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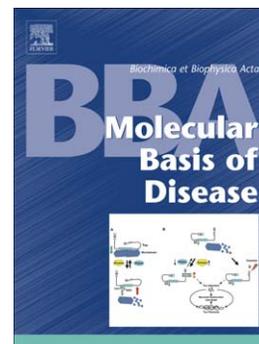
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**Factors that affect postnatal bone growth retardation in the twitcher murine model
of Krabbe disease**

Miguel Agustin Contreras, William Louis Ries, Srinivasan Shanmugarajan, Gonzalo
Arboleda¹, Inderjit Singh and Avtar Kaur Singh^{a,*}

Department of Pediatrics, Darby Children's Research Institute, Medical University of
South Carolina, and ^aDepartment of Pathology and Laboratory Medicine, Ralph H.
Johnson Veteran's Administration Medical Center, Charleston, SC.

(*) Corresponding author:

Avtar K. Singh, M.D.

516 Darby Children's Research Institute

Medical University of South Carolina

173 Ashley Avenue,

Charleston, SC 29425

Phone: (843) 789-7534.

Fax: (843) 792-7130.

E-mail address: avtar.singh@med.va.gov

⁽¹⁾ **Present address:** Departamento de Patologia, Facultad de Medicina, Universidad
Nacional de Colombia, Cr 30 No 45-03, Bogota, Colombia. gharboledab@unal.edu.co

Abbreviations: CM: ceramide; GH: growth hormone; IGF-1: insulin-like growth factor-
1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; qPCR: quantitative polymerase
chain reaction; TRAP: tartrate resistant acid phosphatase; SM: sphingomyelin; Sph:
sphingosine; Sph-1P: sphingosine-1-phosphate; Twi: twitcher; Wt: wild type.

Abstract

Krabbe disease is an inherited lysosomal disorder in which galactosylsphingosine (psychosine) accumulates mainly in the central nervous system. To gain insight into the possible mechanism(s) that may be participating in the inhibition of the postnatal somatic growth described in the animal model of this disease (twitcher mouse, *twi*), we studied their femora. This study reports that *twi* femora are smaller than of those of wild type (*wt*), and present with abnormality of marrow cellularity, bone deposition (osteoblastic function), and osteoclastic activity. Furthermore, lipidomic analysis indicates altered sphingolipid homeostasis, but without significant changes in the levels of sphingolipid-derived intermediates of cell death (ceramide) or the levels of the osteoclast-osteoblast coupling factor (sphingosine-1-phosphate). However, there was significant accumulation of psychosine in the femora of adult *twi* animals as compared to *wt*, without induction of tumor necrosis factor-alpha or interleukin-6. Analysis of insulin-like growth factor-1 (IGF-1) plasma levels, a liver secreted hormone known to play a role in bone growth, indicated a drastic reduction in *twi* animals when compared to *wt*. To identify the cause of the decrease, we examined the IGF-1 mRNA expression and protein levels in the liver. The results indicated a significant reduction of IGF-1 mRNA as well as protein levels in the liver from *twi* as compared to *wt* littermates. Our data suggest that a combination of endogenous (psychosine) and endocrine (IGF-1) factors play a role in the inhibition of postnatal bone growth in *twi* mice; and further suggest that derangements of liver function may be contributing, at least in part, to this alteration.

Keywords: Galactosylsphingosine; Insulin-like growth factor-1; Krabbe disease; lysosomal disorders; osteopenia; psychosine; twitcher mice

1. Introduction

The infantile form of Krabbe disease (Globoid cell leukodystrophy) is the most common, a severe, rapidly progressing, neurodegenerative and invariably fatal, inherited metabolic disease produced by mutations on the β -galactosylcerebrosidase gene, which expresses a lysosomal enzyme that participates in the degradation of several glycosphingolipids [1, 2]. As a consequence, galactosylsphingosine (psychosine, also a substrate of the enzyme [3]) accumulates in the nervous system [3] and peripheral organs [4]. The availability of a mutant mouse (twitcher mouse; *twi*), that is a morphologically, genetically and enzymatically authentic murine model of the human disease [5], has been a valuable tool to advance our understanding of the development/progression of the disease in the central nervous system. In the nervous system, accumulation of psychosine exerts a cytotoxic effect especially on myelin-producing cells (oligodendrocytes) [6], with further induction of myelin degeneration, astrocytic gliosis, axonal loss and presence of multinucleate giant (globoid) cells [1]. Psychosine-induced local inflammatory response in the nervous system (induction of proinflammatory cytokines and intermediates)[7-10] plays a role in the early apoptotic loss of oligodendrocytes [11, 12] observed in Krabbe patients [13, 14] and twitcher mice [15] brains.

Twitcher mouse develops clinical signs by day 20th, characterized by stunted growth, twitching and hind leg weakness; and the disease progresses to a near-terminal stage by day 40-45 [1]. The twitching sign is associated with the loss of oligodendrocytes [16-18]; however, the cause of decreased somatic growth is unclear. Multiple etiologies could underlie this growth defect; but alteration of hypothalamic-hypophysial axis

function may play a causative role. Growth hormone (GH), secreted by the anterior pituitary into the circulation, has long been recognized as one of the principal factors that control postnatal growth [19]. GH acts as a promoter of skeletal long bone growth and development as well as chondrocyte maturation [20, 21], by receptor mediated induction of the transcription of genes whose expressed proteins are important in mediating its diverse effects on body growth and metabolism [22]. GH also stimulates the expression of hepatic and extrahepatic insulin-like growth factor-1 (IGF-1), that plays an essential GH-mediated anabolic role on skeletal and non skeletal tissues [23-28].

