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Osteocalcin is a stress-responsive neuropeptide

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Abstract. Osteocalcin (OC) is a small, acidic extracellular protein synthesized by osteoblasts during bone formation. 3 residues of gamma-carboxy glutamic acid, formed in a vitamin K dependent process, enable highly specific binding to ionic or bone mineral calcium. Some OC is released to circulation (pOC) and can serve as a biomarker of bone turnover. A series of experiments indicated that OC is stress-responsive in ways that vary with the type of stressor. Those in which the HPA axis predominates slowly decrease OC synthesis and secretion while sympathetic neural activation rapidly increases pOC. The advent of an OC null mutant mouse (KO) led to discovery of several functions for the protein outside the skeleton, most notably in regulation of energy metabolism. The KO mouse also exhibits numerous behavioral traits that are characteristic of sensory impairment. The discovery of OC protein in sensory ganglia stimulated further investigation of the interaction of sensory responses and both OC gene expression and OC protein in trigeminal and dorsal root ganglia. A recently discovered G-coupled protein receptor has been suggested as a potential OC receptor in combination with calcium ions. Because of the importance of ionic calcium to signal transduction in the nervous system, the presence of this unique calcium binding protein in neurons led to the hypothesis that OC functions as a neuropeptide. Implications of this potential new function are discussed.

Keywords. calcium binding protein, ionized calcium, gamma-carboxy glutamic acid, trigeminal ganglia, dorsal root ganglia, uncarboxylated osteocalcin, GPRC6A

Researchers studying "Catecholamines and Other Neurotransmitters in Stress" are not likely to consider the skeleton as an endocrine organ or as the possible origin of anything relevant to their interests. However, as we first proposed in 1995, osteocalcin (OC), widely believed to be primarily a bone protein, might be a stress hormone (Patterson-Buckendahl et al., 1996). Indeed, there are increasing data to support extra-skeletal roles for osteocalcin (OC). For those outside the field of bone research, some background information is needed.

What is osteocalcin?

Osteocalcin (also known as bone-Gla protein or BGP) is a small (44 to 50 amino acid residues), acidic, calcium-binding protein found primarily in bone. It was first identified following the discovery that prothrombin activity required the vitamin K-dependent post-translational modification of several glutamic acid residues to gamma-carboxy glutamic acid (Gla) for calcium binding to initiate the blood clotting cascade (Nelsestuen and Suttie 1973; Stenflo 1974; Stenflo et al. 1974). Given that most of the calcium in the vertebrate body is in the skeleton, the next logical place to look for Gla was in bone. Hauschka et al. (1975) and Price et al. (1976) reported the presence of a small protein, 45-50 amino acids, including 3 Gla residues that enabled highly specific binding to the hydroxyapatite mineral crystal as it was deposited during bone formation by osteoblasts. The OC amino acid sequence, especially in the Gla region, is highly conserved across more than 20 vertebrate species

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studied to date, ranging from fish to human (Cancela et al. 1995). Interestingly, the elasmobranch fish (sharks and rays) do not express the protein, and do not have hydroxyapatite in their cartilaginous skeletons.

Among the characteristics of OC are (1) its inhibition of mineral crystal growth in super-saturated calcium phosphate solutions suggesting regulation of mineral deposition, and (2) chemo-attraction of human peripheral blood monocytes and related polymorphonuclear leucocytes involved in bone resorption. In addition, subcutaneous implants of devitalized bone particles from rats depleted of OC by treatment with warfarin, a vitamin K inhibitor, were poorly reabsorbed, suggesting a requirement for OC to recruit bone resorbing osteoclasts (Hauschka et al. 1989).

Although most OC is incorporated into bone as a stable component of the protein matrix, a relatively constant portion of newly synthesized OC (pOC, about 10 to 30% depending on age) is immediately released to circulation rather than being incorporated into bone (Hauschka et al. 1989). This pOC has been well correlated with histological indices of osteoblastic activity and is useful as a biomarker of bone turnover (Gundberg 2000).

Osteocalcin synthesis is highly regulated

Virtually anything that affects bone metabolism, whether age, gender, exercise, weight bearing or disuse, pharmacological agents, disease, etc, is likely to have an effect on OC. This led to the assumption that OC functioned primarily in bone; thus, initial research focused on conditions affecting bone metabolism. Most endocrine and signaling systems known to influence bone also regulate OC synthesis and/or release directly or indirectly, including vitamin D (Hauschka and Reid 1978), parathyroid hormone and glucocorticoids (Beresford et al. 1984; Morrison et al. 1989) the pro-inflammatory cytokines IL-6 and TNF-alpha (Li and Stashenko 1992, 1993) and cyclic-AMP (Theofan and Price 1989). Lian et al. (1998) have extensively studied the promoter region of the rat osteocalcin gene, finding many regulatory elements that control suppression of the gene in osteoprogenitor cells and others that lead to development of the osteoblast phenotype. The mouse osteocalcin promoter region also contains transcriptional factors that regulate osteoblast differentiation and bone formation (Xiao et al. 2005; Yu et al. 2008).

