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Phenotypic characterization of *Grm1crv4* mice reveals a functional role for the type 1 metabotropic glutamate receptor in bone mineralization.

Ilaria Musante[a], Deborah Mattinzoli[b,c], Lavinia Alexandra Otescu[d], Simone Bossi[a], Masami Ikehata[b,c], Chiara Gentili[e], Giuliana Cangemi[f], Cinzia Gatti[f], Laura Emionite[g], Piergiorgio Messa[b,c], Roberto Ravazzolo[a,h], Maria Pia Rastaldi[b,c], Daniela Riccardi[d], Aldamaria Puliti[a,h]

[a] DiNOGMI, University of Genoa, via Gaslini 5, 16148 Genoa, Italy;

[b] Renal Research Laboratory, Fondazione IRCCS Ospedale Maggiore Policlinico, via Pace 9, 20122 Milan, Italy;

[c] Fondazione D'Amico per la Ricerca sulle Malattie Renali, via Pace 9, 20122 Milan, Italy;

[d] School of Biosciences, Cardiff University, Cardiff, United Kingdom;

[e] Laboratory of Regenerative Medicine, DIMES, University of Genoa, IRCCS A.O.U. San Martino-IST Largo Rosanna Benzi 10, Genoa, Italy.

[f] Clinical Pathology Laboratory Unit, Istituto Giannina Gaslini, via Gaslini 5, 16148 Genoa, Italy;

[g] Animal Facility, IRCCS A.U.O. San Martino-IST, Largo Rosanna Benzi 10, Genoa, Italy;

[h] Medical Genetics Unit, Istituto Giannina Gaslini, via Gaslini 5, 16148 Genoa, Italy

Corresponding author: Aldamaria Puliti

Medical Genetics Unit, G. Gaslini Institute,

via G. Gaslini 5, 16148 Genoa, Italy

Tel:+39-010-56362802 FAX:+39-10-3779797

e-mail: apuliti@unige.it

ABSTRACT 275

Recent increasing evidence supports a role for neuronal type signaling in bone. Specifically glutamate receptors have been found in cells responsible for bone remodeling, namely the osteoblasts and the osteoclasts. While most studies have focused on ionotropic glutamate receptors, the relevance of the metabotropic glutamate signaling in bone is poorly understood. Specifically type 1 metabotropic glutamate (mGlu1) receptors are expressed in bone, but the effect of its ablation on skeletal development has never been investigated. Here we report that $Grm1^{crv4/crv4}$ mice, homozygous for an inactivating mutation of the mGlu1 receptor, and mainly characterized by ataxia and renal dysfunction, exhibit decreased body weight, bone length and bone mineral density compared to wild type (WT) animals. Blood analyses of the affected mice demonstrate the absence of changes in circulating factors, such as vitamin D and PTH, suggesting renal damage is not the main culprit of the skeletal phenotype. Cultures of osteoblasts lacking functional mGlu1 receptors exhibit less homogeneous collagen deposition than WT cells, and present increased expression of osteocalcin, a marker of osteoblast maturation. These data suggest that the skeletal damage is directly linked to the absence of the receptor, which in turn leads to osteoblasts dysfunction and earlier maturation. Accordingly, skeletal histomorphology of Grm1crv4/crv4 mice suggests that the absence of the mGlu1 receptor causes enhanced bone maturation, resulting in premature fusion of the growth plate and shortened long bones, and further slowdown of bone apposition rate compared to the WT mice.

In summary, this work reveals novel functions of mGlu1 receptors in the bone and indicates that in osteoblasts mGlu1 receptors are necessary for production of normal bone matrix, longitudinal bone growth, and normal skeletal development.

1. Introduction

The metabotropic glutamate (mGlu) receptors belong to family C G-protein coupled receptors. mGlu receptors are a heterogeneous subfamily with different subtypes and are divided into three groups based on sequence homology and signal transduction pathways. Group I consists of the mGlu1 and mGlu5 receptors, coded by the *Grm1* and the *Grm5* genes, respectively. mGlu1 receptor is primarily coupled to Gq/G11 protein and its activation stimulates phospholipase C β (PLC β) with the ensuing formation of the intracellular second messengers, inositol-1,4,5-trisphosphate (Ins-1,4,5-P$_3$) and diacylglycerol (DAG) (reviewed in [1]). In turn, Ins-1,4,5-P$_3$ production leads to the release of Ca^{2+} from intracellular stores, whereas DAG activates protein kinase C. The mGlu1 receptor is present in a number of key central nervous system (CNS) structures including the hippocampus, cortex, thalamus and cerebellum [2]. The receptor plays an important role in neuronal cells by triggering various signaling pathways that modulate neuron excitability, synaptic plasticity, and mechanisms of feedback regulation of neurotransmitter release [1, 3]. mGlu1 receptor is also expressed in non-neuronal tissues, *e.g.* skin, pancreas, liver, heart, retina and bone [4-9]. In the kidney, mGlu1 receptors are present particularly in glomerular podocytes, where they play an important role in podocyte signaling and glomerular intercellular communication [8, 10].

The implication of the glutamate signaling pathways in bone mass regulation has been studied extensively during the past years. Accumulating evidence supports the expression in bone cells of all functional elements of glutamate signaling [11-15], particularly in cells responsible for bone remodeling, such as osteoblasts and osteoclasts. In addition, osteoblasts have been found to be able to release and recycle glutamate using mechanisms identical to those of neuronal cells [16, 17]. Finally, functional mGlu1 receptor has been detected in primary culture of rat femoral osteoblasts, as demonstrated by the fact that its activation leads to an elevation of intracellular Ca^{2+} levels [4].

We previously described a mouse carrying a spontaneous recessive mutation in the *Grm1* gene (*crv4*)[18]. The *crv4* germline mutation affects constitutively all tissues and gives rise to a complex phenotype, mainly characterized by ataxia and tremor due to impaired cerebellar activities, renal

dysfunction, and kyphoscoliosis particularly evident in female mice [8, 18, 19].

As for the skeletal phenotype, it could be an indirect consequence of neuronal and renal changes, or it can be directly caused by *Grm1* expression absence in bone cells.

Based on the evidence that mGlu receptors are expressed in bone, we hypothesized that absence of *Grm1* expression from osteoblasts could lead to impaired glutamate signaling and consequently to pathological changes in bone remodelling. Therefore, the aim of the current study was to conduct a detailed analysis of the skeletal phenotype of the $Grm1^{crv4}$ mice. To determine the impact of mGlu1 receptors absence on the skeletal phenotype *in vivo*, we analysed body weights and lengths of affected and wild type animals, and carried out bone histomorphology and blood biochemical analyses. The direct effects of constitutive *Grm1* from osteoblasts were determined *in vitro*, in osteoblasts isolated from mice lacking *Grm1*, and from wild-type control animals. The results suggest that absence of mGlu1 receptor from osteoblasts directly contributes to the skeletal abnormalities present in $Grm1^{crv4/crv4}$ mice.