We have previously reported that psychosine accumulation mediates the induction of cytokines in *twi* liver, thus compromising some of its functions [29]. In the present report, , we studied the presence of endogenous (psychosine; Sph-1P) and humoral (IGF-1) factors that may be responsible for the stunted growth of femora of *twi* mice, to help identify possible mechanisms that may be playing a role in the postnatal somatic growth retardation of *twi* mouse. Our data indicate that: i) psychosine accumulates in femora of *twi* mice; ii) osteoclast function of *twi* femora (bone resorption) is enhanced; iii) tartrate resistant acid phosphatase (TRAP) activity is increased in the growth plate; iv) *twi* mouse femora present alteration of sphingolipid homeostasis, without signs of change in Sph-1P or a significant decrease in sphingomyelin or an increase in ceramide pool; v) liver secreted IGF-1 level is drastically reduced in *twi* mice plasma, and vi) IGF-1 transcripts and protein levels are significantly down regulated in *twi* liver. This study reports for the first time evidence that psychosine, acting either endogenously in the bone (local accumulation) or in conjunction with a disrupted endocrinal signal (IGF-1), are

potential factors that mediate the alteration in bone growth, and hence the somatic growth of *twi* mice.

2. Material and methods

2.1. Animal model

All animal work for this study was performed under a protocol approved by the Institutional Animal Care and Use Committee. *Twitcher* heterozygote breeding pairs (C57BL/6J $twi^{+/-}$) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and $twi^{(-/-)}$ (*twi*) and age-matched littermates wild type $wt^{(+/+)}$ (*wt*) were used for this study at postnatal day 33-34. *Wt* and *twi* animals were sacrificed with an overdose of anesthetic and tissues were removed, frozen in liquid nitrogen and stored at -70°C .

2.2. Femora analysis of psychosine and sphingolipid levels by HPLC-mass spectrometry

For lipidomic analysis, bones (left and right femur) were defrosted in ice-cold phosphate buffered saline and the muscle tissue removed manually. Bones were weighed, enveloped in aluminum foil, frozen in liquid nitrogen and crushed in a mortar. Quantitative high pressure liquid chromatography-tandem mass spectrometry analysis of psychosine and bioactive sphingolipids were performed on organics solvent extracts derived from *wt* and *twi* crushed bones, at the University Lipidomics Core facility [30].

2.3 Calcein bone labeling

To define abnormal bone growth in the *twi* mouse, a modified histomorphometric analysis was performed using double labeling with calcein as a marker [31]. Adult mice were given two intraperitoneal injections of calcein 5 days apart (postnatal days 26 and 31). Calcein was prepared in a 2% solution of sodium bicarbonate, and injected at a dose of 20 mg/kg of body weight. Animals were killed 2 days after the last injection (postnatal day 33). Femora were isolated and calcein was assessed in undecalcified bones embedded in methacrylate. Sections 4-6 μm thick (Leica RM 2155 rotary microtome; Leica Microsystems, Ontario, Canada) were examined under a fluorescence microscope (Olympus BX-60; excitation at 485 nm and emission at 510 nm), and the acquired images were rendered using Adobe Photoshop 7.0.

2.4. Histology and tartrate-resistant acid phosphatase (TRAP) staining analysis

Wild-type and *twi* mice bone specimens were fixed in 4% paraformaldehyde and dehydrated with serial changes in 70-100% ethanol. The specimens were embedded in methacrylate and serial 4-6 μm sections were stained by Goldner's trichrome method for light microscopy analysis [32] or stained for TRAP activity using a histochemical kit (Sigma-Aldrich) as described elsewhere [33].

2.5. Plasma and liver analysis of Insulin-like Growth Factor-1

Blood from *wt* and *twi* mice was collected in EDTA-K containing vacutainer tubes (BD Biosciences, Franklin Lakes, NJ), and the plasma was separated by centrifugation at 900xg for 10min (Allegra X-15R centrifuge, Beckman-Coulter,

Fullerton, CA) and stored at -70°C . Levels of IGF-1 were determined using an ELISA kit (Quantikine, mouse IGF-1 immunoassay according to manufacturer instructions (R&D Systems, Minneapolis, MN).

2.6. Bone analysis of cytokines (Tumor Necrosis Factor-alpha and Interleukin-6):

Expression of cytokines (TNF- α and IL-6) was detected by ELISA in the supernatant of lysis buffer bones' extracts (RIPA buffer, Thermo Scientific Inc., Waltham, MA). Bones were individually processed as indicated for the mass spectrometric analysis and extracted with 120 μL of lysis buffer. ELISA analysis was performed according to manufactured instructions (TNF- α : eBioscience, San Diego, CA; IL-6: BD Biosciences, San Jose, CA) using 50 μL of bone's extract. Plates' readings were performed in a Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA).

2.7. Quantitative polymerase chain reaction

Liver total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and the purified RNA used for the synthesis of cDNA. Single-strand cDNA was synthesized from 2 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc, Hercules, CA) according to manufacturer's instructions. qPCR was conducted using iCycler (iCycler iQ, Bio-Rad), specific primers for mouse IGF-1 (Cat. number PPM03387E; SABiosciences, Frederick, MD) and iQ SYBR Green Supermix (Bio-Rad). Thermal cycling conditions were as follows: activation of DNA polymerase at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 30s and 60°C for 30s. The expressions of target genes were normalized with respect to the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All samples were run in

duplicate. Statistical analysis was performed by student-t test (Graph Path InStat3, GraphPath Software Inc., San Diego, CA) and the difference at $p < 0.05$ was considered statistically significant.