Similar to many hormones, pOC exhibits a circadian rhythm, although this may reflect the rhythmicity of overall bone metabolism. That of OC is associated with the well-known rhythm of glucocorticoids, but in an inverse pattern delayed by approximately 2 hours. There is a further circadian relationship between plasma OC, ionized calcium, and parathyroid hormone (Nielsen et al. 1991). The circadian rhythmicity, small size, short half-life (5 to 20 minutes depending on species), control of synthesis, concentration in plasma, and conserved sequence of OC are characteristics of a hormone. Although an OC-specific receptor has yet to be conclusively identified, there is a strong candidate among the family C, G protein-coupled receptors. This protein, termed GPRC6A, is closely related to the better known calcium-sensing receptor first discovered in parathyroid glands (CASR), but is also activated by OC. GPRC6A is broadly expressed in many tissues including brain and bone (Pi et al. 2005).

Osteocalcin is stress responsive

Prolonged and intense sensory input induces the physiological response that we know as "stress". Physiological mechanisms have evolved for coping with various external stimuli such as temperature change, pheromones, strange environments, painful injury, annoying noises or vibrations that may stimulate metabolic but not "fight-or-flight" responses. These stimuli must first be sensed by well-known means (sight, hearing, touch, smell, taste, etc.) and then interpreted and translated to action.

Because glucocorticoids inhibit transcription of the OC gene (Morrison et al. 1989), it is a prime target for the effects of increased levels of glucocorticoids during stress. Indeed, some changes observed in pOC in both humans and experimental animals were associated with environmental conditions considered to be emotionally or mentally stressful, resulting in alertness and increased anxiety but not alarm responses, and inducing an elevation of glucocorticoids. Common experimental conditions experienced by both wild and laboratory animals, including transfer to a novel environment, sudden noise, vibration, cold exposure, water immersion, and light cycle disruption have been shown to induce elevations of rat corticosterone. We observed that some of these induced a decrease of pOC of up to 25 % compared to non-stressed rats. The alteration of pOC could be replicated by administration of physiological to low pharmacological doses of corticosterone in a time and dose related fashion. Furthermore, adrenalectomy resulted in a marked increase in plasma OC of close to 40%