2. Materials and methods

2.1 Animal model and ethics statement

The *crv4* mutation is a spontaneous recessive mutation occurred in the BALB/c/Pas inbred strain that disrupts the *Grm1* splicing and causes absence of the mGlu1 receptor protein [18]. Affected ($Grm1^{crv4/crv4}$) and control ($Grm1^{+/+}$) mice are maintained on the same genetic background by intercrossing $Grm1^{crv4/+}$ mice at the animal facility of the IRCCS San Martino-IST (Genoa, Italy). Mice are given free access to water. Normal chow food is placed on the floor to ensure that all mice are fed in equal measure. To obtain the genotype of the mouse progeny, DNA was extracted from ear clippings according to the manufacturer's protocol (KAPA Mouse Genotyping Kits, Kapa Biosystems, Woburn, MA, USA) and amplified *via* polymerase chain reaction (PCR) using specific primers as previously described [20]. In this study a total of 52 $Grm1^{crv4/crv4}$, 42 $Grm1^{crv4/+}$, and 52 $Grm1^{+/+}$ (WT) mice were used, with an equal number of females and males *per* genotype.

All procedures involving animals were performed in the respect of the National and International current regulations (D.lvo 27/01/1992, n° 116, European Economic Community Council Directive 86/609, OJL 358, Dec. 1, 1987) and were reviewed and approved by the licensing and Ethical Committee of the IRCCS-AOU San Martino-IST National Cancer Research Institute, Genoa, Italy, and by the Italian Ministry of Health.

2.2 Mouse body weight and body length measurements

Body weights and lengths of 10 males and 10 females WT and *Grm1*^{crv4/crv4} were measured at 24 weeks of age. Body length was determined by measuring nasal-to-anal distance using a caliper immediately after the mice were killed before organ collection.

2.3 X-ray analysis, imaging techniques and image processing

Hind limbs of 12 to 28 weeks old sex- and genotype- matched mice were analysed. Mouse hind limbs were X-rayed and bone length and density were measured using the Bruker In-Vivo FX PRO imaging system (Foley et al, application note, in preparation and [21]). Briefly, the X-ray energy was calibrated to 12.85 – 13.03 Kev, thus the X-ray quantum efficiency is maximal at energy levels between 10 and 15 Kev [22]. Imaging and image processing were performed via Carestream (Bruker) Molecular Imaging Software v5.0.2. The software interface enables estimation of bone mineral density in a certain region of interest (ROI) selected by the user from the X-ray image [22]. The X-ray images were processed with the Carestream software, rotated and mirrored, so that the femoral axis was as horizontal as possible and the images had the same orientation (the condyle on the left hand side and the femoral head on the right hand side). A standard size ROI was selected in the most cylindrical region of the bone. Bone length and surface density were calculated with the same tool. Femur length was measured by drawing a straight horizontal line from the most exterior point of the femoral head, to the most exterior point of the condylar extremity. The tibia was measured in a comparable manner.

2.4 Bone histomorphometry

Histomorphology of the hind limbs of 12 and 24 weeks old mice (N = 3 per genotype) was analysed

by hematoxylin and eosin (H&E) staining. The femora were isolated and immersed in 342 mM EDTA, 7.2 pH for 21 days. Following demineralization, the tissue was paraffin-embedded and 5 µm thick sections were mounted on Plysine microscope slides. Sections were subjected to H&E staining and toluidine blue staining, performed with an R.A. LAMB Histomate and the slides were mounted with DPX.

2.5 Histomorphometric analysis of bone formation

To assess bone formation indices, mice received two intra-peritoneal injections of calcein (10 mg/kg dissolved in 2% Na_2HCO_3 in PBS) (Sigma Chemical Company, St. Louis, USA) 14 and 3 days before sacrifice. Calcein double labelling was quantified in methylmethacrylate embedded sections. Femora from WT and Grm1$^{crv4/crv4}$ mice were recovered and infiltrated with the light-curing resin Technovit 7200VLC (Kulzer, Wehrheim, Germany) for 21 days under vacuum with resin replaced every 7 days. Samples were polymerized by the EXAKT 520 polymerization system (EXAKT Wehrheim, Bio-Optica, Italy) with curing performed with 450 nm light at temperature bellow 40°C. The specimens were then prepared to be cut, according to the precision paralleling-guide procedure protocol, using the precision presses Exakt 401 and 402 Vacuum Adhesive Press (EXAKT Wehrheim, Bio-Optica, Italy). Sections were cut using the EXAKT 310 CP cutting unit (EXAKT Wehrheim, Bio-Optica, Italy). Obtained sections were approximately of 150 µm in thickness. Sections were then grinded to 20–30 µm thickness using the EXAKT 400 CS micro grinding unit (EXAKT Wehrheim, Bio-Optica, Italy). Sections were stained with Stevenel's/Van Gieson. For all processed samples, images were taken using Axiovert 200M microscope (Zeiss, Germany) [23]. Mineralizing surface (MS) and mineral apposition rate (MAR, µm/d) were measured from unstained sections, and bone formation rate was calculated (BFR=MS/BS $*$ MAR, µm2/µm3/d)[24].

2.6 Immunohistochemistry and immunocytochemistry

For immunohistochemistry (IHC), tibia recovered from 4 weeks old *Grm1$^{crv4/crv4}$* and WT mice were fixed in 4% formaldehyde/phosphate-buffered saline. Paraffin-embedded sections were decalcified (HCl 37% / Formic acid 85% /distilled water), dewaxed and incubated with the following primary

antibodies: rabbit anti-mGlu1 receptor, NB300-123 NOVUS; rabbit anti-collagen I, ab292 ABCAM. Then IHC was performed using super picture HRP polymer conjugated broad spectrum detection kit (Invitrogen), diaminobenzidine (DAKO). Sections were counterstained with Brilliant cresyl blue (BCB). Then IHC was performed using super picture HRP polymer conjugated broad spectrum detection kit (Invitrogen), diaminobenzidine (DAKO). Sections were counterstained with Brilliant cresyl blue (BCB). Double immunofluorescence staining was performed using mouse femur frozen tissue sections fixed in cold acetone. In brief, after blocking with BSA 1%, the tissue was incubated with the primary antibodies as follows: mouse anti-RUNX2 (Abcam), rabbit anti-mGlu1 receptor, (Novus Biologicals), and rat anti-CD68 (Serotec), followed by fluorescently labeled secondary antibodies Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 546 goat anti-mouse IgG, and Alexa Fluor 546 anti- rat IgG (Molecular Probes). For osteoblasts and osteoclasts count, experiments were conducted on 3 mice replicates WT and $Grm1^{crv4/crv4}$, taking 2 bone diaphysis and 2 epiphysis for each condition, and using 10 ROIs for each image.

For immunocytochemistry, the cells fixed in acetone were incubated with the primary antibody, a rabbit anti-collagen I (ab292 ABCAM), and Alexa Fluor 546 goat anti-rabbit IgG highly cross adsorbed (Invitrogen) as a secondary rhodamine-labelled antibody.

