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Neuropeptide Y Y2 antagonist treated ovariectomized mice exhibit greater bone mineral density

K.L. Seldeen[b,e,1], P.G. Halley[a,1], C.H. Volmar[a], M.A. Rodríguez[c,f], M. Hernandez[c,f], M. Pang[b,e], S.K. Carlsson[a], L.J. Suva[d], C. Wahlestedt[a], B.R. Troen[b,e], S.P. Brothers[a,*]

[a] Center for Therapeutic Innovation, Department of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, Miami, FL, USA
[b] Division of Geriatrics and Palliative Medicine, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, NY, USA
[c] Bruce W. Carter VA Geriatric Research Education and Clinical Center (GRECC), Miami, FL, USA
[d] Department of Orthopaedic Surgery, Centre for Orthopaedic Research, University of Arkansas Medical School, Little Rock, AR, USA
[e] Research Service, Veterans Affairs Western New York Healthcare System, Buffalo, NY, USA
[f] University of Miami Miller School of Medicine, Miami, FL, USA

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ABSTRACT

Osteoporosis, a disease characterized by progressive bone loss and increased risk of fracture, often results from menopausal loss of estrogen in women. Neuropeptide Y has been shown to negatively regulate bone formation, with amygdala specific deletion of the Y2 receptor resulting in increased bone mass in mice. In this study, ovariectomized (OVX) mice were injected once daily with JNJ-31020028, a brain penetrant Y2 receptor small molecule antagonist to determine the effects on bone formation. Antagonist treated mice had reduced weight and showed increased whole-body bone mineral density compared to vehicle-injected mice. Micro computerized tomography (micro-CT) demonstrated increased vertebral trabecular bone volume, connectivity density and trabecular thickness. Femoral micro-CT analysis revealed increased bone volume within trabecular regions and greater trabecular number, without significant difference in other parameters or within cortical regions. A decrease was seen in serum P1NP, a measure used to confirm positive treatment outcomes in bisphosphonate treated patients. C-terminal telopeptide 1 (CTX-1), a blood biomarker of bone resorption, was decreased in treated animals. The higher bone mineral density observed following Y2 antagonist treatment, as determined by whole-body DEXA scanning, is indicative of either enhanced mineralization or reduced bone loss. Additionally, our findings that *ex vivo* treatment of bone marrow cells with the Y2 antagonist did not affect osteoblast and osteoclast formation suggests the inhibitor is not affecting these cells directly, and suggests a central role for compound action in this system. Our results support the involvement of Y2R signalling in bone metabolism and give credence to the hypothesis that selective pharmacological manipulation of Y2R may provide anabolic benefits for treating osteoporosis.

1. Introduction

In adults, bone remodeling is necessary to maintain the shape, structure and strength of bone in response to mechanical stress. This homeostatic process occurs when bone tissue is broken down by resorptive osteoclasts and is replaced by bone-forming osteoblasts (Aguila and Rowe, 2005; Raggatt and Partridge, 2010). However, a greater rate of bone resorption and/or a decreased rate of bone formation result in osteoporosis, a disease characterized by progressive bone loss and increased risk of fracture (Duque et al., 2009; Raisz, 1999, 2005; Troen, 2003). Osteoporosis is most prevalent in post-menopausal women, and estrogen has been shown to prevent bone resorption by inhibiting osteoclast activity (Jilka et al., 1992; Kameda et al., 1997; Troen, 2003).

NPY is processed from a 94–95-amino acid prohormone and has been classified into the same family as peptide YY and pancreatic polypeptide (Balasubramaniam, 1997; Tatemoto et al., 1982). Since its original isolation, it has been linked to numerous processes such as inhibiting anxiety and depression (Heilig, 2004), regulating learning-associated synaptic plasticity (Redrobe et al., 2004; Thorsell et al., 2000), and modulating circadian rhythm (Medanic and Gillette, 1993; Yannielli and Harrington, 2001), vascular smooth muscle contraction (Glenn and Duckles, 1994; Pernow and Lundberg, 1988) and pain

* Corresponding author at: Center for Therapeutic Innovation (CTI), Dept. of Psychiatry and Behavioral Sciences, University of Miami Miller School of Medicine, 1501 NW 10th Ave, BRB #416 (M860), Miami, FL 33136, USA.
E-mail address: sbrothers@med.miami.edu (S.P. Brothers).
[1] These authors contributed equally to this work.