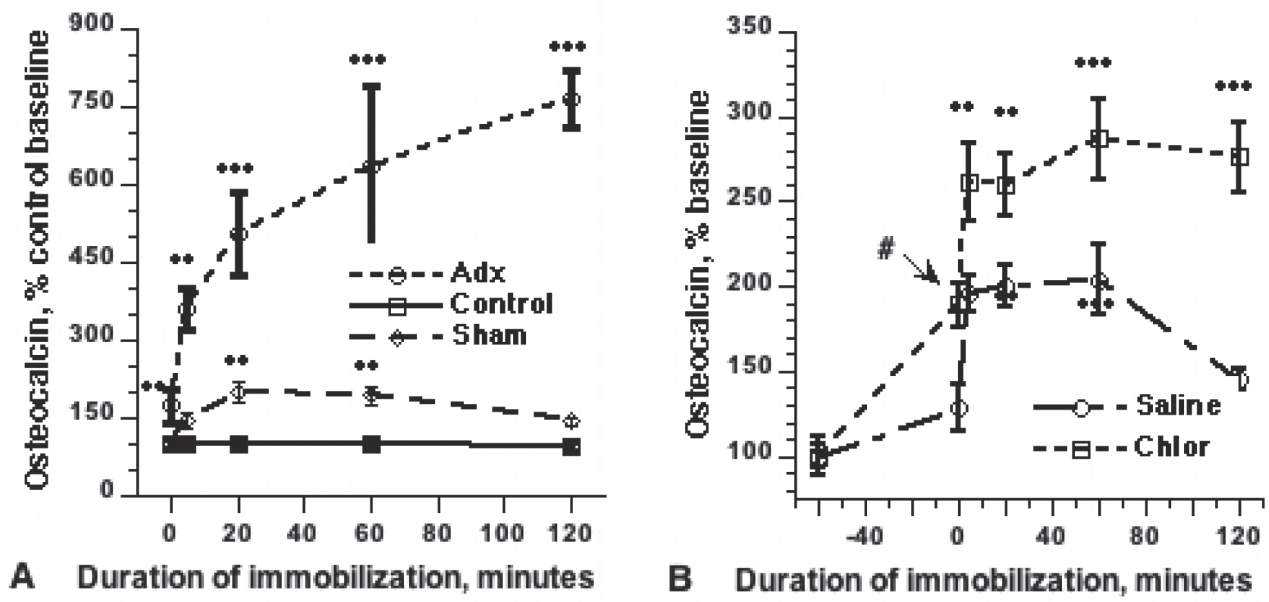

Fig. 1. (A) The effect Immo on plasma osteocalcin in adrenalectomized and sham operated rats, compared to non-Immo control rats.
(B) Effect of administration of saline or chlorisondamine, a blocker of ganglionic transmission, one hour prior to Immo. Data are expressed as a percent of baseline level for each animal. Note difference in scale between A and B. ** and * indicate significant differences from pre-Immo levels of p<0.01 and p<0.001 respectively; # indicates effect of chlorisondamine prior to Immo.**

(Patterson-Buckendahl et al. 1988). The data implied that activation of the hypothalamic-pituitary-adrenal axis by stressful environmental stimuli classified as anxiety producing exerted negative control of plasma OC.

In sharp contrast, fight-or-flight activation of the sympathetic nervous system, modeled by foot restraint immobilization (Immo), is a well characterized model of acute, severe stress developed by Kvetnansky and Mikulaj (1970). Cannulation of the rat tail artery one day prior to Immo allowed blood to be collected remotely during 2 hours of IMMO for analysis of corticosterone, catecholamines, and pOC. As depicted in Fig. 1, pOC was rapidly increased rather than slowly decreased in these animals as previously seen in rats exposed to milder stressors (Patterson-Buckendahl et al. 1995), while pOC in non-Immo rats sampled in the same manner did not change. If all adrenal hormones were removed by adrenalectomy (ADX), basal pOC concentration was significantly elevated, and Immo induced changes increased more than 5 fold over basal levels. Removal of only the adrenal medulla, thus eliminating epinephrine but not norepinephrine, had no effect on pOC. On the other hand, administration of chlorisondamine, a nicotinic acetylcholine receptor antagonist that eliminates norepinephrine release, resulted in a near-doubling in basal pOC, and an Immo induced increase that failed to return toward normal as seen in rats administered saline only (Patterson-Buckendahl et al. 1995).

Because the extremely high pOC observed in the ADX rats is rarely seen except in renal failure, we investigated plasma clearance of radioiodinated OC in control and Immo rats. As can be seen in Fig. 2, clearance is rapid in both groups, but unaltered by Immo.

Brief cold exposure of rats aged 1.5 to 10 months for 1.5 to 4 hr, or of 6 month old rats continuously for up to 3 weeks decreased pOC in a strong relationship to both increased plasma corticosterone and adrenal weight and decreased thymus weight (Patterson-Buckendahl et al. 1988). As shown in Fig. 3, 24 hours cold exposure at 4°C similarly decreased basal pOC, but did not prevent increase during Immo (Patterson – Buckendahl et al., 1995).

We have also determined that ethanol consumption induces a significant elevation in rats' basal catecholamine production and blunts their response to Immo (Fig. 4). Catecholamines were elevated in baseline samples of ethanol consuming rats and significantly exceeded those of either pairfed or ad libitum fed controls. The response of pOC to this treatment did not differ significantly from ad libitum fed controls except at the

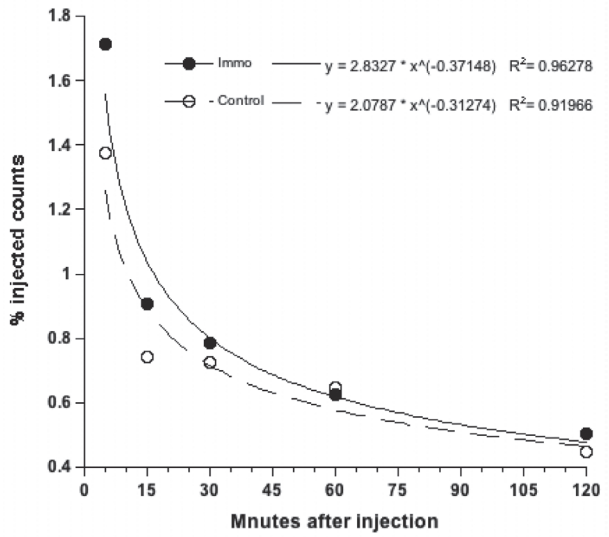

Fig. 2. Plasma clearance of radioiodinated osteocalcin by control and Immo rats (n = 6 each). Cannulae were placed in tail artery for blood sampling and in jugular vein to enable injection of labeled protein (1 µCi/100 g rat, specific activity, 6.8 µCi/µg) without handling the animals. Values shown are mean cpm/ml as % of initial injectate ± SEM. Where error bars are not seen, they are within the symbol. Best-fit equations are shown for each group. Repeated measures ANOVA indicated there was no difference in clearance between control and Immo rats.

