PHYSIOLOGY AND PATHOPHYSIOLOGY OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR

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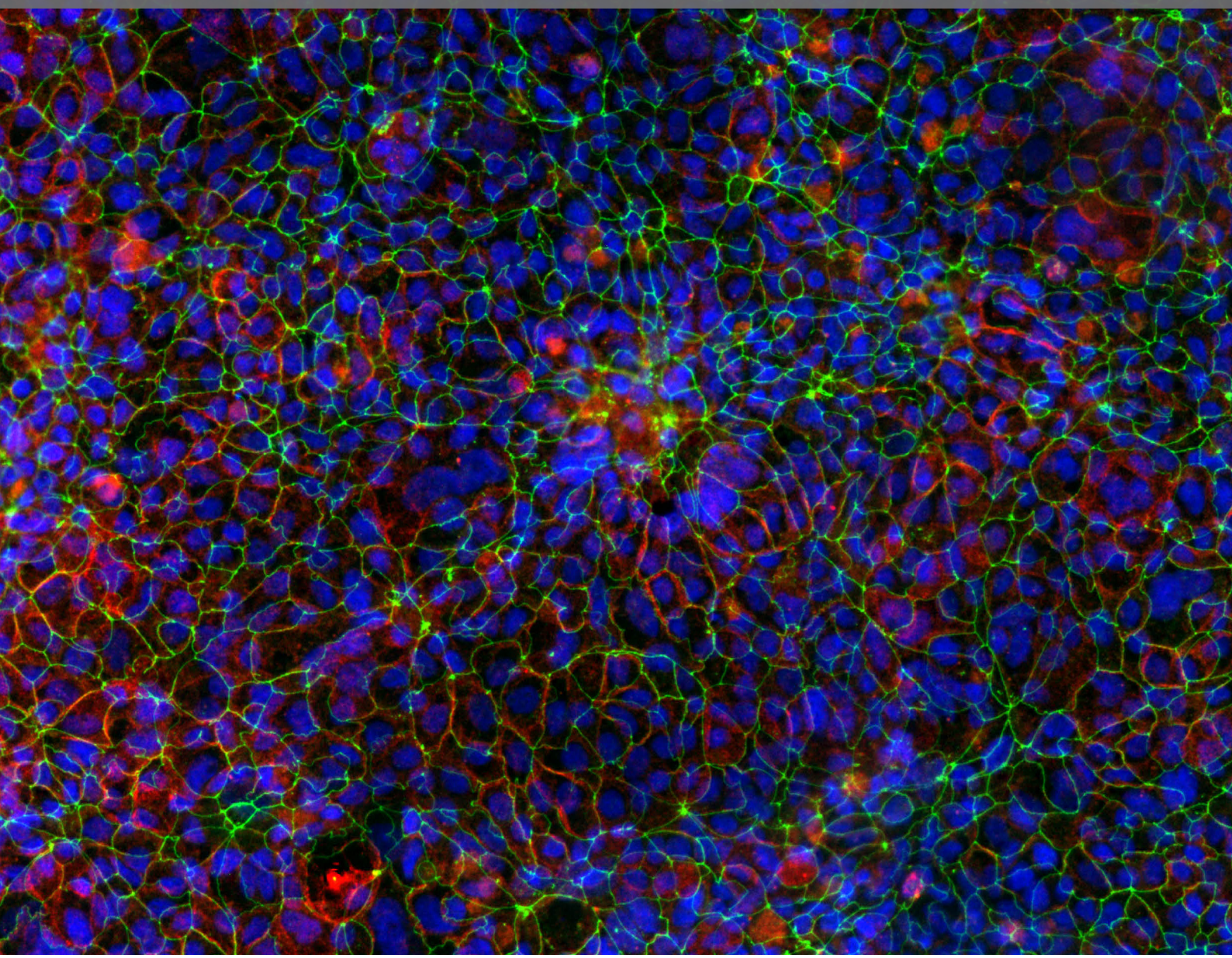

