effects may be influenced by drug dose, with only a single dose being explored for each agent in the current study. The investigation of a dose response may influence the skeletal effect size; however, it is not anticipated to change the direction of the skeletal effect for each agent. Supporting this is the fact that the skeletal phenotypes observed with each psychotropic agent are consistent with previous preclinical [23-26] and preliminary clinical data [6,8-10,12,13,42,43]. Clinical use of SSRIs has been shown to be associated with increased bone loss [12], decreased BMD [13] and increased fracture risk [42]. In particular, Diem et al. [12] found SSRIs to double the rate of hip bone loss among elderly women when assessed longitudinally over an average period of 4.9 yr, while Richards et al [42] found SSRIs to be associated with a two-fold increase in the risk for incident clinical fragility fractures. These effects are greater in magnitude and more consistent than observed in users of TCAs [6,8,9,12]. In terms of lithium, there is preliminary evidence that it improves femoral neck and spine BMD [43], and decreases fracture risk [6,10].

In conclusion, the current study demonstrates that psychotropic drugs with differing underlying mechanisms of action have contrasting skeletal effects on the growing mouse skeleton and that these effects do not result indirectly via the generation of animal physical inactivity. Pharmacological inhibition of the 5-HTT using a clinically popular SSRI (fluoxetine hydrochloride) had anti-anabolic skeletal effects, whereas GSK-3 β inhibition using lithium was anabolic. There were minimal skeletal effects of norepinephrine transporter inhibition using a TCA (desipramine hydrochloride). The observed anabolic effect of GSK-3 β inhibition using lithium reconfirms the importance of Wnt/ β -catenin signaling in the skeleton and its recent targeting in drug discovery efforts. Meanwhile, the negative skeletal effects of 5-HTT inhibition, combined with recent findings of direct inhibitory effects of 5-HT on bone formation, are of interest given the frequent prescription of SSRIs for the treatment of depression and other affective disorders. As the current study eliminates physical inactivity as a contributing factor for the negative skeletal phenotype observed with SSRIs, other potential indirect and possibly direct mechanisms for the bone changes associated with 5-HTT inhibition need to be explored.

Acknowledgments

The authors thank Keith W. Condon for assistance with tissue processing. This work was supported by the National Institutes of Health (R01 AR052018 and S10 RR023710).