3. Results

3.1 Retarded growth of long bones (femora) of Twitcher mouse and Galactosyl-sphingosine (psychosine) accumulation

Postnatal growth curves show that *twi* mouse body weight is severely inhibited by day 20 (Fig. 1A), as reported by several laboratories [34-36]. Taking long bones (femora) as representatives of the animals' growth, we analyzed their physical differences between *wt* and *twi* mice. The analysis of femora of *wt* and *twi* at post natal day 32-33 indicated that *twi* bones have smaller size (Fig. 1B) and weighed 27% less than *wt* mice bones (Fig. 1C). Furthermore, determination of psychosine levels by mass spectrometry indicated that psychosine accumulates in *twi* bones of adults animals (33 days old), at levels 14 fold higher than the levels found in *wt* (Table 1).

3.2 Histomorphologic analysis of Twitcher femora

Examination of stained *wt* and *twi* femora under light microscopy revealed differences in cortical bone thickness, cellular organization (isogenous groups) and width of the growth plate, between *wt* and *twi* animals (Fig. 2A, B). The metaphyseal region of *twi* bones also showed marked structural differences, with reduction of trabecular bone (stained in green) mainly in the region of the secondary spongiosa (Fig. 2C, D). Overall,

there was a marked decrease in cortical bone thickness and the number of trabeculae, indicating the *twi* mouse shows an osteopenic phenotype.

3.3. Bone growth/remodeling functions (osteoblastic deposition and osteoclastic resorption) in femora of wild type and twitcher mice

To gain insight into the possible cause of reduced bone in *twi* femora, the bone-forming capacity of osteoblasts was examined by the deposition of calcein. The calcein labeling along the cortical bone (seen as two layers of fluorescent green lines under the fluorescence microscopic analysis) showed no difference between *wt* and *twi* (Fig. 3A, B), indicating no differences in the mineral apposition rate. However, there was a substantial reduction of bone deposition (green fluorescence) in the metaphyseal and epiphyseal region of *twi* femora (Fig. 3A, B). To evaluate whether enhanced osteoclastic activity is the cause of the observed reduced calcein deposition in both regions, we performed histochemical analysis for tartrate-resistant acid phosphatase (TRAP) activity. TRAP-positive osteoclasts were identified along the base of the growth plate and most of the trabeculae and on the endosteal surface of the diaphysis in both *wt* and *twi* mice bone showed activity (Fig. 4A, B). However, TRAP staining was stronger in *twi* bone, and included the narrow growth plate of the *twi* femur. These data indicate that psychosine-induced alterations in *twi* bone preferentially affects the function of osteoclasts and suggests that enhanced osteoclastic resorption may be playing a role in the bone growth retardation observed in *twi* animals.

3.4. Sphingolipid homeostasis and cytokine markers in femora of twitcher mouse

To evaluate possible changes in the osteoclast-osteoblast coupling factor sphingosine-1 phosphate (Sph-1P)[37] and to better understand the cause of reduced cellularity reported in *twi* bone marrow [36], we studied the levels of sphingolipid in femora. The analysis of the *wt* and *twi* femora by mass spectrometry indicated a significant increase (1.3 fold) in the total sphingomyelin (SM) pool found in the *twi* (341 ± 10 nmoles/g) when compared to *wt* bones (263 ± 11 nmoles/g; $p < 0.0001$)(Fig. 5A). The observed increase is mainly a contribution of the saturated species C16:0-SM (1.3 fold), C18:0-SM (1.2 fold), C20:0-SM (1.4 fold), C22:0-SM (1.4 fold), and C24:0-SM (1.2 fold); and of the unsaturated species C22:1-SM (1.4 fold), C24:1-SM (1.4 fold) and C26:1-SM (1.1 fold) (Table 2). On the other hand, the analysis of the ceramide (CM) pool did not indicate a significant difference between *wt* and *twi* bones (Fig. 5B). However, significant changes were observed in the individual saturated species C20:0-CM (0.7 fold) and C22:0-CM (1.4 fold), DHC16:0 (0.8 fold), Sph (1.4 fold), and the unsaturated species C24:1-CM (1.3 fold) (Table 2). Interestingly, the most abundant species (C24:1) in SM as well as in CM was significantly elevated in both pools; and the dihydro-C16:0-CM (DHC16:0; a *de novo* CM synthesis indicator [38]) was significantly decreased. Taking into account that the changes observed in the total SM pool and SM species represent an increase in concentrations in *twi* as compared with *wt*, and minor variations in some CM saturated and unsaturated species, these data suggest that psychosine accumulation in *twi* bones is interfering with SM and CM turnover.

To rule out a possible psychosine-induced inflammatory process as demonstrated in the brains and livers of twitcher mice [8, 29], we determined the bone's levels of the cytokines TNF- α and IL-6 (Table 4). Because the cytokine analysis did not indicate a significant difference between wild type and twitcher mice (Table 4), the results suggest that psychosine is not exerting its effect by inducing the expression of mediators of inflammation.

3.5 Insulin-like growth factor-1 (IGF-1) levels in plasma and mRNA expression and protein levels in liver

IGF-1 is a GH-dependent growth factor produced mainly by the liver, and it is known to regulate growth and bone remodeling [39]. ELISA analysis of plasma levels of IGF-1 in *wt* and *twi* animals indicated that IGF-1 levels were significantly reduced by 37.6% in *twi* animals as compared to *wt* (234.4 ± 26.4 vs 148.7 ± 20.5 ng/ml in *wt* and *twi* respectively; $p < 0.002$)(Fig. 6), and decreases as the disease progresses (Fig. S1A, B). Because plasma circulating IGF-1 is of hepatic origin and liver is also affected by psychosine induced-disease in *twi* mice [29], we investigated the mRNA expression and protein levels in liver of *wt* and *twi* mice. The hepatic expression of IGF-1 mRNA was down regulated by 59.3% in adult animals (1.9 ± 0.4 in *wt* vs. 0.8 ± 0.3 folds in *twi*; $p < 0.005$)(Fig. 7A), whereas IGF-1 protein levels were decreased by 60.4% (1.7 ± 0.2 in *wt* vs. 0.7 ± 0.3 ng/mg of protein in *twi*; $p < 0.0001$)(Fig. 7B) in *twi* liver as compared with *wt*. This result indicated a strong alteration of the liver functions in *twi* animals.

