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NEWS

Conversations with pioneers in the bone field: Edward M Brown

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Professor Brown recounts the cloning of the calcium-sensing receptor and discusses the research to which that seminal discovery gave rise

Editors' Note: This is the third in a new series of interviews with investigators who have made groundbreaking contributions to understanding endocrinology, bone health and bone disease. See previous interviews with T John Martin (http://www.nature.com/bonekey/knowledgeenvironment/2013/130424/bonekey201373/full/bonekey201373.html) *and Stavros Manolagas* (http://www.nature.com/bonekey/knowledgeenvironment/2013/130904/bonekey2013139/full/bonekey2013139.html).

Edward Brown, MD, is Professor of Medicine at Brigham and Women's Hospital in Boston, Massachusetts, USA. In 1993, Professor Brown reported the cloning and characterization of an extracellular calcium-sensing receptor from bovine parathyroid glands. This seminal discovery set the stage for a surge of interest in studying the function of the receptor in the parathyroid, kidney, bone and a host of other tissues, and also spurred the development of calcimimetics. Professor Brown has spent a distinguished career investigating how cells sense calcium and how calcium serves as an extracellular messenger in health and disease. He spoke recently with Neil Andrews, *BoneKEy* Features Editor, to discuss the circumstances surrounding the cloning of the receptor, the research it inspired and what there is still left to learn. An edited version of their conversation appears below.

BoneKEy: What was your path to medicine?

Edward Brown: I have many doctors in my family. My father was an orthopedist, and his father was an orthopedist, and in part because of that I swore I wanted to have nothing to do with bone! Things turned out otherwise. My maternal grandfather was also a physician who did research, but I found out only after I was well into my career that he had worked on calcium metabolism in cows, so I guess it is in the genes. The only other possibility I considered was getting a PhD, but I wanted to have contact with patients; I have always felt, particularly once I went into research, that if you understand the normal it can tell you what can go wrong, but if you study the abnormal it can also tell you how things go right, and I felt that was helpful in the research that I have carried out.

BoneKEy: What was the genesis of your interest in the calcium-sensing receptor?

Edward Brown: When I was a resident in the 1970s at the Peter Bent Brigham Hospital (now Brigham and Women's Hospital) in Boston, I saw some patients with calcium disorders such as primary hyperparathyroidism, and I started to become interested in the parathyroid cell. Back then, most of us interested in research went to the NIH [National Institutes of Health] after we did our residencies, and when I talked to people there, one of the real stalwarts in the calcium field who impressed me very much was Gerald Aurbach, who was doing interesting work on calcium metabolism and hyperparathyroidism. But when I actually went down there for a fellowship, he had switched research interests to studying the beta-adrenergic receptor in turkey erythrocytes. Fortunately, he let me develop a system of dispersed parathyroid cells because I thought that if I studied what happened inside the cell when I changed calcium outside the cell, that might give some clues into the mechanism of receptor function. That general approach drove my research over the next several years.

When I came back to the Brigham in 1979, I continued to look at aspects of the parathyroid cell and how it responded to calcium that suggested it might be responding through a receptor-like mechanism. Others were working in this same general area, particularly Dolores Shoback, who had been a fellow with me, and Edward Nemeth, the father of calcimimetics

and calcilytics, and also a group in Uppsala, Sweden. By the early 1990s, we were a select group of people who were 'true believers' in the existence of a calcium-sensing receptor—most people thought that it would be a calcium channel or some other mechanism that somehow sensed calcium, but we believed there was a type of G-protein-coupled calcium-sensing receptor.

I became aware that others were trying to clone the receptor, and I just couldn't sit on the sidelines and watch that happen. I was very fortunate that Steven Hebert, a nephrologist who had developed a very successful expression cloning program in *Xenopus laevis* oocytes cloning renal transporters/cotransporters and ion channels, was working at the Brigham. I walked into his office one day, and he was talking to a fellow, but I interrupted, introduced myself, and asked if he would be interested in trying to clone a calcium-sensing receptor in the parathyroid cell. He said yes on the spot and that got us started.

BoneKEy: What was your approach to cloning the receptor?

Edward Brown: We were trying to turn the Xenopus oocyte into a parathyroid cell by injecting messenger RNA from parathyroid glands, and if the G-protein-coupled receptor is then expressed by the oocyte, that cell should become responsive to calcium under conditions where normally it is not responsive. We used electrophysiological measurements of a calcium-activated chloride channel as a readout, providing the 'signature' of the calcium-sensing receptor. We found, however, that high calcium concentrations had no effect on the cells, and we were somewhat taken aback, but earlier work we had done showed that trivalent cations such as gadolinium activated the receptor and were more potent than calcium. We decided that we would try gadolinium instead of calcium as our receptor agonist, and fortunately the oocytes responded to it. But it was an act of faith that if we cloned the gadolinium receptor it would turn out to be the calcium-sensing receptor.