REFERENCES

- [1]. Arroll B, Macgillivray S, Ogston S, Reid I, Sullivan F, Williams B, Crombie I. Efficacy and tolerability of tricyclic antidepressants and SSRIs compared with placebo for treatment of depression in primary care: a meta-analysis. *Ann Fam Med* 2005;3:449–56. [PubMed: 16189062]
- [2]. Hetrick S, Merry S, McKenzie J, Sindahl P, Proctor M. Selective serotonin reuptake inhibitors (SSRIs) for depressive disorders in children and adolescents. *Cochrane Database Syst Rev*. 2007 CD004851.
- [3]. Storosum JG, Wohlfarth T, Schene A, Elferink A, van Zwieten BJ, van den Brink W. Magnitude of effect of lithium in short-term efficacy studies of moderate to severe manic episode. *Bipolar Disord* 2007;9:793–8. [PubMed: 18076528]
- [4]. Vestergaard P. Skeletal effects of central nervous system active drugs: anxiolytics, sedatives, antidepressants, lithium and neuroleptics. *Curr Drug Saf* 2008;3:185–9. [PubMed: 18690999]
- [5]. Haney EM, Warden SJ. Skeletal effects of serotonin (5-hydroxytryptamine) transporter inhibition: Evidence from clinical studies. *J Musculoskelet Neuronal Interact* 2008;8:133–45. [PubMed: 18622082]
- [6]. Bolton JM, Metge C, Lix L, Prior H, Sareen J, Leslie WD. Fracture risk from psychotropic medications: a population-based analysis. *J Clin Psychopharmacol* 2008;28:384–91. [PubMed: 18626264]
- [7]. Vestergaard P, Rejnmark L, Mosekilde L. Anxiolytics, sedatives, antidepressants, neuroleptics and the risk of fracture. *Osteoporos Int* 2006;17:807–16. [PubMed: 16520889]
- [8]. Vestergaard P, Rejnmark L, Mosekilde L. Selective serotonin reuptake inhibitors and other antidepressants and risk of fracture. *Calcif Tissue Int* 2008;82:92–101. [PubMed: 18219438]
- [9]. Ziere G, Dieleman JP, van der Cammen TJ, Hofman A, Pols HA, Stricker BH. Selective serotonin reuptake inhibiting antidepressants are associated with an increased risk of nonvertebral fractures. *J Clin Psychopharmacol* 2008;28:411–7. [PubMed: 18626268]
- [10]. Vestergaard P, Rejnmark L, Mosekilde L. Reduced relative risk of fractures among users of lithium. *Calcif Tissue Int* 2005;77:1–8. [PubMed: 16007481]
- [11]. Mezuk B, Eaton WW, Golden SH. Depression and osteoporosis: epidemiology and potential mediating pathways. *Osteoporos Int* 2008;19:1–12. [PubMed: 17763997]
- [12]. Diem SJ, Blackwell TL, Stone KL, Yaffe K, Haney EM, Bliziotes MM, Ensrud KE. Use of antidepressants and rates of hip bone loss in older women: the study of osteoporotic fractures. *Arch Intern Med* 2007;167:1240–5. [PubMed: 17592096]
- [13]. Haney EM, Chan BK, Diem SJ, Ensrud KE, Cauley JA, Barrett-Connor E, Orwoll E, Bliziotes MM. Association of low bone mineral density with selective serotonin reuptake inhibitor use by older men. *Arch Intern Med* 2007;167:1246–51. [PubMed: 17592097]
- [14]. Landi F, Onder G, Cesari M, Barillaro C, Russo A, Bernabei R. Psychotropic medications and risk for falls among community-dwelling frail older people: an observational study. *J Gerontol A Biol Sci Med Sci* 2005;60:622–6. [PubMed: 15972615]
- [15]. Thapa PB, Gideon P, Cost TW, Milam AB, Ray WA. Antidepressants and the risk of falls among nursing home residents. *N Engl J Med* 1998;339:875–82. [PubMed: 9744971]
- [16]. Battaglini R, Fu J, Spate U, Ersoy U, Joe M, Sedaghat L, Stashenko P. Serotonin regulates osteoclast differentiation via its transporter. *J Bone Miner Res* 2004;19:1420–1431. [PubMed: 15312242]
- [17]. Bliziotes M, Eshleman A, Burt-Pichat B, Zhang XW, Hashimoto J, Wiren K, Chenu C. Serotonin transporter and receptor expression in osteocytic MLO-Y4 cells. *Bone* 2006;39:1313–1321. [PubMed: 16884969]
- [18]. Bliziotes MM, Eshleman AJ, Zhang X-W, Wiren KM. Neurotransmitter action in osteoblasts: expression of a functional system for serotonin receptor activation and reuptake. *Bone* 2001;29:477–486. [PubMed: 11704501]
- [19]. Gustafsson BI, Thommesen L, Stunes AK, Tommeras K, Westbroek I, Waldum HL, Slordahl K, Tamburstuen MV, Reseland JE, Syversen U. Serotonin and fluoxetine modulate bone cell function in vitro. *J Cell Biochem* 2006;98:139–51. [PubMed: 16408289]
- [20]. Westbroek I, van der Plas A, de Rooij KE, Klein-Nulend J, Nijweide PJ. Expression of serotonin receptors in bone. *J Biol Chem* 2001;276:28961–8. [PubMed: 11387323]