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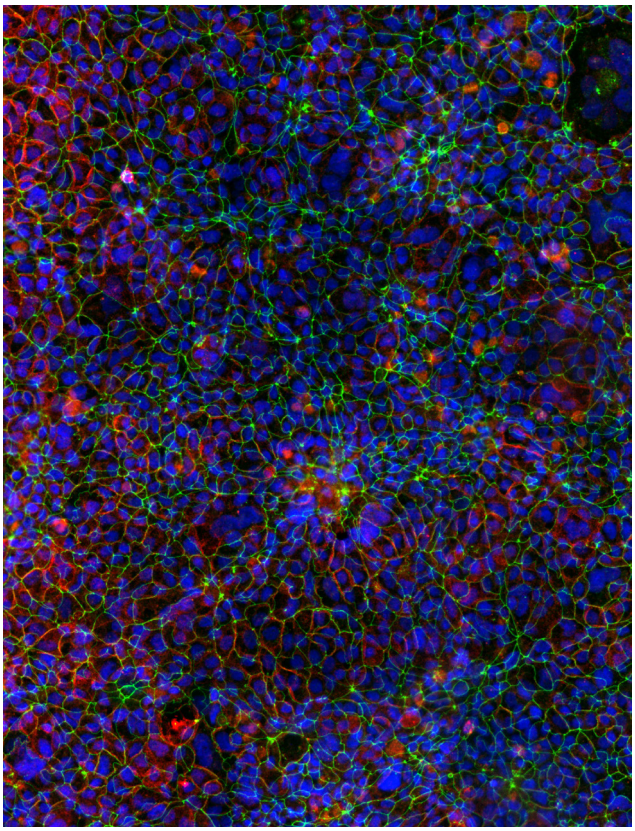
PHYSIOLOGY AND PATHOPHYSIOLOGY OF THE EXTRACELLULAR CALCIUM-SENSING RECEPTOR

Topic Editor:
Enikö Kallay, Medical University of Vienna, Austria



Human colorectal cancer cells (Caco-2) were grown for a month on 0.4 µm polyester membranes. The cells were fixed with methanol at -20°C over night. Double-immunofluorescence staining was performed to identify the calcium-sensing receptor (red) and the tight junction protein zonula occludens (green). The nuclei were stained with DAPI (blue). Image: Himadri Bahadur Thapa Bsc and Dr. Martin Schepelmann.

Calcium is vital for human physiology; it mediates multiple signaling cascades, is critical for cell survival, differentiation, or death both as first and as second messenger. The role of calcium as first messenger is mediated by the G protein-coupled receptor, the extracellular calcium-sensing receptor (CaSR). The CaSR is a multifaceted molecule that senses changes in the concentration of a wide variety of environmental factors including di- and trivalent cations, amino acids, polyamines, and pH. In calcitropic tissues with obvious roles in calcium homeostasis such as parathyroid, kidney, and bone it regulates circulating calcium concentrations. The germline mutations of the CaSR cause parathyroid disorders demonstrating the importance of the CaSR for the maintenance of serum calcium homeostasis. The CaSR has an important role also in a range of non-calcitropic tissues, such as the intestine, lungs, central and peripheral nervous system, breast, skin and reproductive system, where it regulates molecular and cellular processes such as gene expression, proliferation, differentiation and apoptosis; as well as regulating hormone secretion and lactation.

This Research Topic is an overview of the CaSR and its molecular signaling properties together with the various organ systems where it plays an important role. The articles highlight the current knowledge regarding many aspects of the calcitropic and non-calcitropic physiology and pathophysiology of the CaSR.

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EDITORIAL
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Editorial: Physiology and Pathophysiology of the Extracellular Calcium-Sensing Receptor

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Keywords: calcium-sensing receptor, physiology, structure-activity relationship, mutations, G protein-coupled receptor

Editorial on the research topic

Physiology and Pathophysiology of the Extracellular Calcium-Sensing Receptor

Calcium is a universal signal carrier for biological information, being one of the most specific and most selective messengers in nature. It is involved in multiple signaling cascades - critical for cell survival, differentiation, or death. Within the cell, calcium controls several different signaling pathways, including those that regulate cell growth and cell death. The extracellular calcium-sensing receptor (CaSR) is the fundamental tool used by cells to detect subtle changes in extracellular calcium (Ca^{2+}) and has been the subject of intense research activity since it was first cloned 25 years ago (Brown et al., 1993).

The class C G protein-coupled receptor CaSR plays a pivotal role in systemic calcium homeostasis by regulating parathyroid hormone (PTH) secretion and urinary calcium excretion. There is emerging evidence that the CaSR represents a key molecule in physiology, as it controls such diverse processes as hormone secretion, ion channel activity, gene expression, modulation of inflammation, proliferation, differentiation, and apoptosis, depending on cell type.