Images were acquired using a Zeiss Axioscope 40FL microscope, equipped with AxioCam MRc5 digital videocamera and immunofluorescence apparatus and AxioVision software 4.3 (Carl Zeiss SpA, Arese, Mi, Italy).

2.7 ALP and TRACP staining

The activity staining of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRACP) was carried out separately on mouse femur frozen tissue sections using a commercially available kit (Takara Bio) in which chromogenic substrates for alkaline phosphatase, an enzyme marker of osteoblasts, and tartrate-resistant acid phosphatase, an enzyme marker of osteoclasts, are used. Briefly, after fixation with 45% citrate buffer and 10% acetone, the enzyme substrates were added on separate slides and incubated at 37°C.

2.8 Blood and Serum Biochemistry

Blood samples were obtained from each mouse through retro-orbital veins. Baseline serum parameters were evaluated in 20 weeks old WT and *Grm1$^{crv4/crv4}$* mice. Murine C-terminal telopeptides of collagen type I (CTX-I) were determined in serum by using the commercial kit Ratlaps EIA (Immunodiagnostic systems Ltd, Frankfurt am Maim, Germany). 1,25-Dihydroxy Vitamin D was purified and determined in serum by using the IDS 1,25-Dihydroxy Vitamin D (Immunodiagnostic systems Ltd) commercial kit. Parathyroid hormone (PTH) was measured by the Mouse PTH 1-84 ELISA Kit (Immutopics, San Clemente, CA, USA). Calcium and phosphate were determined in plasma by using a COBAS C600 (Roche, Milan, Italy) analyzer.

2.9 Osteoblast cultures and treatments

Primary osteoblasts were isolated from 4 weeks old mice of the three genotypes (WT, *Grm1$^{crv4/+}$* and *Grm1$^{crv4/crv4}$*), according to a well-established procedure [25]. Briefly, calvaria were removed, parietal bones were cut in two halves, and subjected to four sequential digestion steps (15 min each, at 37 °C) in PBS with 0.05 % CollagenaseP (Roche) and 0.1 % Trypsin (Thermo Fisher Scientific, MA, USA). Cells from the first digestion were discarded, cells from the 2nd to the 4th digestion were placed in alpha-MEM medium (Thermo Fisher Scientific) supplemented with 10 % FBS and 1 % streptomycin/penicillin, 50 μg/mL ascorbic acid and 3 mM glycerol 2-phosphate disodium salt hydrate (Sigma- Aldrich, Milan, Italy). After 24 h medium was changed, thereafter, medium was changed every other day. When cells reached confluence, they were trypsinised and seeded at a density of 10,000 cells/cm^2 to get second passage cells. Cells were monitored daily to assess morphology, and experiments performed after 10 days of cultures. Notably, mGlu1 receptor expression is retained by WT cells (RT–PCR method described in the Supporting information and results shown in Supplementary figure 4). Cell viability was assessed by Trypan blue uptake (Trypan Blue Solution, Sigma-Aldrich) in three wells per genotype after 24, 48 and 72 hours after plating. In order to assess DNA synthesis and cell proliferation, BrdU incorporation was measured using the BrdU cell Proliferation assay kit (Cell Signaling Technology, Danvers, Massachusetts, USA)

according to the manufacturer's instructions. Twenty-four hours after plating, BrdU reagent was added to the wells and incubated for 24 h, 48 h or 72 h. Three wells per genotype per time point were analysed.

Pharmacological inhibition of mGlu1 receptors in osteoblasts was also performed. Osteoblasts isolated from WT animals were treated with 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt)(Hello-bio, Portishead, Bristol, UK), a known selective mGlu1 receptor antagonist [20], at a final concentration of 10 μM in 0.01% DMSO or with vehicle (DMSO 0.01%) only. Treatment was repeated on alternate days for a total of 10 days, when osteoblasts reached confluence. All experiments were performed in triplicate.

2.10 Real-time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) assay

Total RNA was extracted using Trizol (Invitrogen) from osteoblasts kept in culture for 10 days, as described above. cDNA was prepared from 3μg RNA using the iScript Select cDNA Synthesis Kit and oligo(dt)20 primers (Bio-Rad, Segrate, Milan, Italy). Gene expression levels were quantified using gene-specific primers (Supplementary file). Real Time RT-PCR was run with iQ Sybr Green Supermix (Bio-Rad) on a MyIQ instrument (Bio-Rad) and data were analysed by the IQ5 Bio-Rad Software.

The relative expression levels of mRNA from $Grm1^{crv4/crv4}$ osteoblast cells were calculated using the ΔΔCt method [26] normalizing to Gapdh and relative to WT cells. Three replica *per* mouse, and three mice *per* genotype were analysed.

Analogously, the relative expression levels of mRNA from CPCCOEt treated osteoblast cells were obtained using the ΔΔCt method [26] normalizing to Gapdh and relative to DMSO treated cells.

2.11 Alizarin red staining and extraction methods

Alizarin red staining solution (1 mg/mL, pH 5.5, Sigma-Aldrich) was added to ethanol-fixed osteoblasts then cells were destained by 5% perchloric acid. Optical density of the supernatant was evaluated by spectrophotometry at 450 nm. To normalize, Yanus Green Whole-cell Stain was added for 10min followed by Elution buffer and absorbance was read at 615nm. All experiments were

carried out using 3 replicates per each treatment.

2.12 ALP activity

To evaluate ALP activity in cultured cells, the medium was removed, the wells rinsed once with PBS, and 0.2 ml p-nitrophenyl phosphate (pNPP) (Sigma-Aldrich) was added to the wells and incubated for 30 seconds at room temperature in the dark. The reaction was stopped by the addition of 3 N NaOH and the sample was analyzed at 405 nm by the GloMax-Multi Detection System (Promega, Madison, WI, USA). The amount of protein was determined using a Pierce BCA Protein Assay kit (Thermo-Fischer Scientific), and the absorbance was read at 560 nm with the GloMax-Multi Detection System. ALP activity was defined by dividing the values from the pNPP assay by the values from BCA total protein to represent normalized alkaline phosphatase activity per unit total protein.

2.13 Sirius Red staining

Osteoblast cells were fixed in 4 % paraformaldehyde, then Sirius red solution (Polysciences, inc, Warrington, PA, USA) was added and washed with tap water after 1 hour incubation. Cells were destained by NaOH 0.1M. Optical density of the supernatant was evaluated by spectrophotometry at 540 nm wavelength. To normalize, Yanus Green Whole-cell Stain (Fisher Scientific, Rodano Milan, Italy) was added for 10min followed by Elution buffer and absorbance was read at 595 nm. All experiments were carried out using 3 replicates per each mouse, and three mice per genotype.

2.14 Statistics

Analysis of variance (ANOVA) was used for continuous, normally-distributed variables; Tukey post-hoc test was used to determine significance between multiple groups. Comparisons between two groups were carried out using an unpaired, two-tailed Student's t test. The Kruskal Wallis nonparametric test was used when the normality assumption was violated, in which case the Wilcoxon-Mann-Whitney test was used for pairwise comparisons. Between subjects t-test using Levine's test for equality of variances was used to assess differences in bone parameters. For all statistical analyses, a p-value less than 0.05 was considered to be statistically significant.