- [21]. Yadav VK, Ryu JH, Suda N, Tanaka KF, Gingrich JA, Schutz G, Glorieux FH, Chiang CY, Zajac JD, Insogna KL, Mann JJ, Hen R, Ducy P, Karsenty G. Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell* 2008;135:825–37. [PubMed: 19041748]
- [22]. Warden SJ, Haney EM. Skeletal effects of serotonin (5-hydroxytryptamine) transporter inhibition: Evidence from in vitro and animal-based studies. *J Musculoskelet Neuronal Interact* 2008;8:121–32. [PubMed: 18622081]
- [23]. Warden SJ, Robling AG, Sanders MS, Bliziotes MM, Turner CH. Inhibition of the serotonin (5-hydroxytryptamine) transporter reduces bone accrual during growth. *Endocrinology* 2005;146:685–693. [PubMed: 15539550]
- [24]. Warden SJ, Nelson IR, Fuchs RK, Bliziotes MM, Turner CH. Serotonin (5-hydroxytryptamine) transporter inhibition causes bone loss in adult mice independently of estrogen deficiency. *Menopause* 2008;15:1176–83. [PubMed: 18725867]
- [25]. Bonnet N, Bernard P, Beaupied H, Bizot JC, Trovero F, Courteix D, Benhamou CL. Various effects of antidepressant drugs on bone microarchitecture, mechanical properties and bone remodeling. *Toxicol Appl Pharmacol* 2007;221:111–118. [PubMed: 17383703]
- [26]. Clement-Lacroix P, Ai M, Morvan F, Roman-Roman S, Vayssiere B, Belleville C, Estrera K, Warman ML, Baron R, Rawadi G. Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci U S A* 2005;102:17406–11. [PubMed: 16293698]
- [27]. Owens MJ, Morgan WN, Plott SJ, Nemeroff CB. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J Pharmacol Exp Ther* 1997;283:1305–22. [PubMed: 9400006]
- [28]. Cryan JF, O’Leary OF, Jin SH, Friedland JC, Ouyang M, Hirsch BR, Page ME, Dalvi A, Thomas SA, Lucki I. Norepinephrine-deficient mice lack responses to antidepressant drugs, including selective serotonin reuptake inhibitors. *Proc Natl Acad Sci U S A* 2004;101:8186–91. [PubMed: 15148402]
- [29]. Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology* 2004;29:1321–30. [PubMed: 15085085]
- [30]. Alvarez JC, Gluck N, Fallet A, Gregoire A, Chevalier JF, Advenier C, Spreux-Varoquaux O. Plasma serotonin level after 1 day of fluoxetine treatment: a biological predictor for antidepressant response? *Psychopharmacology* 1999;143:97–101. [PubMed: 10227085]
- [31]. Ferguson JM, Hill H. Pharmacokinetics of fluoxetine in elderly men and women. *Gerontology* 2006;52:45–50. [PubMed: 16439824]
- [32]. Quiroz JA, Gould TD, Manji HK. Molecular effects of lithium. *Mol Interv* 2004;4:259–72. [PubMed: 15471909]
- [33]. Holmes A, Yang RJ, Murphy DL, Crawley JN. Evaluation of antidepressant-related behavioral responses in mice lacking the serotonin transporter. *Neuropsychopharmacology* 2002;27:914–23. [PubMed: 12464448]
- [34]. Morey-Holton ER, Globus RK. Hindlimb unloading of growing rats: a model for predicting skeletal changes during space flight. *Bone* 1998;22:83S–88S. [PubMed: 9600759]
- [35]. Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci Biobehav Rev* 2005;29:571–625. [PubMed: 15890404]
- [36]. Yadav VK, Oury F, Suda N, Liu ZW, Gao XB, Confavreux C, Klemenhagen KC, Tanaka KF, Gingrich JA, Guo XE, Tecott LH, Mann JJ, Hen R, Horvath TL, Karsenty G. A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell* 2009;138:976–89. [PubMed: 19737523]
- [37]. Zolkowska D, Baumann MH, Rothman RB. Chronic fenfluramine administration increases plasma serotonin (5-hydroxytryptamine) to nontoxic levels. *J Pharmacol Exp Ther* 2008;324:791–7. [PubMed: 18032571]
- [38]. Westbroek I, Waarsing JH, van Leeuwen JP, Waldum H, Reseland JE, Weinans H, Syversen U, Gustafsson BI. Long-term fluoxetine administration does not result in major changes in bone architecture and strength in growing rats. *J Cell Biochem* 2007;101:360–8. [PubMed: 17163489]