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transmission (Naveilhan et al., 2001; Solway et al., 2011). In humans, NPY mediates its effects by interacting with a diverse family of G-protein coupled receptors (Y1, Y2, Y4, Y5), which are highly expressed across a number of different brain regions and throughout the sympathetic nervous system (Blomqvist and Herzog, 1997; Brothers and Wahlestedt, 2010; Fetissov et al., 2004b; Jacques et al., 1997; Nozdrachev and Masliukov, 2011; Redrobe et al., 2002). While Y1, Y4 and Y5 receptors are generally considered to be post-synaptically expressed, Y2 receptors (Y2R) are primary located pre-synaptically and function as autoreceptors to inhibit NPY release, along with other neurotransmitters such as noradrenaline, glutamate and GABA (Chen and van den Pol, 1996; Chen et al., 1997; King et al., 1999; Martire et al., 1995; Silva et al., 2007; Wahlestedt et al., 1986). Importantly, Y2 receptors are highly abundant in the hypothalamus, where they regulate body weight, feeding, and energy homeostasis (Baldock et al., 2007; Fetissov et al., 2004a; Sainsbury et al., 2002; Sainsbury and Zhang, 2010).

Over the last decade, there has been an increasing body of experimental evidence to suggest that bone remodeling is under central, hypothalamic and amygdalar regulation (for review see (Driessler and Baldock, 2010)). Results from a number of studies, using transgenic mouse models, now contradict the traditional view that bone formation is primarily controlled by autocrine or paracrine mechanisms (Shi and Baldock, 2012). Altering the signalling of neuropeptide Y (NPY), a 36-amino acid peptide that primarily acts as a neurotransmitter in the brain and autonomic nervous system, can also independently affect bone physiology (Gu et al., 2016; Liu et al., 2016; Xiao et al., 2016; Baldock et al., 2002, 2005, 2006; Dumont et al., 1992; Heilig and Widerlov, 1995). A role for NPY Y2 receptors in bone metabolism was first reported by Baldock and colleagues, who demonstrated increased trabecular bone volume, trabecular number and trabecular thickness in germ line Y2R knockout mice compared to wild-type control animals (Baldock et al., 2002). Y2 receptors are not apparently present in either wild-type mouse bone tissue including osteoblasts, thus excluding the possibility that these changes in bone structure were the result of direct Y2R-mediated effects on bone cells. A central mechanism was confirmed, as selective deletion of hypothalamic Y2 receptors in adult mice also led to an identical two-fold increase in bone volume, relative to experimental control animals (Baldock et al., 2002). Indeed, similar enhancement of bone mass and bone formation has been observed in subsequent studies, thus positioning the Y2 receptor as a potential drug target for enhancing bone formation (Baldock et al., 2006; Lundberg et al., 2007). However, it has also been shown that germ line NPY Y1 receptor knockout mice display a comparable increase in cancellous bone volume, along with greater trabecular number and thickness (Baldock et al., 2007). In contrast, these effects were not centrally mediated, as selective knockout of hypothalamic Y1 receptors did not recapitulate this phenotype (Baldock et al., 2007). Instead the increase in bone volume appeared to be the result of altered direct NPY activity on Y1 receptors expressed on osteoblasts (Baldock et al., 2007). Reduced Y1 receptor expression has also been seen in bone stromal cells isolated from Y2R knockout mice (Lundberg et al., 2007), possibly an adaptive response to an increase in NPY due a complete lack of Y2R-mediated feedback. More recently, a novel inducible Y2 receptor system was used to show central disruption of Y2 signalling in the brain decreases bone mineral content (Qi et al., 2016).