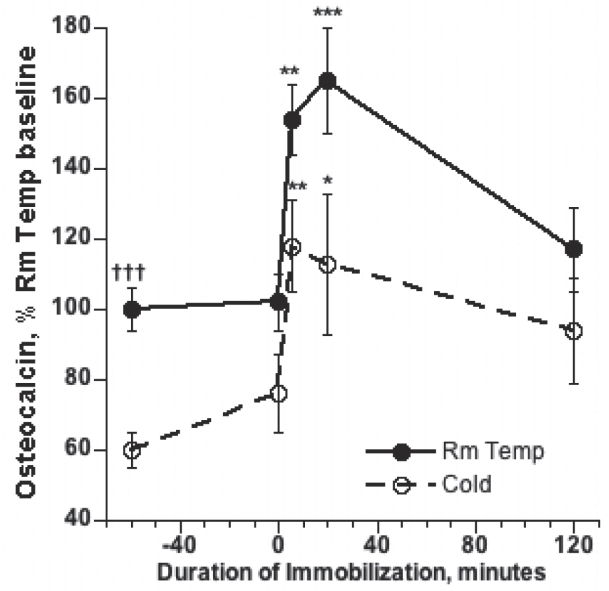

Fig. 3. Effect of 24 hours cold exposure on subsequent response of pOC to Immo, mean ± SEM. ††† indicates difference of p < 0.001 between cold and RT rats. *, **, * indicate differences from baseline sample taken 60 min prior to Immo within treatment group of p < 0.05, 0.01, 0.001 respectively.**

5 minute time point, but did differ from pairfed from 5 through 60 min Immo suggesting that food restriction had a greater effect on Immo response than high ethanol intake (Patterson-Buckendahl et al. 2005).

Although few studies have been conducted in other species to determine the acute effects of intense stressors, there are two striking studies reporting an increase in pOC similar to that of Immo rats (See Fig. 1). Hotchkiss et al. (1998) compared pOC, ionized calcium, and parathyroid hormone in blood from cynomolgous monkeys subjected to several types of anesthetics, including isoflurane, ketamine, and ketamine combined with atropine. Blood was collected at baseline, and after 15, 60, and 120 minutes of treatment. Isoflurane anesthetic caused a marked change in all three parameters, with decreased ionized calcium, and increased parathyroid hormone and pOC. The authors speculated that inorganic fluoride in the blood might have altered the calcium levels and that the pOC and parathyroid changes were secondary to the ionized calcium decline (Hotchkiss et al. 1998). An alternative explanation may be that the volatile isoflurane as well as halothane directly affects neuronal excitability involving movement of cytosolic Ca2+ (Xu et al. 2000), which may in turn involve interaction with OC.

A similar response of PTH, ionized Ca and OC was observed in adult human subjects while watching an audiovisually provocative TV program that had earlier been blamed for convulsive seizures in 680 children. The experimental subjects exhibited a nearly linear rise in pOC in samples taken at baseline, 10, 20, 60 and 120 min, to a level that was nearly 20% higher than baseline by 120 min. Ionized calcium and parathyroid hormone also showed responses similar to the monkeys under isoflurane anesthesia. Again, the authors related the changes to decreased blood ionized calcium. They proposed as a counter-measure a highly biologically available calcium preparation to be given as a precaution before undergoing especially emotional experiences (Fujita et al. 1999). If hypocalcaemia is artificially decreased by either citrate (Gundberg et al. 1991) or EDTA administration (Thomas et al. 1990), both PTH and pOC are acutely increased.

Weight bearing, whether increased or decreased, is a strong regulator of bone metabolism and the associated synthesis of OC. It is well known that a prolonged decrease in weight bearing, whether due to illness or the microgravity of spaceflight, leads to bone loss, termed disuse osteoporosis. This has been modeled in healthy humans by complete bed rest (Pavy-Le Traon et al.