3. Results

3.1 Grm1$^{crv4/crv4}$ mice exhibit impaired skeletal development in vivo compared to WT animals

The body weight of homozygous mutant mice was reduced with respect to that of WT mice of about 25%. Analogously, body length of *Grm1$^{crv4/crv4}$* mice was about 10% shorter than that of WT mice. Heterozygous *Grm1$^{crv4/+}$* did not exhibit any differences in weight or length compared to that of WT animals (Figure 1 A-C, Supplementary table 1 and Supplementary table 2).

Analysis performed on sex-matched WT, *Grm1$^{crv4/+}$* and *Grm1$^{crv4/crv4}$* mice of pooled ages (12 – 28 weeks) showed a statistically significant decrease of femoral length both in female and in male *Grm1$^{crv4/crv4}$* mice when compared to WT females and males (Figure 1D). Femur length of *Grm1$^{crv4/crv4}$* female and male mice was shorter also with respect to heterozygous *Grm1$^{crv4/+}$* female and male counterparts (Figure 1D). Tibial length of the homozygous mutant female and male mice was shorter than that of WT females and WT males, respectively, the difference being statistically significant only in female animals (Figure 1D). Femoral length and tibia length of heterozygous *Grm1$^{crv4/+}$* mice did not differ statistically from those of WT animals. Indeed, anterior-posterior femoral width was reduced in female and not in male *Grm1$^{crv4/crv4}$* mice relative to heterozygous and WT animals (Supplementary Figure 1A, 1B).

Bone mineral density of male *Grm1$^{crv4/crv4}$* mice showed a statistically significant reduction when compared to WT and *Grm1$^{crv4/+}$* mice (Figure 1E). Similarly, bone surface density of male *Grm1$^{crv4/crv4}$* mice was significantly reduced with respect to that of WT and of *Grm1$^{crv4/+}$* mice (Figure 1E). In the female animals no difference in bone mineral density and bone surface density could be detected when mice of pooled ages (12 – 28 weeks) were analysed (Supplementary Figure 1C, 1D). When only animals of 24 weeks of age were considered, a statistically significant reduction in bone mineral density of female *Grm1$^{crv4/crv4}$* with respect to WT mice and, accordingly, bone surface density in *Grm1$^{crv4/crv4}$* with respect to WT mice was observed (Figure 1E). There was no statistically significant difference of either bone mineral density or surface density between heterozygous *Grm1$^{crv4/+}$* and WT mice (not shown).

Levine's test for equality of variances showed no statistically significant difference in tibia length (mm) (t(4) = 2.026, p = 0.113), bone mineral density (g/cm^3) (t(4) = 0.961, p = 0.391) and bone surface density (g/cm^2) (t(3) = 1.744, p = 180) of WT male mice of 12 and 20 weeks age. Similarly, no statistically significant difference was detected in tibia length (mm) (t(4) = 0.210, p = 0.844), bone mineral density (g/cm^3) (t(4) = 1.273, p = 0.272) and bone surface density (g/cm^2) (t(4) = 1.626, p = 0.179) of WT female mice of 12 and 24 weeks old.

All together, these observations indicate growth retardation and bone mineralization defects in *Grm1*$^{crv4/crv4}$ mice, which were not present in heterozygous *Grm1*$^{crv4/+}$ animals.

H&E staining of the hind limbs of 12 weeks old WT mice (Supplementary Figure 2A-C) and *Grm1*$^{crv4/crv4}$ mice (Supplementary Figure 2D-F) showed that the growth plate in the distal epiphysis of the femora was active at this age. However, when observing the 24 weeks old *Grm1*$^{crv4/crv4}$ mice (Figure 2A and Supplementary Figure 2J-L), it was noted that the process by which chondrocytes are embedded into calcified matrix was completed and thereby the growth plate transformed into epiphyseal line, whereas in the 24 weeks WT mice epiphysis (Figure 2A and Supplementary Figure 2G-I) secondary ossification centers were still present, osteoblasts were still invading the cartilage matrix, causing ossification, indicating that the epiphyseal line has not yet fused. Thus, longitudinal bone growth is still an on-going process in 24 weeks old WT mice, while in the 24 weeks old *Grm1*$^{crv4/crv4}$ mice the growth plate fused, ceasing the longitudinal bone growth. Overall, these data suggest that bone growth terminates earlier in the *Grm1*$^{crv4/crv4}$ than in the WT mice.

We wondered if this observation was associated to an imbalance in the number of osteoblasts/osteoclasts in the mutated bone tissues. By using enzymatic histochemistry we evaluated the presence of ALP-positive osteoblasts and TRACP-positive osteoclasts at bone epiphysis of WT and *Grm1*$^{crv4/crv4}$ mice. This double staining analysis did not reveal any difference in number and/or distribution of osteoblasts and osteoclasts in the mutated with respect to the WT tissues (Figure 2B).

Indeed, we performed double immunofluorescence analyses with antibodies specific for Runx2, as marker for osteoblasts, and the mGlu1 receptor in diaphysis (Figure 3A-C). As expected, Runx2 and mGluR1 double positive cells were clearly observable in the wild type tissues and absent in the mutated bone. To note, no difference in the number of osteoblasts (Runx2-positive cells) was observable between mutated and wild type tissues. We than analysed bone epiphysis of WT and mutated mice by double immunofluorescence using antibodies specific for the osteoclast marker, CD68, and for the mGlu1 receptor (Figure 2D-G). No difference in the number of osteoclasts (CD68-positive cells) was observable between mutated and wild type tissues. Of note, a few CD68-expressing osteoclasts also expressed mGlu1 receptors (Figure 2E).

We next carried out histomorphometry on toluidine blue-stained bone sections from 6-week-old wild type and Grm1$^{crv4/crv4}$ mice (Figure 4A-B). Here, we observed a moderate enrichment of the bone surface and of the trabecular number in the mutated relative to wild type animals.

We than investigated whether the bone formation could be different in the affected animals (Figure 4C-D). By 20 weeks, MAR and BFR/BS were both significantly decreased (MAR: 34%; BFR/BS: 40%) in the femora of Grm1$^{crv4/crv4}$ compared to those from WT mice.

3.2 No evident serum biochemical alterations are present in the Grm1$^{crv4/crv4}$ mice

Giving the renal impairment observed in *Grm1$^{crv4/crv4}$* mice [8], we measured a series of circulating markers to assess whether systemic effects could account for the skeletal observed abnormalities in these mice. Serum analyses of 10 animals of 24-weeks old, sex-matched WT and 10 *Grm1$^{crv4/crv4}$* mice demonstrated comparable serum levels of Calcium, Phosphorus, Magnesium and 1,25-dihydroxyvitamin D (Table1) therefore excluding any renal involvement in the observed phenotypic changes seen in mutant mice. In addition, serum levels of CTX (Table1) were also comparable between WT and *Grm1$^{crv4/crv4}$*. Due to renal dysfunction, it might have been possible that serum

calcium and 1,25-dihydroxyvitamin D levels were being maintained by elevated PTH, which would have skeletal effects. We thus evaluated serum PTH level in 10 WT and 10 *Grm1$^{crv4/crv4}$* mice and no statistically significant difference was evidenced (Supplementary figure 3).