4. Discussion

Twitcher mouse is an animal model of the genetic disorder Globoid cell leukodystrophy (Krabbe disease) [5, 40, 41]. These animals develop clinical signs by day 20 (stunted growth, twitching, hind leg weakness), and they reach a near-terminal stage by day 40-45 [1]. While the twitching sign is associated with oligodendrocytes (myelin-forming cell) loss [16-18], the cause of *twi* mouse decreased growth is unclear. It is well established that psychosine accumulates in nervous tissue [3], and exerts an inflammatory response characterized by induction of proinflammatory cytokines, inducible nitric oxide synthase, oxidative imbalance and early oligodendrocyte cell death [8, 11-15, 42]. As the disease pathology progresses, the inflammatory process compromises further brain homeostasis, altering subcellular functions of peroxisomes [42, 43] and possibly the mitochondria [44, 45]. In parallel with the central nervous system events, psychosine also accumulates in non-nervous tissues/organs, affecting their homeostasis [29, 36]. Our data demonstrated that psychosine also accumulates in long bones (femora) of twitcher mice, where it may exert cytotoxic effects, thus supporting the notion that globoid cell leukodystrophy (Krabbe disease) is a generalized storage disease [4], and adding another tissue to the list of those that accumulate psychosine [29, 36, 46-48]. Such accumulation, however, does not compare to the levels found in *twi* liver or brain (3 and 27 folds higher than in bone, respectively) [29].

Many local [37, 49-51], humoral/endocrine [20, 21, 26] and central and peripheral nervous system-derived factors [39, 52] regulate the functions of the specialized cells such as osteoblasts (bone deposition) and osteoclasts (bone resorption) responsible for

bone growth/remodeling [50]. Morphologic and functional analysis of *wt* and *twi* femora demonstrated reduced content of cortical and trabecular bone indicating that the *twi* mouse shows an osteopenic phenotype, possibly a consequence of enhanced osteoclastic bone resorption (TRAP activity) without much alteration of osteoblastic function (calcein deposition). These data indicate that psychosine-induced alterations in *twi* bone may uncouple bone deposition from bone resorption. It affects preferentially osteoclastic function and suggests that over time an enhanced osteoclastic resorption may be playing a role in the premature closure of the growth plate; and hence is a contributing factor to the reduction in bone growth in *twi* animals. Interestingly, in another animal model of lysosomal disease, mucopolysaccharidosis type VII (deficiency of the enzyme β -glucuronidase), there is metabolite accumulation mediated bone alteration by impairment of both osteoclastic and osteoblastic functions [53].

In most tissues, SM degradation (hence increase in CM levels) is associated with activation of stress response/cell death [54], and Sph-1P increase is associated with antiapoptotic/cell survival signal [55, 56]. In addition, in bone Sph-1P is a factor that regulates the osteoclast-osteoblast coupling during bone growth/remodeling [37, 51]. In general, our sphingolipid analysis resembles the changes reported for *twi* liver [29]; with insignificant changes in known apoptotic (C16:0-CM) or antiapoptotic (Sph-1P) sphingolipid fractions [38, 57]. However, an increase was found in total SM fraction, and in most of the SM and CM containing saturated-unsaturated fatty acids, including the most abundant species (C24:1) in SM and in CM. On the other hand, the dehydro-C16:0 (DHC16:0; an indicator of *de novo* CM synthesis [38]) was significantly decreased and

Sph accumulated in *twi* mice. These data suggest that psychosine accumulation may interfere with SM degradation and CM synthesis in *twi* bones. Furthermore, the lipidomic analysis showing no significant changes on Sph-1P between wt and *twi* mice bone rules out that this sphingolipid may be playing a role in the osteoclast-osteoblast uncoupling [37]. These results suggest that the levels of psychosine may not reach a threshold that is enough to trigger stress response/cell death in *twi* bones. Consistent with these results, analysis of the inflammation marker TNF- α and IL-6 were found not to be altered, which strongly suggests that the psychosine-induced pathology in twitcher bones is not dependent or potentiated by the induction of mediators of inflammation, as demonstrated in the brain and liver of those animals [8, 29] or studies on cell culture [13]. On the other hand, psychosine accumulation may be interfering with cell signaling at the level of the receptor sites (lipids rafts), as suggested recently [58]. Future studies should be aimed to identify the cellular distribution of the accumulated psychosine in long bones.

Liver is the main organ that contributes to the blood circulating IGF-1 pool [27, 28, 59]; with IGF-1 playing an important role in bone physiology [25, 60, 61]. Taking into consideration that liver functions are compromised by psychosine-induced inflammation in *twi* animals [29], it was not surprising that mRNA expression and protein levels of IGF-1 were found to be down regulated in the liver of *twi* mice, supporting the drastic decrease in IGF-1 plasma levels observed in those mice. These results indicate an additional psychosine-mediated metabolic dysfunction in *twi* liver (synthesis of IGF-1), and suggest that reduction in IGF-1 plasma levels may be an additional factor contributing to the observed impaired balance (uncoupling) between osteoclast-osteoblast

function, and hence to the bone growth stunting in *twi* mice. Studies performed in double gene disrupted mice (liver IGF-1-deficient and acid labile subunit (ALS) knockout) support our results [62]; dramatic decrease in IGF-1 levels in both animals is consistent with decreased bone growth. Indeed, the fewer trabeculae in the metaphysis is a sign of osteopenia; and the smaller growth plate in both cases is consistent with the narrowing of the growth plate observed with aging, which is associated with decrease in circulating and local production of IGF-1 [63-65]. Furthermore, additional factors such as the endogenous presence of cytokines (as seen in brain and liver)[8, 29] or alterations in the hypothalamic-pituitary axis in the nervous system [66], may also play a role in the derangements of bone growth. Recent studies have reported drastic changes in *twi* mice bone marrow functions [36].