Initially, the studies on the CaSR were focusing on calcitropic tissues with obvious roles in calcium homeostasis such as parathyroid, kidney, and bone. By now it has become clear that the receptor is expressed in many other tissues, without any obvious role in this process, such as neurons, lung, skin, placenta, breast, endothelium. Abnormal CaSR expression and function is implicated not only in calcitropic disorders such as hyper- and hypoparathyroidism, but also in diseases linked to non-calcitropic systems, such as the nervous, reproductive, and respiratory system, and even in diseases such as chronic inflammation and cancer (Brennan et al., 2013).

The CaSR binds different ligands and interacts with multiple heterotrimeric G protein subtypes thereby regulating highly divergent downstream signaling pathways, depending on the cellular context. A broad understanding of the genetic, molecular, and cellular regulation of CaSR expression and signaling is crucial to comprehend both its importance in normal physiology and to devise unique, targeted drugs to treat diseases linked to impaired CaSR expression or function.

Since 1993, when the CaSR was first cloned and characterized, more than 3,600 citations are listed in PubMed under the search term "calcium-sensing receptor." The aim of this research topic is to group research articles and reviews that highlight the latest discoveries on the multifaceted roles of the CaSR both in health and disease.

The excellent overview of Conigrave describes how CaSR regulates parathyroid function in health and disease and how studies of this receptor have led to finding new approaches to treat various disorders of parathyroid function and calcium metabolism. He provides an account of the first characterization of the pivotal parathyroid Ca^{2+} sensing mechanism and how key biochemical features of the signaling mechanisms were exploited to clone the CaSR.

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In order to understand the function of the CaSR it is important to know its structure and the factors responsible for its regulation. The crystal structure of the extracellular domain (ECD) of the CaSR has been deciphered only recently (Geng et al., 2016; Zhang et al., 2016). In the present research topic Zhang et al. review the newest insights into the molecular basis of the structure and functional cooperativity of the CaSR, describing the structure–function relationships of the various domains of the CaSR protein. Numerous mutations of the *CASR* gene leading to human diseases were identified. The paper from Zhang et al. summarizes the four major types of mutations that lead to calcitropic diseases, a subject that is considered in more detail by Roszko et al. In their paper Roszko et al. describe two forms of hypoparathyroidism: autosomal dominant hypocalcaemia (ADH) type 1, caused by activating mutations of the *CASR* gene and ADH type 2, caused by gain-of-function mutations in the alpha subunit of the G protein G11 (Gα11), a key signaling partner of the CaSR.

The level of the CaSR mRNA and protein is very different in the different tissues. Hendy and Canaff reviewed the efforts made by numerous groups to unveil the mechanisms that regulate CaSR expression. The multiple promoters offer the potential for tissue-specific regulated expression from one promoter vs. ligand-specific responsiveness from the other. This up-to-date review of the structure and regulation of the CaSR describes that besides epigenetic mechanisms, increased levels of different pro-inflammatory cytokines, and active vitamin D_3, calcitriol modulate the expression of the CaSR. In the contribution of Aggarwal and Kállay the link between the CaSR and vitamin D is further analyzed, showing a multifactorial cross-talk between the two systems and demonstrating synergism in regulating multiple pathways involved in carcinogenesis. As Bikle et al. further show, mice lacking both the CaSR and the vitamin D receptor (VDR) in their epidermis, develop tumors spontaneously if fed low calcium diet, underlining the importance of both CaSR and VDR for keratinocyte differentiation and skin cancer prevention.

Next, the group of Carmen de Torres describes the role of the CaSR for the normal development of the central and peripheral nervous system, and its involvement in neuroblastoma differentiation. They suggest that pharmacological modulation of this GPCR might be beneficial in the treatment of neuroblastoma and other developmental diseases of the nervous system. Indeed, the CaSR is a key target for Ca_o^{2+} signaling in neurons, as it is so well described by Jones and Smith, who show that the CaSR regulates intrinsic excitability, synaptic transmission, and neuronal activity and it is involved in the pathogenesis of acute neurological diseases like stroke, traumatic brain injury, and epilepsy. Moreover, the CaSR seems to be involved also in the pathophysiology of Alzheimer's disease. Chiarini et al. show that in the late-onset form of the disease exogenous amyloid-β_{42} oligomers are able to bind to the CaSR on neurons and astrocites, inhibit the proteolysis of the intracellular amyloid-β_{42} oligomers, leading to their accumulation. They found that selective allosteric CaSR antagonists were able to suppress these neurotoxic effects. Tharmalingam and Hampson focus on the role of the CaSR in cell differentiation and migration, and the mechanisms underlying these processes in the developing central nervous system and in cancer, suggesting that the CaSR may serve either as a tumor suppressor or oncogene. They address recent developments that show that the CaSR interacts with integrins and that the CaSR/integrin complexes may function as a universal cell migration or homing complex.