- [39]. Warden SJ, Robling AG, Haney EM, Turner CH, Bliziotes MM. The emerging role of serotonin (5-hydroxytryptamine) in the skeleton and its mediation of the skeletal effects of low-density lipoprotein receptor-related protein 5 (LRP5). *Bone*. 2009 doi: 10.1016/j.bone.2009.06.029.
- [40]. Dawson LA, Nguyen HQ, Smith DL, Schechter LE. Effect of chronic fluoxetine and WAY-100635 treatment on serotonergic neurotransmission in the frontal cortex. *J Psychopharmacol* 2002;16:145–52. [PubMed: 12095073]
- [41]. Koch S, Perry KW, Nelson DL, Conway RG, Threlkeld PG, Bymaster FP. R-fluoxetine increases extracellular DA, NE, as well as 5-HT in rat prefrontal cortex and hypothalamus: an in vivo microdialysis and receptor binding study. *Neuropsychopharmacology* 2002;27:949–59. [PubMed: 12464452]
- [42]. Richards JB, Papaioannou A, Adachi JD, Joseph L, Whitson HE, Prior JC, Goltzman D. Effect of selective serotonin reuptake inhibitors on the risk of fracture. *Arch Intern Med* 2007;167:188–94. [PubMed: 17242321]
- [43]. Zamani A, Omrani GR, Nasab MM. Lithium's effect on bone mineral density. *Bone* 2009;44:331–4. [PubMed: 18992857]

Fig. 1.

Effect of: A) activity and drug intervention on final body mass, and; B) drug intervention on physical activity levels. UNLOAD, tail suspended group; CON, cage control group; V, vehicle treated group; S, selective serotonin reuptake inhibitor treated group; T, tricyclic antidepressant treated group; L, lithium chloride treated group. Bars represent mean $\pm$ SD. There was no activity $\times$ drug interaction on body mass, but significant main effects for both activity and drug as determined by two-way factorial ANOVA followed by Fisher's PLSD for pairwise comparisons. There was also a significant drug effect on physical activity levels as determined by one-way factorial ANOVA followed by Fisher's PLSD for pairwise comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2.

Effect of activity and drug intervention on change in *in vivo* measures of: A) tibial length; B) hindlimb areal BMD [aBMD]; C) midshaft tibial volumetric BMD [vBMD], and; D) proximal tibial vBMD. UNLOAD, tail suspended group; CON, cage control group; V, vehicle treated group; S, selective serotonin reuptake inhibitor treated group; T, tricyclic antidepressant treated group; L, lithium chloride treated group. Bars represent mean $\pm$ SD. There were no activity $\times$ drug interactions, but significant main effects for activity on change in hindlimb aBMD, and both activity and drug on change in hindlimb aBMD and proximal tibial vBMD, as determined by two-way factorial ANOVA followed by Fisher's PLSD for pairwise comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3. Effect of activity and drug intervention on *ex vivo* assessment of: A) femoral areal BMD [aBMD]; B) spine aBMD; C) midshaft femur cortical area [Ct.Ar]; D) fifth lumbar [L5] vertebrae bone volume [BV/TV]; E) representative three-dimensional reconstructions of L5 trabecular architecture; F) femoral midshaft ultimate force, and; G) L5 vertebrae ultimate force. UNLOAD, tail suspended group; CON, cage control group; V, vehicle treated group; S, selective serotonin reuptake inhibitor treated group; T, tricyclic antidepressant treated group; L, lithium chloride treated group. Bars represent mean $\pm$ SD (body-weight corrected means are provided in C, D, F and G). There were no activity $\times$ drug interactions, but significant main

effects for both activity and drug, as determined by two-way factorial ANOVA followed by Fisher's PLSD for pairwise comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fig. 4.

Effect of activity and drug intervention on indices of: (A) femoral midshaft periosteal bone formation (bone formation rate normalized for bone surface [BFR/BS]), and; (B) distal femoral bone resorption (osteoclast number normalized for trabecular bone surface [N.Oc/BS]). UNLOAD, tail suspended group; CON, cage control group; V, vehicle treated group; S, selective serotonin reuptake inhibitor treated group; T, tricyclic antidepressant treated group; L, lithium chloride treated group. Bars represent mean $\pm$ SD. There were no activity $\times$ drug interactions or activity main effects, but a significant main effect for drug on bone formation, as determined by two-way factorial ANOVA followed by Fisher's PLSD for pairwise comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Effect of drug intervention on: A) serum and B) platelet-free plasma levels of 5-HT in cage control animals. V, vehicle treated group; S, selective serotonin reuptake inhibitor treated group; T, tricyclic antidepressant treated group; L, lithium chloride treated group. Bars represent mean $\pm$ SD, with values expressed relative to V. There were significant drug effects as determined by one-way factorial ANOVAs followed by Fisher's PLSD for pairwise comparisons. *** $p < 0.001$.