Our data and that of others demonstrate the complexity of the development and progression of the psychosine-induced pathology in the nervous system [11-13, 36, 43] and in peripheral organs [29, 36], and highlight the importance of peripheral organs for the overall organism homeostasis [29]. Based on these observations, we hypothesize that progression of inflammation in the *twi* central nervous system could affect hormone secretion which may play a role in the homeostasis of peripheral organs, such as in bone growth regulation [67, 68].

In summary, we have identified two potential factors that may be affecting bone growth in *twi* mice. We report that psychosine accumulates in bones, where either maybe by itself as an endogenous factor or in conjunction with a decrease of plasma IGF-1 levels, preferentially impaired osteoclastic function, and hence stunted bone growth.

Therefore, recent therapeutic treatments [69, 70] might consider the supplementation/induction of endocrinous/paracrine factors (i.e., GH-IGF-1). Combination therapy may prove to be of benefit for the central nervous system [71-73] and peripheral organs, and ultimately for the amelioration of the development and progression of the disease pathology in *twi* mice and Krabbe patients.

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Figures captions:

Figure 1. Growth curves and differences in femora size and weight between wild type and twitcher mice. Significant differences in body weight (A), and length and weight (B) between femora from wild type and littermate twitcher mice is evident in adult animals. Graph represents the average \pm SD for $n = 6-7$ animals per group. ***: Asterisks denote significant statistical differences from wild type littermates ($p < 0.0001$). WT: wild type mice; TW: Twitcher mice.

Figure 2. Morphologic differences between the femur from wild type and twitcher mice. Light microscopic study of Goldner's trichrome stained sections derived from wild type (A, C) and twitcher (B, D) mouse bone identified differences in thickness of green-stained cortical bone (CB), organization and morphology of the chondrocytes and width of the growth plate (GP) and reduction of green-stained trabecular bone (TB) (A, B: 40x magnification; line represents 2 mm; C, D: 60x magnification, line represents 200 μ m).

Figure 3. Bone deposition (osteoblasts activity) and resorption (osteoclasts activity) functions in the femora of twitcher mice. Osteoblasts function in wild type (A) and twitcher (B) was determined by double calcein labels (green fluorescence) during new bone formation/remodeling. Osteoclasts function, as TRAP-positive osteoclasts (red) can be seen along the base of the growth plate as well as on most of the trabeculae (TB) and on the endosteal surface of the cortex in both wild type (C) and twitcher (D) mice. Note the drastic reduction of labeling in twitcher mouse trabeculae (trabecular bone, TB) (40x magnification; line represents 2 mm)(B) and the intense TRAP activity of the growth

plate in twitcher mice (D). TRAP assay was done as indicated in Material and methods (40x magnification).

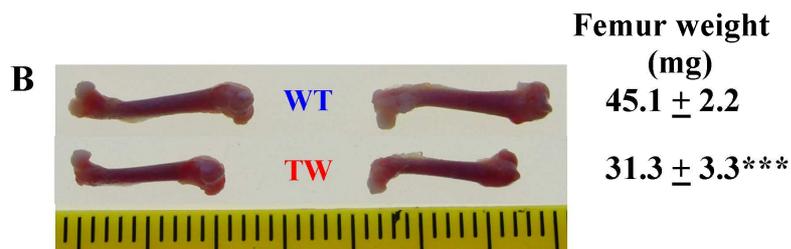
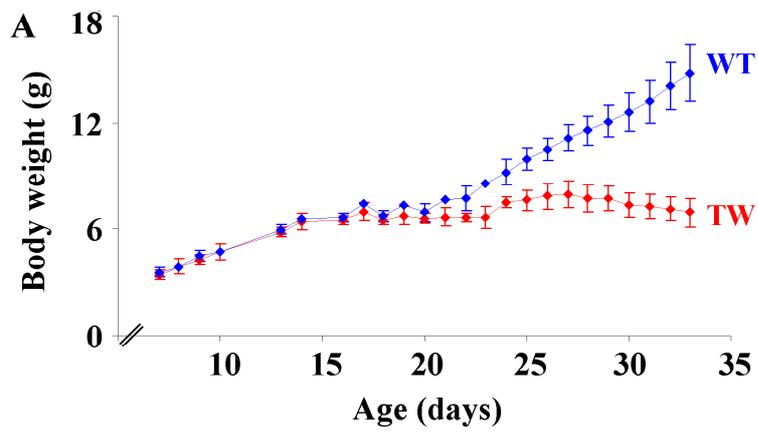
Figure 4. Sphingolipid concentration in wild type and twitcher femora. The levels of sphingomyelin (SM) and ceramide (CM) (A); sphingosine (Sph) and sphingosine-1-phosphate (Sph-1P) (B) fractions were determined by HPLC-mass spectrometry in organic extracts from pairs of femora of wild type (white bars) and twitcher mice (black bars), as indicated in Materials and methods. Values in (A) were obtained from the summary of the individual SM and CM fractions (including saturated and unsaturated fatty acids fractions). Graph represent the average \pm SD for $n = 4$ samples per group. Asterisks denote significant statistical differences from wild type littermates (*: $p < 0.01$; **: $p < 0.0001$).