2007) and in laboratory animals by hind-limb unloading (Morey-Holton et al. 2005). Rats subjected to hind-limb unloading for 2 to 28 days had acutely decreased pOC that returned toward normal after 2 weeks suggesting decreased bone formation or turnover. They also had bones that weighed 20 % less than normally loaded rats after 10 to 28 days and contained less bone OC than controls, indicating that both pOC and bone OC are sensitive indicators of disruption of osteoblastic activity during altered loading (Patterson-Buckendahl et al. 1989). Decreased bone formation in real and simulated spaceflight has been verified by histomorphometry in numerous experiments (Globus et al. 1986; Morey-Holton and Globus 1998) and by gene expression (Cavolina et al. 1997). Rats that experienced actual spaceflight aboard NASA Spacelab 3 also had decreased pOC and bone mass following 7 days in microgravity. While the pOC might have been related to post-flight transport stress, the bone changes were attributed to decreased bone formation and growth during flight (Patterson-Buckendahl et al. 1985, 1987). Human subjects followed for 6 months following spinal cord injury had a minor increase in bone formation markers, including alkaline phosphatase and pOC (Roberts et al. 1998). Rats receiving a complete spinal cord severance had a slight but significant increase in pOC 21 days post injury, accompanied by greatly decreased bone mineral density, assessed by microCT, and biomechanical properties (Jiang et al. 2006).

Exercise is widely studied for its effects on the skeleton, which are generally considered beneficial to the preservation of bone mass. However, rarely if ever, are truly acute blood samples obtained. Most studies collect blood hours to days after the end of the exercise period. Biomarkers of bone metabolism, including OC, are highly variable but do tend to reflect the increase bone turnover that is stimulated by exercise to an extent that varies with the type and intensity of exercise (Banfi et al. 2010). Maimoun et al. (2006) compared numerous biomarkers in cyclists exercising below or above the ventilatory threshold. They reported changes in pOC in blood samples obtained at 30 min intervals during the cycling activity similar to those seen in our Immo studies (see Figs. 1, 3, and 4). Exercise training using electrical stimulation cycle ergometry with spinal cord injured individuals induced significantly increased pOC after 6 months, indicating an increase in bone turnover (Bloomfield et al., 1996). Resistance training of human subjects participating in a simulated microgravity study also induced an increase in pOC along with other bi-

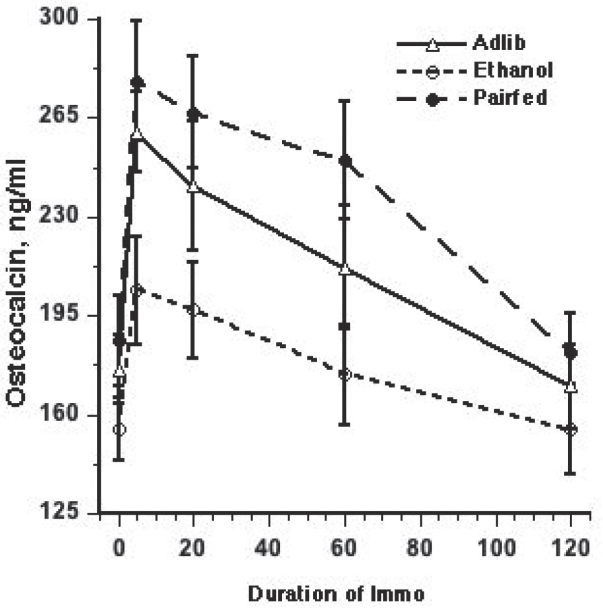

Fig. 4. Effect of 1 week of ethanol liquid diet (5% EtOH, 35% calories) on subsequent pOC response to Immo compared to controls fed liquid diet either ad libitum or in quantities equal to the average amount consumed by ethanol rats on previous day. Values are mean ± SEM.

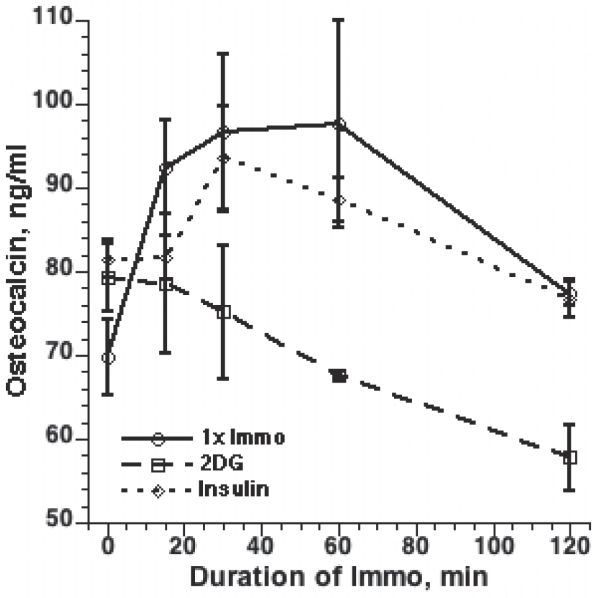

Fig. 5. Response of pOC to Immo ± insulin & 2DG, mean ± SEM.. Mice were sampled remotely via catheter implanted in the tail artery 24 hr prior to Immo.

omarkers, accompanied by beneficial effects on lumbar spine and hip bone mineral density, suggesting that this

type of exercise was an effective countermeasure for disuse (Shackelford et al. 2004).