3.3 Absence of mGlu1 receptor impairs osteoblast differentiation and mineralization in vitro

To test whether decreased bone formation was directly caused by absence of mGlu1 receptor in osteoblasts, we studied the effects of genetic inactivation of mGlu1 receptor on osteoblast differentiation and mineralization *in vitro*. Expression of mGlu1 receptor was confirmed in WT, but not in *Grm1$^{crv4/crv4}$* mouse tibia osteoblasts (Supporting information, Supplementary figure 4). Osteoblasts obtained from WT and *Grm1$^{crv4/crv4}$* showed similar level of cell survival and proliferation rate (Supplementary figure 5), however osteoblasts obtained from mutant mice showed impaired mineralization, compared to that observed in WT mouse osteoblasts (Figure 5). Alizarin staining of the bone matrix demonstrated that the matrix mineralization was abundant in WT cells while it significantly decreased in heterozygous *Grm1$^{crv4/+}$* cells ($p<0.05$), and was almost completely absent in *Grm1$^{crv4/crv4}$* cells ($p<0.01$) (Figure 5A-B). Accordingly, ALP activity was strongly reduced in *Grm1$^{crv4/crv4}$* cells with respect to WT cells ($p<0.05$) (Figure 3C).

As collagen is the major matrix component released from osteoblasts, we examined the possibility that the quantity of collagen released from *Grm1$^{crv4/crv4}$* mice osteoblasts was reduced with respect to the quantity released from WT cells. To this purpose, we first evaluated the SiriusRed staining, which specifically stains for collagen regardless of its different isoforms, and did not detect statistically significant differences between WT and mutated cells (Figure 5D). We then examined the expression of type I collagen, the prevalent component of bone matrix in homeostatic conditions, and found an abnormal distribution of type I collagen in the matrix of the mutant as compared to the WT cells (Figure 5E). Specifically, osteoblasts derived from *Grm1$^{crv4/crv4}$* mice showed a more scattered collagen distribution and less nodular appearance than those obtained from WT animals.

Next, to confirm a direct role of mGlu1 receptors in osteoblasts mineralization, we assessed the ability of WT osteoblasts to form mineralized matrix in the presence of a selective non-competitive antagonist of mGlu1 receptor, CPCCOEt. Matrix mineralization observed in treated mouse WT osteoblasts was significantly decreased compared to that seen in the presence of DMSO vehicle ($p < 0.01$) and to that of untreated control cells ($p < 0.05$). No statistically significant difference between untreated osteoblast control and DMSO-treated osteoblastic cells was observed (Figure 5F-G). Furthermore, qRT-PCR revealed a reduction of Collagen type 1 expression in osteoblasts treated with CPCCOEt when compared to control cells ($p < 0.05$) (Figure 5H), confirming that mGlu1 receptor activity is required for osteoblast homeostasis. Finally, we performed real-time qRT-PCR to evaluate whether any differences were present in the expression of collagen type 1, collagen type 3, and the osteoblast markers Runx2, Osterix (Sp7) and Osteocalcin (Bglap) at mRNA level (Figure 6). No differences were evident except for osteocalcin, which was significantly more expressed in osteoblasts of $Grm1^{crv4/crv4}$ with respect to WT cells.

4. Discussion

While it is well known that glutamate signaling pathways play a major role in the regulation of bone formation and dynamics, most studies have focused on ionotropic glutamate receptors. In contrast, the relevance of metabotropic glutamate signaling on bone mass regulation has not been investigated. In humans, *GRM1* inactivating mutations lead to a form of recessive ataxia known as *SCAR13* and, thus far, all patients reported to be affected by *SCAR13* exhibit below the normal range head circumference and weight, both at birth and later during infancy; indeed, they show short stature in adult age (143-154 cm) [27, 28]. Consistent with these findings, previously we have shown that mice in which *Grm1* is mutated exhibit cerebellar ataxia, impaired gait, kyphoskolyosis and reduced body weight and length compared to WT animals [18]. While the skeletal phenotype could be the direct consequence of both neuronal and renal abnormalities seen in these mice, as the bone is equipped

with all functional elements of the glutamate signaling, we hypothesized that the *GRM1* ablation could account for the skeletal phenotype both seen in *SCAR13* patients and in $Grm1^{crv4/crv4}$ mice. To test this hypothesis directly, we carried out a detailed characterisation of the skeletal phenotype of the $Grm1^{crv4/crv4}$ mice, specifically directed at testing the contribution that mGlu1 receptor makes to osteoblast function.

In adult female and male $Grm1^{crv4/crv4}$ mice we mainly observed reduced femoral length, reduced bone mineral density and bone surface density when compared to age-matched WT animals. Differences in bone mineral and bone surface density were predominantly visible in older mice, suggesting that the observed reduction in bone mass is age-related. Accordingly, $Grm1^{crv4/crv4}$ mice exhibited reduced mineralization and bone apposition rates compared to WT mice at 24 weeks of age.

Unlike in humans, in rodents the longitudinal bone growth continues even after they reach sexual maturity [29]. When analysing the morphology of the 12 and 24 weeks bone in $Grm1^{crv4/crv4}$ and WT mice, it was discovered that the growth plate, still very much active in both genotypes at 12 weeks of age, at 24 weeks of age in $Grm1^{crv4/crv4}$ mice the transformation of the growth plate into epiphyseal line was already completed while longitudinal bone growth in WT mice has still not ceased. Histomorphometry of the tibia from $Grm1^{crv4/crv4}$ mice indicated a slight increase of bone surface and trabecular number compared to WT mice at 6 weeks of age, features suggestive of enhanced bone maturation at this age. We performed toluidine blue staining to distinguish in epiphyseal plate metachromatic cartilage (blue dark border) from trabecular bone tissue; these data suggest that the absence of the mGlu1 receptor causes a premature cessation of the epiphyseal plate in the $Grm1^{crv4/crv4}$ mice, with subsequent reduction of bone length in the $Grm1^{crv4/crv4}$ compared to the WT mice.

It is well acknowledged that renal damage interferes with bone apposition, resulting in reduction of bone density and diameter. In particular, blood and urine sampled from patients with chronic renal failure present elevated levels of serum phosphorus and decreased levels of Ca^{2+} and vitamin D, feature markers that indicate the onset of bone dystrophy [30]. Of note, the $Grm1^{crv4/crv4}$ mice exhibit an impaired renal function [8], which is also necessary for the preservation of bone integrity.