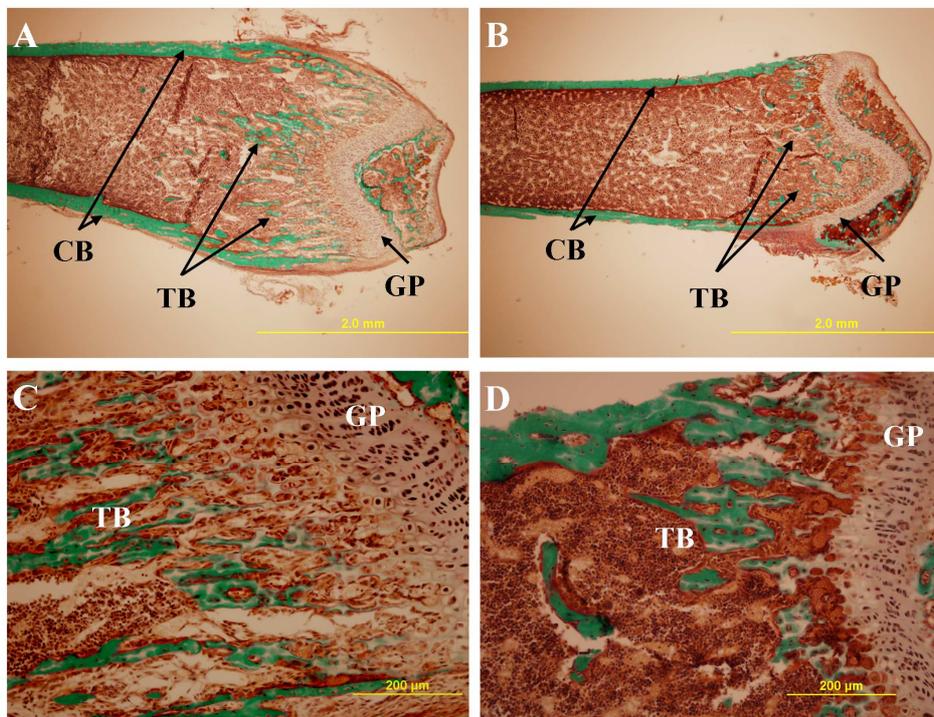
Figure 5. Insulin-like growth factor-1 levels in the plasma of twitcher mice. Plasma levels of IGF-1 in wild type and twitcher mice were determined by ELISA assay at postnatal day 33. Graph represents the average \pm SD for $n = 4$ animals per group. Asterisks denote significant statistical differences from wild type littermates (**: $p < 0.002$).

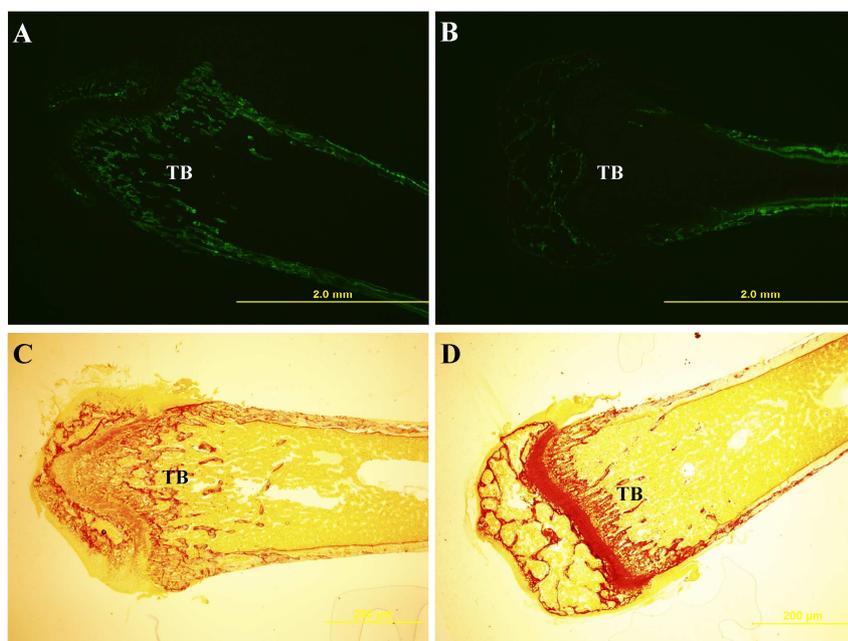
Figure 6. Expression and protein level of insulin-like growth factor-1 in twitcher mice liver. Wild type and twitcher mouse IGF-1 mRNA expression were determined by quantitative PCR (A), and IGF-1 protein levels in liver homogenate soluble fractions

were determined by ELISA assay (B), as described in Materials and Methods. Graph represents the average \pm SD for the number of animals indicated per group. Analysis was performed in mice 33-34 days old. **: $p < 0.005$ ($n = 3$); ***: $p < 0.0001$ ($n = 6$). Asterisks denote significant statistical differences from wild type littermates. WT: wild type mice; TW: Twitcher mice.