Osteocalcin has hormonal functions

The development of the OC null mutant mouse (OC-KO) has made it possible to explore in greater depth the possibility that OC has hormonal functions. Ducy et al. (1996) reported that the OC-KO mouse had denser, stronger bone after reaching full adulthood, suggesting that OC might exert autocrine control of the osteoblast. At the time, no other obvious differences were reported. However, the same research group recently reported that the OC-KO mice were obese, hyperglycemic, and insulin resistant, all characteristics of type-2 diabetes mellitus. This was the first indication that OC might have extraskeletal hormonal influence (Lee et al. 2007). They further showed that OC could stimulate insulin expression in cultured pancreatic beta cells and adiponectin expression in adipocytes. Ferron et al. (2008) extended this line of work to determine that effective doses of OC to stimulate beta-cells were in the picomolar range, while nanomolar concentrations were required to affect adipocytes. Curiously, they also determined that for OC to have these effects, its Gla residues must be decarboxylated.

Possible support for a positive relationship between undercarboxylated OC (ucOC) and glucose metabolism was found in a report noted by Levinger et al. (2010), who analyzed the relative effects 45 min to 2 hr of aerobic or power exercise on glucose, adiponectin, OC and ucOC. Aerobic exercise significantly increased both forms of OC as well as adiponectin, while power exercise had a limited effect, although both forms of exercise reduced serum glucose levels. However, these authors did not report the percent of total OC represented by ucOC. This is an important consideration because carboxylated and uncarboxylated forms are highly correlated due to regulation of the carboxylase enzyme.

Yet another possible hormonal influence of OC was reported by Oury et al. (2011). OC-KO mice were said to be less fertile and have smaller testes and lower testosterone than their wild-type littermates. Binding of OC to a G protein-coupled receptor, GPRC6A that is expressed in Leydig cells exerted regulatory influence on expression of enzymes needed for testosterone synthesis. Conversely, males of a GPRC6A null mutant mouse strain exhibit osteopenia, feminization, and metabolic syndrome (Pi et al. 2008).

Most of the evidence for hormonal influence of OC on extraskeletal targets comes from studies of the OC-KO mouse. Studies of Immo effects on rats typically show decreased plasma insulin, decreased insulin binding to fat and skeletal muscle cells, and increased plasma glucose. Such responses would be needed if the animal were to mount an appropriate fight or flight response (Macho et al. 1999, 2003). We have also documented in numerous studies the significant increase in pOC under the same conditions. One must question whether the assertion that extra-skeletal OC induces an increase in insulin and a decrease in glucose and insulin resistance are in conflict with the Immo data. We have not evaluated whether the increased pOC during Immo is fully carboxylated or not. However, it is not clear that the osteoclast resorption mechanism proposed by Ferron et al (Feron et al. 2010) for releasing uncarboxylated OC from the bone can occur rapidly enough to account for the rapid increase in pOC that occurs during Immo. Acid decarboxylation of OC in vitro may take weeks rather than minutes as would be required during Immo (Hauschka et al. 1989). Furthermore, the acid concentration required for decarboxylation in vitro (1 M) is far higher than that generated by the osteoclast (Hauschka et al. 1980), and OC bound to bone hydroxyapatite is strongly protected from decarboxylation (Poser and Price 1979). A microelectrode study determined that the acidic environment beneath the osteoclast when firmly attached to bone was approximately pH 4 to 6, significantly higher than that of HCl used for in vitro decarboxylation studies (Silver et al. 1988). This does favor the activity of the acid proteases that are also secreted by the osteoclast into its resorption cavity.

A further conflict between these data may be seen in the response of pOC to Immo under conditions of either glucoprivation caused by 2-deoxyglucose injection (2DG, 500 mg/kg body wt) or insulin injection (5 IU/kg body wt). As seen in Fig. 5, injection of insulin had little effect on the response of pOC to Immo, whereas 2DG significantly decreased pOC in a linear fashion from 15 through 120 min Immo ($p< 0.01$). Repeated measures ANOVA indicated that pOC response in 2DG treated mice differed significantly from 1x Immo mice, $F[2,6] = 8.42$, $p<0.05$, whereas insulin had no effect on pOC.