Smith et al. review the role of the CaSR in pulmonary hypertension. They show that the CaSR is upregulated in smooth muscle cells of the pulmonary arteries of patients with pulmonary artery hypertension which leads to more robust CaSR-mediated cytosolic Ca^{2+} increase and enhanced proliferation. However, pharmacological inhibition of the CaSR prevents the progression of experimental pulmonary hypertension, suggesting that targeting the CaSR may be useful as a novel therapeutic approach.

As shown by Kim and Wysolmerski in normal breast epithelial cells the CaSR ensures adequate translocation of Ca^{2+} from the blood to the milk during lactation, while inhibiting parathyroid hormone-related protein (PTHrP) secretion. Interestingly, in breast cancer cells, CaSR activation leads to higher PTHrP secretion due to a switch in G protein coupling, from $G\alpha_i$ to $G\alpha_s$, thus stimulating cell proliferation and inhibiting apoptosis. In her excellent review, Ellinger provides an overview of the roles of the CaSR in the different reproductive organs, bringing evidence that the CaSR is an important regulator of the physiology of mammalian reproductive and developmental processes, pointing to the necessity to explore more in depth the role of the CaSR in male and female infertility.

The role of the CaSR as mediator of adipose tissue dysfunction was examined by Roberto Bravo-Sagua et al. who show that the CaSR is a potential regulator of white adipose tissue physiology. Tang et al. cover the diverse roles of the CaSR as a nutrient sensor in the physiology of the gastrointestinal tract, which include diverse actions from modulation of acid secretion in the stomach, amino acid-stimulated release of gut hormones in the small intestine, suppression of fluid secretion in the colon, regulation of microbiome composition to inhibitory actions on the proliferation of colonic crypt cells. An active CaSR is relevant for the regulation of intestinal barrier integrity and motility. There is evidence that the CaSR may participate in regulation of food intake, and CaSR agonists affect the enteric nervous system, and are able to restore imbalanced immune responses.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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frontiers in Physiology

REVIEW
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The Calcium-Sensing Receptor and the Parathyroid: Past, Present, Future

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Parathyroid hormone (PTH) defends the extracellular fluid from hypocalcemia and has powerful and well-documented actions on the skeleton and renal tubular system. To achieve a satisfactory stable plasma calcium level, the secretion of PTH, and the resulting serum PTH level, is titrated carefully to the prevailing plasma ionized Ca^{2+} concentration via a Ca^{2+} sensing mechanism that mediates feedback inhibition of PTH secretion. Herein, I consider the properties of the parathyroid Ca^{2+} sensing mechanism, the identity of the Ca^{2+} sensor, the intracellular biochemical mechanisms that it controls, the manner of its integration with other components of the PTH secretion control mechanism, and its modulation by other nutrients. Together the well-established, recently elucidated, and yet-to-be discovered elements of the story constitute the past, present, and future of the parathyroid and its calcium-sensing receptor (CaSR).

Keywords: calcium-sensing receptor, parathyroid, phospholipase C, adenylate cyclase, heterotrimeric G proteins, Calcimimetics, calcilytics, mineral metabolism