For the cloning procedure, we made a cDNA library from the parathyroid cells, screened that library using the oocytes, one oocyte at a time, and eventually reached the point where we had one small pool of clones from the library that gave a very positive signal. We used a chart recorder to record calcium-activated chloride currents, and it was a very exciting moment when we added the gadolinium and the pen on the chart recorder went up and down so far that it hit the top of the recorder. We saw the same thing when we added calcium, and when we tried magnesium. Neomycin, which we had shown was a pretty good agonist, also gave a huge signal and even polyarginine, which is also an agonist, did the same thing. Within a few weeks we had the clone, and we published the results in *Nature* in December 1993.[1]

BoneKEy: What happened next?

Edward Brown: During the previous year I had done a short sabbatical with two molecular cardiologists here at the Brigham, Christine and Jonathan Seidman, to learn some genetics and molecular biology. Jon actually wanted me to work on cloning the beta-adrenergic receptor gene, and when I said I was much more interested in the calcium-sensing receptor, he raised the question of what disease would result from inactivation of the receptor. While I was at the NIH, I had worked closely with Stephen Marx, who was a colleague of Gerry Aurbach's and one of the real pioneers in studying the genetic basis of primary hyperparathyroidism. He had seen numerous families with familial hypocalciuric hypercalcemia [FHH] and neonatal severe hyperparathyroidism [NSHPT] with characteristics suggesting that their parathyroids were resistant to calcium, and their kidneys also did not respond normally to calcium. Shortly after we cloned the receptor, Martin Pollak, who was a renal fellow at the Brigham working with the Seidmans, showed that there were inactivating mutations in the calcium-sensing receptor of patients with FHH and NSHPT, but not in unaffected family members.[2]

This research, along with the cloning of the receptor, was the initial body of work that really established this molecule as a calcium-sensing receptor and FHH and NSHPT as prototypes for calcium-sensing receptor diseases.

BoneKEy: When you cloned the receptor, was there anything about it that struck you as unique, in comparison to other cell surface receptors that people knew about at the time?

Edward Brown: When we started to sequence the receptor, we found that it was related to the metabotropic glutamate receptors, which are in a small family (family C) of G-protein-coupled receptors that all have a very large extracellular domain and sense small molecules like glutamate, GABA, pheromones and taste molecules. It was a surprise that the calcium-sensing receptor was similar to a class of receptors that was already known, but no one had any idea that a receptor related to glutamate would also sense calcium. It turned out that almost all the family C receptors do sense calcium, and the calcium-sensing receptor can also sense aromatic amino acids, as shown by the work done by Arthur Conigrave when he was working with me, enabling it to integrate information from several different classes of nutrients and other environmental signals. It was one of the first examples of a G-protein-coupled receptor acting as such an environmental sensor.

BoneKEy: After you cloned the receptor and the first mutations in the receptor were identified, how did your research progress over the next several years?

Edward Brown: As it became clear that the receptor was expressed in tissues beyond those involved in classic calcium homeostasis, I began to look for collaborators who worked with cell types that looked interesting and might be worth examining. For instance, fairly early on we showed that monocytes, which are precursors for osteoclasts, had a calcium-sensing receptor. David Scadden, a hematologist at Massachusetts General Hospital, had worked with me when he was a medical resident and I was an attending. So I contacted him, and we started to look at monocytes and macrophages. It turned out that monocytes and macrophages chemotaxed quite nicely towards calcium; calcium stimulated the production of some chemokines that are important for regulating those types of cells. This was a nice example of a non-homeostatic tissue that seemed to care quite a bit about calcium. David's group also did the work of identifying a role of the receptor in enabling hematopoietic stem cells to be maintained at their niche within the bone marrow.[3]

BoneKEy: Historically, what was the discussion like about the role of the calcium-sensing receptor in bone cells?

Edward Brown: There was a lot of controversy, with some investigators not finding any evidence whatsoever of the presence of the receptor in cells like osteoclasts or osteoblasts; there were people who thought that calcium-sensing by those cells was through totally different classes of molecules. Finally, a couple of research fellows came from Japan who were

interested in bone and the calcium receptor in bone, and they dragged me kicking and screaming into that field! We were able to see the receptor in bone cells, not necessarily that easily but we were able to see it and convince ourselves that it was there; it was certainly present in osteoclast precursors, and in some osteoblast cell lines.

But there were still people who looked for the receptor and reported negative findings against our positive results, and it was really unclear which way things would go. The use of a knockout was an attractive approach to that problem. The first knockout mouse was developed by the Seidmans and was published in 1995.[4] When investigators found no real evidence of bone disease in that mouse, it looked as though maybe the receptor did not do anything in bone after all. However, this was a global knockout involving all tissues, and it turned out that it still allowed for an alternatively spliced receptor to be made that might be physiologically active.

Subsequently, Dolores Shoback, Wenhan Chang and colleagues did conditional knockouts in cartilage cells and in osteoblasts and the mice exhibited dramatic phenotypes.[5] Knocking out the receptor in chondrocytes was embryonic lethal, and the osteoblast knockouts were stunted mice who had many fractures and very poor mineralization. It was hard for me initially to understand why there would be such a contrast between one knockout—admittedly an incomplete knockout—and these conditionals where there was such a severe phenotype. However, the data using the conditional knockouts are very convincing, and my current thought is that the receptor probably does have a very important role in bone cells.