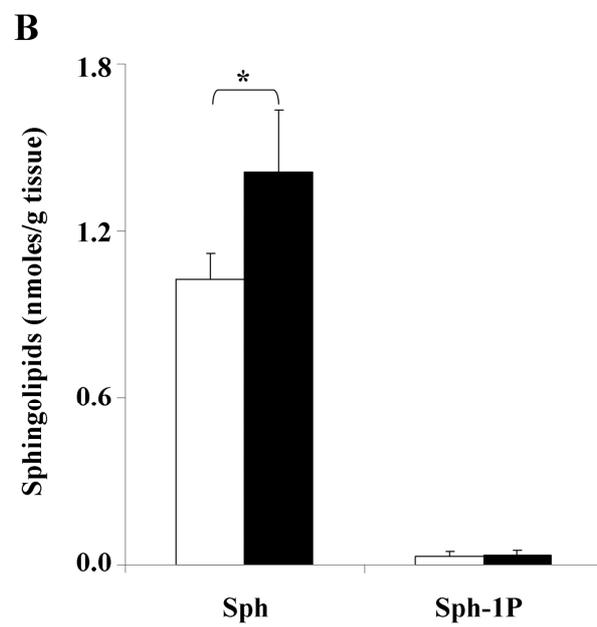
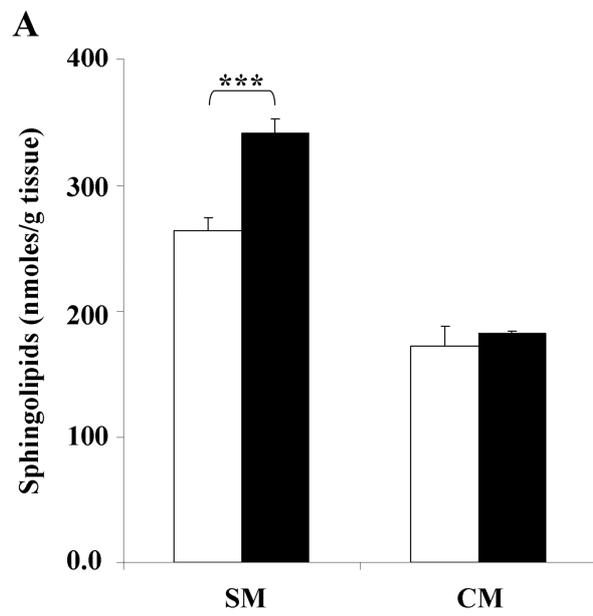
Figure S1. Postnatal differences in femora sizes and protein level of insulin-like growth factor-1 in plasma of wild type and twitcher mice. Postnatal femora from 10 to 45 days of age are shown in (A). White asterisks indicate differences in bones' length between wild type and twitcher animals. Wild type (white bars) and twitcher mouse (black bars) IGF-1 protein levels in plasma were determined by ELISA assay at the age period indicated (B). Graph represents the average \pm SD for the number of animals indicated per group. *: $p < 0.02$; ***: $p < 0.0001$ indicate significant statistical differences from wild type littermates. The number of animals in each age period were: postnatal days 20-25 ($n = 6$ WT, 6TW); 26-30 ($n = 4$ WT, 7TW); 31-35 ($n = 4$ WT, 6TW) and 36-40 ($n = 7$ WT, 11TW). WT: wild type mice; TW: Twitcher mice.