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INTRODUCTION

The parathyroid gland elaborates a peptide hormone, parathyroid hormone (PTH) whose primary role is to prevent and/or reverse acute hypocalcemia. It achieves this by: mobilizing calcium from stores in bone; stimulating renal Ca^{2+} reabsorption; and promoting the production of 1,25-dihydroxyvitamin D_3 to drive intestinal calcium absorption. To prevent uncontrolled elevations in plasma calcium concentration in response to PTH, a molecular feedback mechanism mediated by the extracellular Ca^{2+} ion concentration (Ca^{2+}_o) suppresses PTH secretion from the cells of the gland (review: Conigrave and Ward, 2013). While this mechanism operates primarily on parathyroid chief cells, which are the most numerous cell type and major site of PTH production, it may also operate on a second less numerous cell type, the parathyroid oxyphil cells (Ritter et al., 2012). In addition to providing acute control of PTH secretion from both newly-formed secretory vesicles and stored secretory granules, the Ca^{2+}-mediated feedback mechanism also suppresses the transcription of the PreProPTH gene and cell proliferation (review: Brown and MacLeod, 2001). Herein, I provide an account of how the pivotal parathyroid Ca^{2+} sensing mechanism was first characterized and how key biochemical features of the signaling mechanisms were exploited to clone the class C G-protein coupled receptor (GPCR) we now know as the calcium-sensing receptor (CaSR). I go on to describe how studies of this receptor in these cells have led to deep understandings of parathyroid function in health and disease and new approaches to therapies for various disorders of calcium metabolism and parathyroid function.

THE PAST

In vivo and *In vitro* Evidence for a Parathyroid Ca^{2+} Sensing Mechanism

Surgical removal of the parathyroid glands, whether intentional or inadvertent, induces acute, and in some cases catastrophic, hypocalcemia in experimental animals and in humans (e.g., MacCallum and Voegtlin, 1909; MacCallum et al., 1914; Westerdahl et al., 2000; Vasher et al., 2010; Salinger and Moore, 2013). In addition, perturbations of the plasma ionized calcium concentration *in vivo* by intravenous infusions of calcium salts to induce hypercalcemia or Ca^{2+} chelators such as citrate or EGTA to induce hypocalcemia provoke rapid negative and positive changes in the serum PTH concentration respectively (Fox and Heath, 1981; Conlin et al., 1989; Schwarz et al., 1992). These studies demonstrate that the gland is equipped with a Ca^{2+}-sensor that suppresses PTH secretion in response to elevated Ca^{2+} concentration.

The successful preparation of bovine parathyroid cells using collagenase digestion of sliced parathyroid gland tissue provided novel opportunities to assess the cellular Ca^{2+} sensing mechanism *in vitro* (Brown et al., 1976) and similar observations were made for porcine (Morrissey and Cohn, 1978) and also human (Birnbaumer et al., 1977; Brown et al., 1978a, 1979a; Conigrave et al., 2004) parathyroid cells. In all these cases, mammalian parathyroid cells in primary culture supported a robust endogenous secretion of PTH that was promptly shut off upon elevation of $Ca^{2+}{}_o$. In cells prepared from samples of parathyroid tissue derived from patients with primary hyperparathyroidism there was impairment but not complete loss of $Ca^{2+}{}_o$ sensitivity (Brown et al., 1979a,c; Mun et al., 2009). The behavior raises questions about the nature of the extracellular Ca^{2+} sensor. It also raises questions about the nature of the intrinsic/endogenous PTH secretion mechanism.

In the first description of a viable, functional parathyroid cell preparation (Brown et al., 1976) bovine parathyroid cells in primary culture in Eagle's medium (minus bicarbonate) secreted PTH linearly at a rate of 20–30 pmol $cell^{-1}$ h^{-1} for up to 3 h. PTH secretion was suppressed by around 60% at a $Ca^{2+}{}_o$ of 1.5 mM when compared to that observed at 0.5 mM $Ca^{2+}{}_o$. In the presence of 0.5 mM $Ca^{2+}{}_o$, elevated extracellular Mg^{2+} concentration ($Mg^{2+}{}_o$) also suppressed PTH secretion although $Mg^{2+}{}_o$ was less potent than $Ca^{2+}{}_o$. Finally, increases in PTH secretion were observed in response to the β-adrenergic agonist isoproterenol that were partially reversed by the β-adrenergic antagonist propranolol (Brown et al., 1976). Thus, key features of the preparation included: $Ca^{2+}{}_o$- and $Mg^{2+}{}_o$-mediated suppression of PTH secretion, pointing to the existence of an intrinsic divalent cation sensor with a preference for $Ca^{2+}{}_o$ over $Mg^{2+}{}_o$; and stimulation of PTH secretion by cAMP-linked GPCRs including beta-adrenergic, dopaminergic, and prostanoid receptors (Brown et al., 1977a,b; Gardner et al., 1980). These findings pointed to the existence of neuronal, hormonal, and/or local stimulatory control of PTH secretion. Although not clearly identified, the findings also demonstrated the existence of an intrinsic PTH secretion mechanism. According to one interpretation, parathyroid cells are equipped with a constitutive PTH secretion mechanism. According to an alternative interpretation, parathyroid cells respond to an autocrine/paracrine mechanism that supports PTH secretion.