Osteocalcin is expressed in the nervous system

The first indication of the expression of OC outside the skeleton was reported by Fleet and Hock (1994). They extracted RNA from rat bone, liver, kidney, duo-

denum, lung, and brain, reverse transcribed the mRNA to cDNA and probed with OC specific primers by PCR. The products of the PCR reactions were analyzed to confirm that they were the expected OC sequence. All of the non-skeletal tissues expressed OC RNA, though at levels at least 1000 fold lower than in bone. (Frenkel et al. (1997) also reported indications of OC expression in brain, discovered while probing the characteristics of the rat OC promoter region. They created transgenic mice bearing OC promoter-chloramphenicol acetyltransferase (CAT) constructs. Various tissues were taken from the transgenic mice and assayed for CAT activity. In addition to bone, they also analyzed kidney, brain, peripheral blood cells, heart, muscle, liver, and spleen. All bone samples analyzed had CAT activity, while of the non-skeletal tissues, only brain expressed any activity though at a level up to several hundred fold lower than bone.

The presence of OC protein in sensory ganglia was reported first by Ichikawa et al. (1999) in both trigeminal (TG) and dorsal root (DRG) ganglia neurons. The OC was co-localized with parvalbumin in neurons thought to be proprioceptors. Some neurons in the TG co-expressed OC as well as parvalbumin and CGRP and were thought to be mechanoreceptors and nociceptors. Further investigation by Ichikawa and Sugimoto (2002) of OC in DRG and TG indicated co-expression of OC with the capsaicin receptor VR1. Although DRG neurons did not express both proteins, about 14% of TG neurons did express both, and were thus believed to be nociceptors. OC expression was also detected in the medullary dorsal horn of the spinal cord. Later, Ichikawa et al., (2005) indicated the presence of OC containing neurons in vagal and glossopharyngeal sensory ganglia. The data suggested that some OC containing neurons have chemoreceptive functions in the tongue (Ichikawa et al. 2005). Personal communication with Dr. Ichikawa established that, although they had ample evidence of OC protein in the ganglia, they had not attempted to determine OC gene expression in any of their tissues.

We have since confirmed both extractable OC protein in DRG, TG, and parts of the brain, and also established gene expression in both rat and mouse neural tissues. We presented data for rat gene and protein in neural tissues at the annual meeting of the American Society for Bone and Mineral Research in October, 2010. We homogenized rat E14.5 embryo and adult brain and ganglia and extracted total RNA using Qiagen RNeasy reagents and treated with on-column DNase to eliminate genomic DNA prior to reverse transcription to cDNA. We then determined specific expression of rat OC by semi-quantitative RT-PCR with probes from Applied Biosystems (Bglap, Rn01455285_g1). The results indicated OC gene expression in E14.5 rat hindbrain, forebrain, SCG, spinal cord, and DRG. We also detected expression in 18-mo old rat TG, DRG, hypothalamus, hippocampus, brain stem, olfactory bulb, and spinal cord. In these tissues, TG expressed 4 to 10 fold more OC RNA than the other tissues. Embryonic RNA was approximately 100 fold lower than that of the adult female from which they were taken.

Table 1

Osteocalcin protein concentrations in adult and embryonic rat neural tissues

Tissue	OC (ng/mg protein)	Tissue	OC, ng/mg protein
adult hypothalamus	8.5	E14.5 forebrain	96
adult DRG	90	E14.5 spinal cord	287
adult amygdala	6.3	E14.5 hindbrain	118
adult hippocampus	11.2	embryonic cortex	119
adult rat cortex	7.2	E14.5 SCG	31*
		E14.5 DRG	9.2*

Note that E14.5 SCG and DRG contained immunoreactive protein, but that total protein was too low to be determined by UV spectrophotometry. These values are ng/ml of extracted tissue.

We also quantified OC protein in additional portions of the rat tissue. Tissues were homogenized in phosphate buffered saline containing 2 % SDS, 5 mM EDTA, and a protease cocktail. Protein concentrations were determined by UV absorbance at 280 nm and osteocalcin by ELISA. Results are shown in Table 1.

We have performed similar analyses on mouse tissues taken from both wild type and OC-KO mice. In keeping with the null mutation, there was no OC gene expression nor protein detected in neural tissues of the mutants, but there was consistent expression of OC in TG, DRG, and brain tissues of the wild-type mice.