BoneKEy: When you look back at all the work your research inspired, what strikes you the most about what has been learned about the receptor—what was expected and what were the unexpected things that surprised you and your colleagues?

Edward Brown: The receptor mostly turned out to do the expected things in homeostatic tissues like the parathyroid and in the kidney, although there is some controversy about whether the receptor can explain all the effects of calcium on kidney function or whether there may be another receptor that mediates some of those effects.

But a number of fascinating things have also emerged. Sensing of other ligands, such as amino acids, has turned out to be a very interesting mechanism by which the receptor is probably doing things in the GI tract that are quite different than in the parathyroid, for example, because the concentration of amino acids will be quite high inside the lumen of the intestine after proteins are digested. The receptor in that setting is acting much more as a nutrient sensor that integrates information about calcium but particularly about amino-acid status.

There is also interest now in the possible role of the receptor in Alzheimer's disease—and there are those who feel pretty strongly about that. We had shown in the late 1990s that some amyloid beta proteins can activate the receptor in hippocampal neurons, a part of the brain relevant to Alzheimer's. That has raised the question of whether there might be therapeutic approaches for Alzheimer's that are calcium-sensing receptor-based.

There has also been a huge amount of interest in the role of the receptor in cancer. For example, there is a long history of interest in the effects of dietary calcium on colon cancer, and low dietary intake seemed to increase the risk of carcinogenesis. This was all prior to the cloning of the receptor, and there are colon cancer cell lines and normal colon cell lines where calcium does inhibit proliferation and it does look as though the receptor might be important in that regard; some conditional knockouts are being worked on that may help to definitively prove that *in vivo* one way or the other. On the other hand, there are some cells where calcium stimulates proliferation. In terms of therapeutics, it may be a balance between not stimulating some cells under conditions where you are inhibiting other cells.

Also fascinating is work with regard to the breast. During lactation the receptor is upregulated in breast epithelial cells that make milk and it inhibits the production of parathyroid hormone-related protein [PTHrP]. The receptor also promotes movement of calcium into the milk. When calcium is insufficient, PTHrP will go up and that may mobilize calcium from bone and help to keep it from being excreted in the urine. That will tend to raise blood calcium that can then act through the receptor to make sure there is adequate calcium going into the milk. This is an interesting system that is only present in certain parts of the life cycle and was worked out very nicely by John Wysolmerski and Joshua Van Houten at Yale.[6]

Finally, in terms of more recent data, two papers that came out very close together both showed that calcium acts through the calcium-sensing receptor as a 'danger signal' to activate the inflammasome.[7,8]

BoneKEy: What other research on the calcium-sensing receptor intrigues you?

Edward Brown: There is also a lot of interest in important aspects of the basic biochemistry of the receptor. The receptor has a number of protein-binding partners and also traffics to and from the cell membrane. Many receptors, when they are exposed to the ligand that activates them, will downregulate, either by reducing their cell surface expression or reducing the function of the receptor. That would not be a good thing in the parathyroid because you do not want the calcium sensor to downregulate when there is calcium present, so there are mechanisms currently under investigation by Gerda Breitwieser and others for how the receptor can maintain a steady-state level that is appropriate for sensing calcium even with chronic exposure to calcium as a ligand.

There are binding partners that also provide clues into how the receptor signals to various downstream pathways. The receptor connects to a large number of signaling pathways—it has been shown that the receptor can activate most of the known pathways that have been looked at. There is only one isoform of the calcium-sensing receptor, when in contrast there are eight closely related isoforms of the metabotropic glutamate receptor, so presumably it needs to be pretty versatile in its ability to regulate cell function through a variety of pathways that would be appropriate to a particular cell type.

BoneKEy: What is it like to look back now at your research and all the subsequent work it inspired?

Edward Brown: It has been very exciting. It was fun to contribute to the initial work showing the presence and function of the receptor. Then, at a certain point, the field gets larger and larger and you cannot be the one who is pushing things forward, but then it is gratifying and very interesting to see where other people are going with it. We had the first calcium-sensing receptor symposium in December 2012 in Vienna and it was the first time many of us got together to compare notes and listen to presentations from people working on the calcium-sensing receptor in all of these different areas.

It took a while for interest in the calcium-sensing receptor to catch on. When you measure variation in serum calcium—Michael Parfitt measured serum calcium concentration every few minutes, as did a colleague of mine—the percent coefficient of variation is only about 1 or 2%, so it seems like calcium levels are almost constant. How could that be a signal that cells can decode? As a result, it was a foreign idea that an ion could activate a G-protein-coupled receptor, and it took time for people to wrap their minds around the fact that the receptor is doing interesting things, and to start looking at it in their own cell type or tissue of interest. It turned out to be a very fertile area to investigate.

BoneKEy: Thank you so much for speaking to BoneKEy.

Edward Brown: Thank you—it was my pleasure to participate.

Conflict of Interest

The author declares no conflict of interest.

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