The Concept of a Calciostat and an Extracellular Ca^{2+} Set-Point

The Ca^{2+}-sensing mechanism in the parathyroid supports the operation of an extracellular "calciostat" *in vivo*. The set-point for this calciostat occurs at a plasma ionized Ca^{2+} concentration of around 1.1–1.2 mM corresponding to plasma total calcium concentrations of around 2.2–2.4 mM, of which approximately half is in an albumin-bound form. PTH secretion rates rise 2 to 4-fold as $Ca^{2+}{}_o$ drops toward 1.0 mM and are effectively suppressed by >50% as $Ca^{2+}{}_o$ rises toward 1.4 mM (review: Conigrave et al., 2000a). The changes in PTH secretion rate are reflected in consonant changes in the serum PTH level (normal range 1–6 pmol/L). This set-point behavior can be readily demonstrated in perifused parathyroid cell preparations including those prepared from human parathyroid glands (Conigrave et al., 2004; **Figure 1**). $Ca^{2+}{}_o$-dependent inhibitory control of renal Ca^{2+} reabsorption, resulting in elevated renal calcium excretion, also contributes to the calciostat function, providing a key element of the defense against hypercalcemia (Kantham et al., 2009; Loupy et al., 2012).

Extracellular Ca^{2+}-Mediated Signaling Mechanisms

cAMP Promotes PTH Secretion via a Ca^{2+}-Sensitive Pathway

Suppression of cAMP levels accompanies high $Ca^{2+}{}_o$-induced suppression of PTH secretion in parathyroid cells stimulated to secrete by exogenous agonists of G_s-coupled GPCRs (Brown et al., 1977a, 1979b, 1978b, 1985; Windeck et al., 1978) and also in cells not exposed to exogenous GPCR activators, in which intracellular cAMP levels are typically much lower (≤5% of those in stimulated cells; Brown et al., 1978b). Excellent correlations were observed between cAMP levels and PTH secretion rates in these experiments supporting the hypothesis that cAMP is a primary driver of both exogenous GPCR-stimulated and intrinsic PTH secretion (Brown et al., 1978b). Similar results were obtained in a comparative analysis of the effects of divalent and tervalent cations on PTH secretion and cAMP accumulation (Brown et al., 1990). If this is so, the mechanisms of $Ca^{2+}{}_o$-dependent suppression of cAMP levels and PTH secretion are different under the conditions of (i) exogenous, GPCR-stimulated and (ii) spontaneous PTH secretion. This follows because pertussis toxin disabled $Ca^{2+}{}_o$- and divalent/tervalent cation-induced suppression of dopamine-stimulated PTH secretion (Chen et al., 1989; Brown et al., 1990), demonstrating that G_i is required for inhibitory control of PTH secretion downstream of cAMP-linked GPCRs, but pertussis toxin had no dis-inhibitory effect on high $Ca^{2+}{}_o$-mediated suppression of intrinsic PTH secretion i.e., in the absence of exogenous GPCR activators (Brown et al., 1992). Findings in support of the hypothesis that pertussis toxin suppresses both