We have also observed phenotypic behavioral differences between the OC-KO mice and their wild-type counterparts that are consistent with decreased sensory sensitivity. We reported some of these observations at the 2005 meeting of the American Society for Bone and Mineral Research (2005), and have since expanded our studies. We have concluded that sensory stimuli involving thermal, nociceptive, and proprioceptive responses

are impaired in the OC-KO mouse (manuscript in preparation).

The lack of gene and protein detection in sensory ganglia combined with behavioral impairments in the OC-KO mice implies a function of the OC protein in the sensory ganglia. This, in turn, means that there must be some receptor mechanism by which OC can influence the appropriate sensory response. Although no specific receptor has been clearly identified, recent reports suggest a strong candidate. Wellendorph and Brauner-Osborne (2004) cloned the G protein-coupled receptor, GPRC6A, which has striking similarities to the calcium sensing receptor originally isolated from parathyroid glands. They found three isoforms, with isoform one being the most abundant. The authors surveyed both gene expression and protein in tissues where isoform 1 predominated and detected message brain, skeletal muscle, testis, and leucocytes. Lower levels were found in liver, heart, kidney and spleen, still less in lung, pancreas, placenta and ovary (Wellendorph and Brauner-Osborne 2004). More recent in situ hybridization of brain tissue identified GPRC6A mRNA expression in pia mater, Purkinje and pyramidal cells of the cerebellar cortex, but none in neuroglial cells (Luo et al. 2010).

There is conflicting evidence for interaction of GPRC6A with bone and OC. Pi et al reported that it was stimulated by OC in the presence of calcium and might be mediating extracellular calcium-sensing responses in osteoblasts (Pi et al. 2005). Binding of OC to GPRC6A expressed in Leydig cells of the testes was reported to exert regulatory influence on expression of enzymes needed for testosterone synthesis (Oury et al. 2011). Conversely, males of a GPRC6A null mutant mouse strain were said to exhibit osteopenia, feminization, and metabolic syndrome (Pi et al. 2008). However, Wellendorph et al (2004, 2009) found no evidence for a bone phenotype or infertility or breeding problems or obesity in their GPRC6A null mutant mice.

Recently, Oury et al. (2011) indicated that an osteoblast specific deletion of OC decreased pOC levels by approximately half. If pOC does not originate in the bone of these animals, then a much larger amount must come from non-skeletal tissues than has ever been suspected. The authors gave no indication of where they thought that OC might originate. A much earlier paper from this research group indicated gene expression for the OC related gene (ORG) in lung and kidney (Desbois et al. 1994), although this was not known to contribute to circulating OC, as indicated in their original description of the OC KO mouse (Ducy et al. 1996). Given the significant quantities of OC protein and gene expression we have seen in the nervous system, we believe those tissues may be a potential source of pOC, especially under stressful conditions.

It should be noted that hypogonadism is a common symptom of chronic stress in both males (Guay et al. 2010) and females (Chrousos et al., 1998). Environmental stressors in animal housing that may amount to chronic mild stress are often overlooked and can have unintended consequences on experimental data (Riley 1981). Thus, humans and laboratory animals experiencing chronic stress may have both decreased OC and hypogonadism.

Implications of osteocalcin as a neuropeptide

Much work needs to be done to define the role of OC as a neuropeptide and its mechanisms of action. We know that its synthesis and release are stress-responsive and that it is made in neural tissues, as is its potential receptor GPRC6A. In vivo experiments using various stimuli will need to include investigation of both OC and GPRC6a expression in brain, sensory and sympathetic ganglia as well as effects on peripheral tissues that may be targets for OC influence. Experiments in progress in my laboratory are investigating effects of Immo and sensory stimuli on TG and DRG expression of OC and a variety of neuropeptides and receptors known to respond to these stimuli. Tissue immunocytochemistry and in situ hybridization will aid in determining potential targets of OC regulation. Culture of ganglionic neurons will enable determination of specific mechanisms that induce or inhibit OC activity.

The finding that responses to sensory stimuli are decreased in the absence of OC implies an effect on neural function. If OC were to be upregulated in sensory neurons, would a small, painful stimulus be amplified? If so, how might OC synthesis be specifically inhibited in order to decrease hyperalgesia? To date, the known regulators of OC synthesis, including glucocorticoids, vitamin D, and certain cytokines have effects on many other physiological systems.

Another open question is whether OC affects motor neuron function. GPRC6A was abundant in skeletal muscle so interaction with OC is plausible (Wellendorph and Brauner-Osborne 2004). Do mice lacking OC have impaired muscle strength or slower reaction times, particularly during states of CNS arousal? We have observed that OC-KO mice were reluctant to traverse a balance beam and remained immobile significantly longer than WT mice

and also had slower responses in a tail flick test of noxious heat (Buckendahl, unpublished observations).

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