However, blood analyses performed in the *Grm1^{crv4/crv4}* mice indicated normal levels of Ca^{2+}, Pi, vitamin D, and Mg compared to WT animals, thus excluding that the skeletal defects shown by the *Grm1^{crv4/crv4}* mice could be the direct consequence of their renal phenotype. Furthermore, by showing similar levels of PTH in mutant and WT mice, we can rule out the influence of this hormone on serum calcium and 1,25-dihydroxyvitamin D levels.

Motor impairment could instead interfere with bone apposition and with the remodelling cycle, and could hypothetically account for the observed skeletal phenotype. However, at 21 days of age *Grm1^{crv4/crv4}* mice already exhibited reduced body weight and length as compared to WT mice (data not shown). At this developmental stage, the skeletal tissue cannot be affected by the ataxic behaviour. Therefore, a direct role of motor impairment at the origin of the *Grm1^{crv4/crv4}* skeletal phenotype seems to be unlikely. Indeed, normal serum CTX levels in *Grm1^{crv4/crv4}* mice suggest that increased bone turnover was not a major cause for the skeletal phenotype of mutant mice.

Finally, we tested if a decreased activity of bone-forming osteoblasts is responsible for low bone mass in *Grm1^{crv4/crv4}* mice.

By *in vitro* experiments we showed that osteoblasts obtained from *Grm1^{crv4/crv4}* mice have the same survival and proliferation rate as the WT cells. Indeed, no differences in number and distribution of osteoblast cells were evident at the epiphysis of *Grm1^{crv4/crv4}* with respect to WT animals.

Noteworthy, osteoblasts obtained from mutated mice have a reduced ability to form mineralized matrix, when compared to WT cells. In addition, mineralized matrix production by WT osteoblasts was impaired when these cells were treated with a mGlu1 receptor specific inhibitor.

Differences between WT and *Grm1^{crv4/crv4}* mice in collagen release from osteoblasts cultures were not evident. At RNA level, collagen 1 and 3, the most expressed collagens in the bone matrix, do not seem to be more expressed in osteoblasts culture from WT than in *Grm1^{crv4/crv4}* mice cells, but immunostaining showed differences in collagen type I distribution, and a reduced production of collagen I was detected after mGlu1 receptor blockade by CPCCOEt. This first confirms that mGlu1 receptor activity is required for osteoblast functioning, and suggests that congenital absence of the

receptor might induce compensatory mechanisms. Actually, osteoblast cells obtained from $Grm1^{crv4/crv4}$ mice express higher levels of osteocalcin than those obtained from WT animals, suggesting a different maturation pattern. Indeed, osteocalcin is a marker of late osteoblast differentiation [31], which supports a more rapid maturation of mutated cells, and is in line with the observed premature cessation of the epiphyseal plate in the $Grm1^{crv4/crv4}$ mice in vivo.

Overall, our studies show that the impaired bone formation in the $Grm1^{crv4/crv4}$ mice is most likely explained by osteoblast malfunction. This finding is in agreement with the findings reported previously on the ability of osteoblasts to release and recycle glutamate [16, 17].

In the central nervous system, the mGlu1 receptor is primarily coupled to Gq/G11 protein and its activation leads to IP_3 formation and releases of Ca^{2+} from intracellular stores (reviewed in [1]). In primary culture of rat femoral osteoblasts, it has been suggested that activation mGlu1 receptors is linked to an elevation of intracellular Ca^{2+} levels [4]. Molecular mechanisms regulating intracellular calcium homeostasis are fundamental for adequate osteoblast function and differentiation. In the nervous system and in other non-neural tissues Group-I mGlu receptors also signal via activation of the MAPK and PI_3K pathways and ERK1/ERK2 [1], all of which are known important factors contributing to osteoblast function, as evidenced by fact that their genetic inactivation inhibits osteoblast differentiation [32]. Thus, activation of mGlu1 receptor in bone could also lead to activation of these signaling pathways and therefore play a crucial role in bone biology. Impairment of this process could contribute to the observed skeletal phenotype in patients affected by *GRM1* inactivating mutations and in $Grm1^{crv4/crv4}$ mice.

Finally, mGlu receptors are reported to have a role also in the control of neuroendocrine function [33], including modulatory effects on pituitary function [34].

Thus, inactivation of mGlu1 receptor may have many different effects in different tissues and organs, including bone, nervous system, kidney, which may contribute together to the complex phenotype observed in the $Grm1^{crv4}$ mice.

5. Conclusions

In summary, this work reveals a novel role for metabotropic glutamatergic signaling in bone. We show here that in the absence of mGlu1 receptors bone growth is altered, and that osteoblasts mGlu1 receptors are necessary for skeletal development, longitudinal bone growth and production of normal bone matrix. Further experimental work will be necessary to clarify the mechanisms linking mGlu1 receptors and bone physiology and to clarify to what extent mGlu1 receptors may contribute to bone formation and maintenance.

Author's contribution

M.P.R., D.R., and A.P. conceived and designed the experiments. I.M., L.A.O., D.M., S.B., C.G., G.C., Ch.G., M.I. and L.E. performed the experiments. G.C., P.M., R.R., Ch.G., M.P.R., D.R., D.M., and A.P. analysed and discussed the data. I.M., L.A.O., M.P.R., D.R., and A.P. wrote the paper.

Conflict of interest*:* None declared.

References

[1]   F. Nicoletti, J. Bockaert, G.L. Collingridge, P.J. Conn, F. Ferraguti, D.D. Schoepp, J.T. Wroblewski, J.P. Pin, Metabotropic glutamate receptors: from the workbench to the bedside, Neuropharmacology 60 (2011) 1017-41.

[2]   J.P. Pin, R. Duvoisin, The metabotropic glutamate receptors: structure and functions, Neuropharmacology 34 (1995) 1-26.

[3]   V. Musante, E. Neri, M. Feligioni, A. Puliti, M. Pedrazzi, V. Conti, C. Usai, A. Diaspro, R. Ravazzolo, J.M. Henley, Presynaptic mGlu1 and mGlu5 autoreceptors facilitate glutamate exocytosis from mouse cortical nerve endings, Neuropharmacology 55 (2008) 474-482.

[4]   Y. Gu, S.J. Publicover, Expression of functional metabotropic glutamate receptors in primary cultured rat osteoblasts. Cross-talk with N-methyl-D-aspartate receptors, J. Bio.l Chem. 275 (2000) 34252-9.

[5]   D.J. Mason, Glutamate signalling and its potential application to tissue engineering of bone, Eur. Cell. Mater. 7 (2004) 12-25; discussion 25-6.

[6]   R. Morimoto, S. Uehara, S. Yatsushiro, N. Juge, Z. Hua, S. Senoh, N. Echigo, M. Hayashi, T. Mizoguchi, T. Ninomiya, N. Udagawa, H. Omote, A. Yamamoto, R.H. Edwards, Y. Moriyama, Secretion of L-glutamate from osteoclasts through transcytosis, EMBO J 25 (2006) 4175-86.