Experimental approaches using selective Y2 receptor antagonists may help to further elucidate this process by minimizing possible complications associated with chronic gene deletion, such as compensatory signalling responses. One peptidomimmetic Y2 receptor antagonist, BIIE0246, has been used in this capacity in a limited fashion in the past. However, with the emerging evidence of several off-target impacts, lack of brain penetration and the fact that BIIE0246 is an irreversible inhibitor, such studies have had mixed results (Brothers et al., 2010). Traditionally, NPY receptor selective small molecule drugs were difficult to develop due to lack of good screening systems and functional Y2-assays, although, in recent years, an increasing number of small molecule antagonists have been characterized (Bonaventure et al., 2004; Brothers et al., 2010; Doods et al., 1999; Grouzmann et al., 1997). Of these, JNJ-31020028 is a highly selective Y2-antagonist being sufficiently brain penetrant to achieve very high central receptor inhibition (Cippitelli et al., 2011; Shoblock et al., 2010; Swanson et al., 2011). To assess whether central Y2 receptor inhibition represents a potential therapeutic approach for the treatment of osteoporosis, we examined the effects of systemic Y2 receptor antagonism on bone mass in adult female ovariectomized (OVX) mice, a model of post-menopause characterized by osteopenia and osteoporosis phenotypes.

2. Materials and methods

2.1. Measurement of JNJ-31020028 activity in cell lines

The cAMP biosensor assay was performed as previously described (Brothers et al., 2010; Saldanha et al., 2010). Briefly human embryonic kidney (HEK) 293 cells stably expressing a cyclic nucleotide-gated (CNG) channel and either Y2R or Y1R were cultured in T-175-cm^2 flasks at 37 °C and 95% relative humidity. Cells were plated and maintained in growth medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 5 mM L-glutamine, 250 μg/ml G418 (Geneticin), 1 μg/ml puromycin, and 1% antibiotic mix containing penicillin, streptomycin, and neomycin. A 10-point dose-response curve with a 1:3 dilution series was used, with compounds being tested in quadruplicate for each point. For JNJ-31020028 and BIIE0246 (Y2R) or BIBP3226 (Y1R), cells were simultaneously treated with NPY peptide at a concentration of 100 nM for all points (antagonist mode).

2.2. Animals

All animal work was carried out according to protocols reviewed and approved by University of Miami Institutional Animal Care and Use Committee. Ovariectomized female C57BL/6J mice, 10 weeks of age, were purchased from Jackson Laboratories (Stock 000664; JAX Mice and Services, Bar Harbor, Maine, USA). The mice were housed in groups of five animals per standard open-top mouse cage, and maintained on a regular chow diet and tap water. Both food and water were available *ad libitum*.

JNJ-31020028 was synthesized as described in (Shoblock et al., 2010; Swanson et al., 2011) by Reagent 4 Research, LLC (Hangzhou, China) and suspended in a 10% (v/v) vehicle (10% DMSO in saline). All mice received a daily intraperitoneal injection of either JNJ-31020028 (10 mg/kg) or vehicle control, in a final volume of 100 μl, for eight weeks (n = 10 per treatment group). 10 mg/kg is a dose that is expected to have > 90% receptor occupancy in rodent brains (Shoblock et al., 2010). During this treatment period, weights and food intake were recorded every three days. 24 h after the last injection the mice were anesthetised and whole blood was collected (6% EDTA) by cardiac puncture, immediately following this the mice were culled for follow up experiments. Blood plasma was isolated by centrifugation at 1000 × g for 10 min. Percent weight was calculated on a per animal basis for each day. The initial weights were set at 100% and each subsequent weighing was calculated as a percentage of the baseline weight. Percent weight change was calculated by subtracting 100% from the calculated % weight on each treatment day. A negative number indicates weight loss and a positive number indicates weight gain.

2.3. Dual-energy X-ray absorptiometry

Following sacrifice mice were positioned onto sample trays for analysis. Total body BMD, total body bone mineral content (BMC), body fat % and lean mass were determined using a Lunar PIXImus II (GE

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