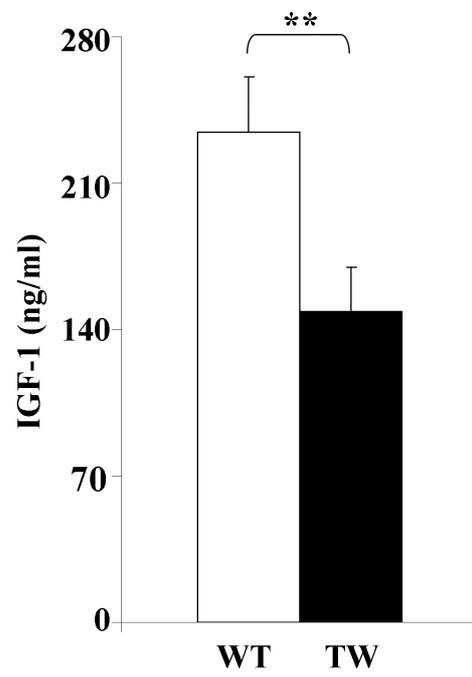


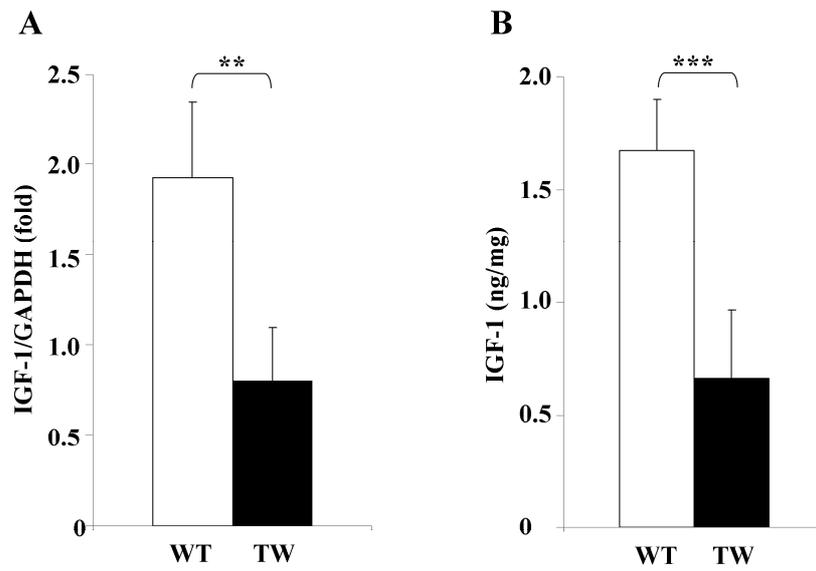




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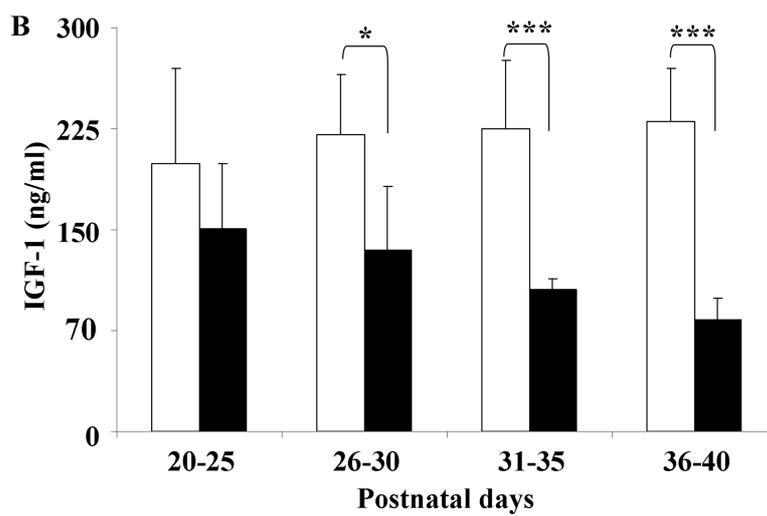
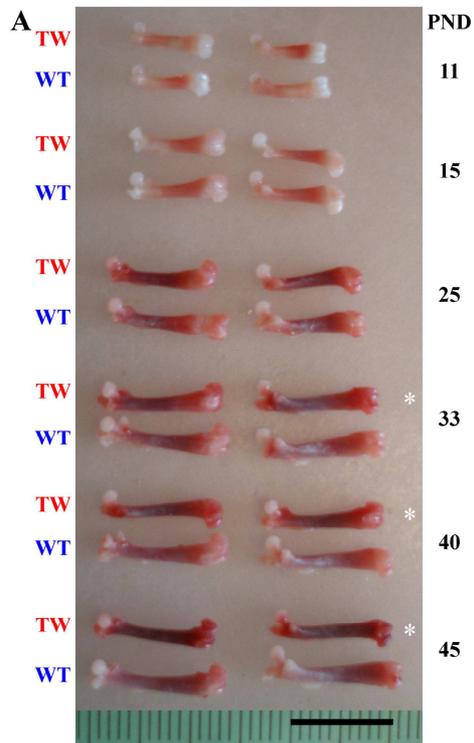


Table 1:

Level of galactosylsphingosine (psychosine) in wild type and twitcher mice femora

	Femora^a (pmoles/g tissue)
Wild type	13 ± 1
Twitcher	187 ± 57**

(^a): Samples represent Mean ± SD of psychosine concentrations found in femora from wild type (n = 3 sets) and twitcher (n = 4 sets) mice, determined at postnatal day 32-33.

(Set includes the left and the right femur from each animal).

***p* < 0.004

Table 2:

Determination of the sphingomyelin fractions composition in wild type and twitcher femora

Sphingomyelin fractions^a (nmoles/g tissue)								
	C14:0	C16:0	C18:0	C20:0	C22:0	C24:0	C26:0	
C18:1	C20:1	C22:1	C24:1	C26:1				
Wild type	1.07 ± 0.05	110 ± 4	39 ± 3	4.7 ± 0.5	14 ± 2	36 ± 2	0.35 ± 0.05	
	2.9 ± 0.3	0.36 ± 0.07	2.6 ± 0.4	52 ± 5	0.75 ± 0.04			
Twitcher	1.23 ± 0.17	143 ± 6	48 ± 2	6.5 ± 0.6	19 ± 2	42 ± 3	0.33 ± 0.05	
	3.2 ± 0.3	0.44 ± 0.06	3.6 ± 0.4	73 ± 4	0.85 ± 0.05			
<i>p</i> <		ns	0.0001	0.001	0.001	0.002	0.005	ns
ns	ns	0.005	0.0002	0.01				

(^a): Individual sphingomyelin fractions were determined by mass spectrometry in defleshed bone (wet weight) organic extracts. Values represent the average ± SD for n = 5 sets per group. *p* <: Statistical significance as indicated, versus wild type; ns: not significant.

Table 3:

Determination of the ceramide fractions composition in wild type and twitcher femora

Ceramide fractions ^a (nmoles/g tissue)									
	C14:0 C20:4	C16:0 C22:1	C18:0 C24:1	C20:0 DHC16:0	C22:0	C24:0	C26:0	C18:1	C20:1
Wild type	6.2 ± 3.4 0.07 ± 0.02	32 ± 8 3.7 ± 0.5	10 ± 2 38 ± 5	10.4 ± 2.1 0.36 ± 0.06	7.5 ± 0.8 22 ± 3	33 ± 4	0.13 ± 0.04	4.2 ± 0.3	2.8 ± 0.4
Twitcher	5.0 ± 2.2 0.04 ± 0.01	34 ± 4 4.0 ± 0.4	10 ± 1 51 ± 4	7.5 ± 1.1 0.32 ± 0.07	10.3 ± 0.5 18 ± 1	35 ± 5	0.11 ± 0.06	3.7 ± 0.6	2.3 ± 0.4
<i>p</i> <	ns	ns	ns	0.03	0.0002	ns	ns	ns	ns
ns	ns	0.001	ns	0.03					

(^a): Individual ceramide fractions were determined by mass spectrometry in defleshed bone (wet weight) organic extracts. Values represent the average ± SD for n = 5 sets per group. *p* <: Statistical significance as indicated, versus wild type; ns: not significant.

Table 4:Level of cytokines (TNF- α and IL-6) in wild type and twitcher mice femora

	TNF-α (pmoles / g tissue) ^a	IL-6
Wild type	86 \pm 56	152 \pm 79
Twitcher	112 \pm 76	149 \pm 63

(^a): Samples represent Mean \pm SD of cytokines concentrations (tumor necrosis factor-alpha, TNF- α ; Interleukin-6, IL-6) found in femora from wild type (n = 4 sets) and twitcher (n = 5 sets) mice, determined at postnatal day 33-35. (Set includes the left and the right femur from each animal).