[7]   F. Nicoletti, G. Battaglia, M. Storto, R.T. Ngomba, L. Iacovelli, A. Arcella, R. Gradini, P. Sale, L. Rampello, T. De Vita, R. Di Marco, D. Melchiorri, V. Bruno, Metabotropic glutamate receptors: beyond the regulation of synaptic transmission, Psychoneuroendocrinology 32 Suppl 1 (2007) S40-5.

[8]   A. Puliti, P.I. Rossi, G. Caridi, A. Corbelli, M. Ikehata, S. Armelloni, M. Li, C. Zennaro, V. Conti, C.M. Vaccari, M. Cassanello, M.G. Calevo, L. Emionite, R. Ravazzolo, M.P. Rastaldi, Albuminuria and glomerular damage in mice lacking the metabotropic glutamate receptor 1, Am. J. Pathol. 178 (2011) 1257-69.

[9]   M.R. Romano, L. Di Menna, P. Scarselli, G. Mascio, M. Madonna, S. Notartomaso, A. Puliti, V. Bruno, G. Battaglia, F. Nicoletti, Type-1, but Not Type-5, Metabotropic Glutamate Receptors are Coupled to Polyphosphoinositide Hydrolysis in the Retina, Neurochemical Research 41 (2016) 924-932.

[10]   M. Li, S. Armelloni, M. Ikehata, A. Corbelli, M. Pesaresi, N. Calvaresi, L. Giardino, D. Mattinzoli, F. Nistico, S. Andreoni, A. Puliti, R. Ravazzolo, G. Forloni, P. Messa, M.P. Rastaldi, Nephrin expression in adult rodent central nervous system and its interaction with glutamate receptors, J Pathol 225 (2011) 118-28.

[11]   C. Chenu, C.M. Serre, C. Raynal, B. Burt-Pichat, P.D. Delmas, Glutamate receptors are expressed by bone cells and are involved in bone resorption, Bone 22 (1998) 295-9.

[12] E. Hinoi, T. Takarada, K. Uno, M. Inoue, Y. Murafuji, Y. Yoneda, Glutamate suppresses osteoclastogenesis through the cystine/glutamate antiporter, Am. J. Pathol. 170 (2007) 1277-90.

[13] C. Itzstein, H. Cheynel, B. Burt-Pichat, B. Merle, L. Espinosa, P.D. Delmas, C. Chenu, Molecular identification of NMDA glutamate receptors expressed in bone cells, J Cell Biochem 82 (2001) 134-44.

[14] D.J. Mason, L.J. Suva, P.G. Genever, A.J. Patton, S. Steuckle, R.A. Hillam, T.M. Skerry, Mechanically regulated expression of a neural glutamate transporter in bone: a role for excitatory amino acids as osteotropic agents?, Bone 20 (1997) 199-205.

[15] A.J. Patton, P.G. Genever, M.A. Birch, L.J. Suva, T.M. Skerry, Expression of an N-methyl-D-aspartate-type receptor by human and rat osteoblasts and osteoclasts suggests a novel glutamate signaling pathway in bone, Bone 22 (1998) 645-9.

[16] R.W. Cowan, E.P. Seidlitz, G. Singh, Glutamate signaling in healthy and diseased bone, Front Endocrinol (Lausanne) 3 (2012) 89.

[17] T. Takarada, Y. Yoneda, Pharmacological topics of bone metabolism: glutamate as a signal mediator in bone, J Pharmacol Sci 106 (2008) 536-41.

[18] V. Conti, A. Aghaie, M. Cilli, N. Martin, G. Caridi, L. Musante, G. Candiano, M. Castagna, A. Fairen, R. Ravazzolo, crv4, a mouse model for human ataxia associated with kyphoscoliosis caused by an mRNA splicing mutation of the metabotropic glutamate receptor 1 (Grm1), International journal of molecular medicine 18 (2006) 593-600.

[19] P.I.A. Rossi, I. Musante, M. Summa, A. Pittaluga, L. Emionite, M. Ikehata, M.P. Rastaldi, R. Ravazzolo, A. Puliti, Compensatory Molecular and Functional Mechanisms in Nervous System of the Grm1(crv4) Mouse Lacking the mGlu1 Receptor: A Model for Motor Coordination Deficits, Cerebral Cortex 23 (2013) 2179-2189.

[20] V. Musante, M. Summa, E. Neri, A. Puliti, T.T. Godowicz, P. Severi, G. Battaglia, M. Raiteri, A. Pittaluga, The HIV-1 viral protein Tat increases glutamate and decreases GABA exocytosis from human and mouse neocortical nerve endings, Cerebral Cortex 20 (2010) 1974-1984.

[21] S.J. Gilbert, L.B. Meakin, C.S. Bonnet, M.A. Nowell, W.C. Ladiges, J. Morton, V.C. Duance, D.J. Mason, Deletion of P58(IPK), the Cellular Inhibitor of the Protein Kinases PKR and PERK, Causes Bone Changes and Joint Degeneration in Mice, Front Endocrinol (Lausanne) 5 (2014) 174.

[22] D.L. Vizard, D.O. Wood, R.V. Papineni, G.D. Feke, S.P. Orton, W.E. McLaughlin, Analytical radiography for planar radiographic images implemented with a multi-modal system, Comput Methods Programs Biomed 99 (2010) 88-97.

[23] B. Canciani, A. Ruggiu, A. Giuliani, D. Panetta, K. Marozzi, M. Tripodi, P.A. Salvadori, M. Cilli, Y. Ohira, R. Cancedda, S. Tavella, Effects of long time exposure to simulated micro- and hypergravity on skeletal architecture, J Mech Behav Biomed Mater 51 (2015) 1-12.

[24] A. Morse, M.M. McDonald, N.H. Kelly, K.M. Melville, A. Schindeler, I. Kramer, M. Kneissel, M.C. van der Meulen, D.G. Little, Mechanical load increases in bone formation via a sclerostin-independent pathway, J Bone Miner Res 29 (2014) 2456-67.

[25] D. Mattinzoli, P. Messa, A. Corbelli, M. Ikehata, C. Zennaro, S. Armelloni, M. Li, L. Giardino, M.P. Rastaldi, A novel model of in vitro osteocytogenesis induced by retinoic acid treatment, Eur. Cell. Mater. 24 (2012) 403-25.

[26] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method, Methods 25 (2001) 402-408.

[27] B. Davarniya, H. Hu, K. Kahrizi, L. Musante, Z. Fattahi, M. Hosseini, F. Maqsoud, R. Farajollahi, T.F. Wienker, H.H. Ropers, H. Najmabadi, The Role of a Novel TRMT1 Gene Mutation and Rare GRM1 Gene Defect in Intellectual Disability in Two Azeri Families, Plos One 10 (2015) e0129631.

[28] V. Guergueltcheva, D.N. Azmanov, D. Angelicheva, K.R. Smith, T. Chamova, L. Florez, M. Bynevelt, T. Nguyen, S. Cherninkova, V. Bojinova, A. Kaprelyan, L. Angelova, B. Morar, D. Chandler, R. Kaneva, M. Bahlo, I. Tournev, L. Kalaydjieva, Autosomal-recessive congenital cerebellar ataxia is caused by mutations in metabotropic glutamate receptor 1, Am J Hum Genet 91 (2012) 553-64.