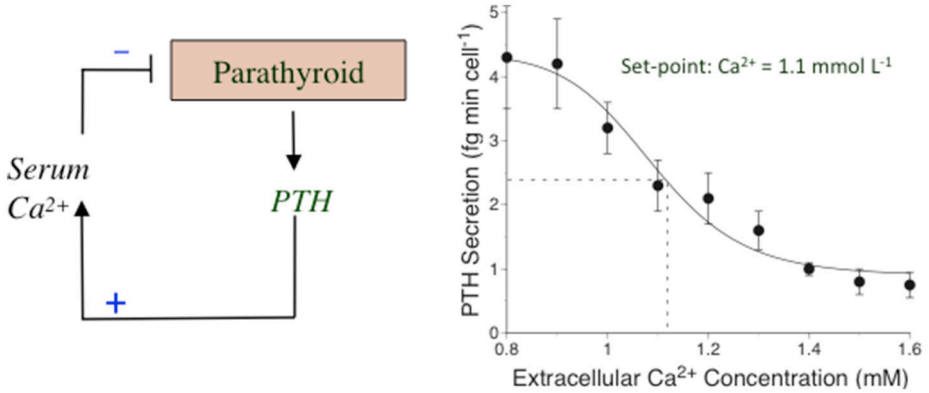

FIGURE 1 | The calciostat in parathyroid cells. Left: A representation of the feedback mechanism by which PTH elevates the serum* Ca^{2+} concentration and Ca^{2+} feeds back on the parathyroid to suppress PTH secretion in a process mediated by the CaSR. **Right:** Human parathyroid cells were perifused with HEPES-buffered physiological saline solutions containing various Ca^{2+} concentrations and samples of perifusate were collected at various times and subsequently analyzed for PTH1–84 as described in Conigrave et al. (2004). The results have been re-drawn. *Total and ionized calcium concentrations are comparable in serum and plasma since the major calcium-binding protein, albumin is present in similar concentrations in both these fluids.

exogenous GPCR-stimulated and endogenous PTH secretion (Fitzpatrick et al., 1986a) have not been confirmed.

The results suggest the existence of an extracellular Ca^{2+} sensor that is capable of activating G_i to suppress cAMP synthesis and, in turn, cAMP-linked PTH secretion in the presence of exogenous agonists that markedly elevate cAMP levels. The lack of association between G_i, cAMP levels, and PTH secretion in parathyroid cells *NOT* exposed to exogenous GPCR activators, on the other hand, points to a distinct biochemical mechanism arising either from a second Ca^{2+} sensor or from a single Ca^{2+} sensor that couples to distinct downstream signaling pathways depending on whether the cells have been stimulated to secrete PTH by exogenous activators or are operating spontaneously (**Figure 2**). Support for the hypothesis that the Ca^{2+} sensing mechanism in parathyroid cells is mediated by Ca^{2+} channels and controlled by the activity of pertussis toxin-sensitive G-proteins (Fitzpatrick et al., 1986a,b) has not been supported by other studies (e.g., Brown et al., 1992). More recent work has implicated $G_{q/11}$ and, possibly, phosphatidylinositol-specific phospholipase C (PI-PLC) and $ERK_{1/2}$ downstream of an extracellular Ca^{2+} sensing GPCR (see below).

Intracellular Ca^{2+} Mobilization and PI-PLC

An alternative signaling pathway, downstream of an extracellular Ca^{2+} sensor was subsequently identified in populations of bovine parathyroid cells loaded with the cell-permeant Ca^{2+}-sensitive fluorophore fura-2AM. The cells exhibited robust intracellular Ca^{2+} transients in response to elevated $Ca^{2+}{}_o$ suggesting the action of a PI-PLC coupled GPCR that senses increases in $Ca^{2+}{}_o$ (Nemeth and Scarpa, 1986, 1987a). Furthermore, they exhibited similar intracellular Ca^{2+} transients in response to elevated Mg^{2+} or Sr^{2+} concentration consistent with the observations referred to above that the parathyroid Ca^{2+} sensing mechanism is promiscuous with respect to divalent cations (Chen et al., 1989; Brown et al., 1990). To investigate whether the parathyroid Ca^{2+} sensor might indeed be a PI-PLC coupled GPCR, further studies demonstrated that Ca^{2+}, Mg^{2+} and other inorganic divalent cations promoted the production of water-soluble [^{3}H]-inositol phosphates from [^{3}H]-inositol labeled cells (Brown et al., 1987; Shoback et al., 1988).

A Promiscuous Divalent/Multivalent Cation Sensor

Investigation of the molecular requirements for divalent cation sensing in parathyroid cell preparations led to some surprising observations. Firstly, tervalent inorganic cations of the lanthanide group including Gd^{3+} and Tb^{3+} were found to be high potency activators ($EC_{50} \approx 5–50\,\mu M$) of parathyroid PI-PLC, suppressors of GPCR-stimulated cAMP accumulation, and inhibitors of PTH secretion (Brown et al., 1990) in a manner analogous to divalent cations. Furthermore, and even more surprisingly, organic multivalent cations including polyarginine, polylysine, and protamine (Brown et al., 1991a), the PLC inhibitor neomycin (Brown et al., 1991b), and polyamines such as spermine (Nemeth and Scarpa, 1987b) stimulated intracellular Ca^{2+} mobilization and inhibited PTH secretion.