[29] H.I. Roach, G. Mehta, R.O. Oreffo, N.M. Clarke, C. Cooper, Temporal analysis of rat growth plates: cessation of growth with age despite presence of a physis, J Histochem Cytochem 51 (2003) 373-83.

[30] S. Moe, T. Drueke, J. Cunningham, W. Goodman, K. Martin, K. Olgaard, S. Ott, S. Sprague, N. Lameire, G. Eknoyan, O. Kidney Disease: Improving Global, Definition, evaluation, and classification of renal osteodystrophy: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO), Kidney Int 69 (2006) 1945-53.

[31] T.A. Franz-Odendaal, B.K. Hall, P.E. Witten, Buried alive: how osteoblasts become osteocytes, Dev Dyn 235 (2006) 176-90.

[32] T. Matsushita, Y.Y. Chan, A. Kawanami, G. Balmes, G.E. Landreth, S. Murakami, Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis, Molecular and cellular biology 29 (2009) 5843-57.

[33] D. Durand, M. Pampillo, C. Caruso, M. Lasaga, Role of metabotropic glutamate receptors in the control of neuroendocrine function, Neuropharmacology 55 (2008) 577-83.

[34] M. Pampillo, T. Scimonelli, B.H. Duvilanski, M.E. Celis, A. Seilicovich, M. Lasaga, The activation of metabotropic glutamate receptors differentially affects GABA and alpha-melanocyte stimulating hormone release from the hypothalamus and the posterior pituitary of male rats, Neurosci Lett 327 (2002) 95-8.

Figure 1. *Grm1$^{crv4/crv4}$* mice exhibit reduced body size, shorter femur length and tibia length, reduced bone mineral density and reduced surface density compared to WT mice. (A) Images of *Grm1$^{crv4/crv4}$* (right) and wt (left) adult male mice from the same progeny. (B) Body weight and (C) body length are presented as mean±SEM of 20 animals (10 females and 10 males) for each genotype. (D) Femur length of females and males, and tibial length of females and males are presented as mean±SD of mice of pooled ages (12 – 28 weeks). (E) bone mineral density (BMD) and bone surface density (BSD) in males (male mice of pooled ages, 12 – 28 weeks) and in females (female mice of 24 weeks of age). Data are presented as mean±SD. $p<0.05$ (*); $p<0.01$ (**).

Figure 2. A. Haematoxylin and eosin (H&E) staining performed on 24 weeks old WT and *Grm1$^{crv4/crv4}$* mice. Staining of the hind limbs of 24 weeks old WT mice (right) show that longitudinal bone growth is still an on-going process in these mice, with secondary ossification centers present in the epiphysis. However, when observing the age-matched *Grm1$^{crv4/crv4}$* mice (left) it was noted that the growth plate fused ceasing the longitudinal bone growth in these mice, there do not appear to be any secondary ossification centers present in the epiphysis of these animals. Images of the growth plate/epiphyseal line were taken at x20 magnification and the scale bars represent 1mm. DC, differentiated chondrocytes; OM, ossified matrix, PC, proliferative chondrocytes, SOC, secondary ossification centres. **B**. Labeling of ALP and TRAP in WT and Grm1$^{crv4/crv4}$ epiphysis bone mice by enzymatic histochemistry. Arrows in the left panel indicate ALP-positive cells, osteoblasts. Arrows in the right panel indicate TRACP-positive cells, osteoclasts. The inset images were taken at 40X magnification and the scale bars represent 50μm.

Figure 3. Osteoblasts and osteoclasts in bone tissue of WT and Grm1$^{crv4/crv4}$ mice **A-B.** Immunofluorescence shows expression of the mGlu1 receptor (green) and Runx2 (red), a marker for osteoblasts, in the bone diaphysis of a wild-type (A) and of a *Grm1$^{crv4/crv4}$* (B) mouse. Runx2-expressing osteoblasts are observable in both WT and mutated tissues (arrows). Green-red double stained cells, indicating the presence of mGlu1 receptor-expressing osteoblasts, are observable in the WT tissue and absent in the mutated mouse bone tissue. **C.** Number of osteoblasts *per* bone surface in WT and mutated bone tissues are presented as mean±SD. **D-F.** Immunofluorescence shows expression of the mGlu1 receptor (green) and CD68 (red), a marker for osteoclasts, in the bone epiphysis of a WT (D-E) and of a *Grm1$^{crv4/crv4}$* (F) mouse. CD68-expressing osteoclasts are observable in both WT and mutated tissues (arrows). Few green-red double stained cells, indicating the presence of mGlu1 receptor-expressing osteoclasts, are observable in the WT tissue and absent in the mutated mouse bone tissue. **G.** Number of osteoclasts *per* bone surface in WT and mutated bone tissues are presented as mean±SD. The inset images were taken at 50X magnification and the scale bars represent 50μm.

Figure 4. Static and dynamic histomorphometry of WT and Grm1$^{crv4/crv4}$ bone tissues. (A) Toluidine blue-stained sections from tibiae of 6-week-old female mice. Scale bar, 200 μm. (B) Analysis of relative bone volume (BV/TV) and trabecular number (Tb.N) is shown. All values are mean ± SEM from three mice per group at 6 weeks of age. (C) Representative calcein-labeled sections of femur bone from WT and Grm1$^{crv4/crv4}$ male mice. Images were taken at x200 magnification. (D) Mineral apposition rate (MAR) and bone formation rate (BFR/BS) calculated from calcein double labelling are shown in WT and Grm1$^{crv4/crv4}$ mice. Bone formation rate is the amount of mineralised bone formed per unit of time per unit of bone surface. All values are mean ± SEM from three mice per group at 24 weeks of age. $p < 0.05$ (*) ; $p < 0.01$ (**).

Figure 5. Mineralization matrix defects are seen in mice lacking mGlu1 receptor. **A.** Light microscopy images of alizarin red staining of primary osteoblasts obtained from WT, *Grm1$^{crv4/+}$* and *Grm1$^{crv4/crv4}$* mice and (**B**) quantification of alizarin extracted from primary cells. **C.** ALP activity is

reduced in *Grm1^{crv4/crv4}* mice with respect to WT mice. **D**. Content of collagen measured by SiriusRed assay and normalized by Janus Green. **E**. Collagen I expression in primary osteoblasts obtained from WT and *Grm1^{crv4/crv4}* mice. Scale bar 50 µm. Magnification: 40X. **F**. Light microscopy images of alizarin red staining of primary osteoblasts obtained from WT mice, cultured with CPCCOEt, DMSO and control (CTRL) and (**G**) quantification of alizarin red extracted from primary cells. **H**. Collagen I mRNA expression of primary osteoblasts of WT mouse treated with CPCCOEt or DMSO (vehicle control). Bars are means ± SD. $p < 0.05$ (*); $p < 0.01$ (**).

Figure 6. Results of real-time qRT-PCR obtained from osteoblast cultures.