Expression Cloning of a Polyvalent Cation-Sensing Receptor from a Bovine Parathyroid cDNA Library

The demonstration that the parathyroid calcium sensor coupled to the activation of PI-PLC and, at least in certain circumstances, to heterotrimeric G_i G-proteins, and was promiscuous with respect to inorganic and organic multivalent cations provided a strategy by which a putative PLC-coupled receptor might be cloned by cellular expression of pools of mRNA derived from a size-fractionated bovine parathyroid cDNA library (Brown et al., 1993). Xenopus oocytes express a large conductance Cl^- channel whose open probability is highly sensitive to changes in intracellular Ca^{2+} concentration (e.g., downstream of GPCR-mediated generation of IP_3 and intracellular Ca^{2+} mobilization).

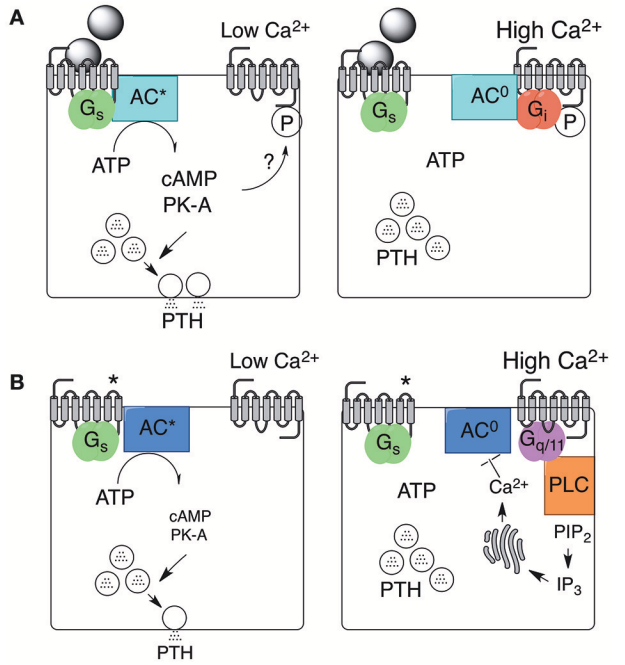

FIGURE 2 | Stimulated and spontaneous mechanisms in support of PTH secretion and its inhibition by high $Ca^{2+}{}_o$. PTH secretion and its inhibition by high $Ca^{2+}{}_o$ arises from two distinct mechanisms. One mechanism is supported by exogenous agonists, including neurotransmitters or hormones, that activate G_S-coupled GPCRs as shown in **(A)** (left and right). PTH secretion continues provided $Ca^{2+}{}_o$ remains low but is promptly inhibited by G_i-dependent inhibition of adenylate cyclase in the presence of high $Ca^{2+}{}_o$. The mechanism by which the $Ca^{2+}{}_o$ sensor, now known to be the CaSR, preferentially binds to G_i in this context is not known but might depend on local protein kinase-A (PK-A) activation. A second mechanism occurs spontaneously and may be supported by constitutive G_S-coupled GPCR activity (as shown in **B**; left and right) or by autocrine/paracrine production of receptor activators. PTH secretion via this second mechanism continues provided $Ca^{2+}{}_o$ remains low but is inhibited by high $Ca^{2+}{}_o$-induced $G_{q/11}$-dependent activation of intracellular Ca^{2+} mobilization or Ca^{2+} influx (not shown). One possible mechanism by which increased intracellular free Ca^{2+} concentration ($Ca^{2+}{}_i$) suppresses PTH secretion is shown via $Ca^{2+}{}_i$-dependent inhibition of adenylate cyclase. *Receptor activated in the absence of neuronal or hormonal stimuli.

In this case, the successful cloning of the novel class C GPCR that is now referred to as "the calcium-sensing receptor" relied on its high degree of sensitivity to Gd^{3+}, which was used to identify "active" pools of mRNA for further separation and purification. Once cloned, the receptor was readily expressed not only in Xenopus oocytes but also various mammalian cell lines including HEK-293 cells and was found to exhibit sensitivity not only to divalent inorganic cations including Ca^{2+} and Mg^{2+}, and tervalent inorganic cations including Gd^{3+} but also to organic cations including the antibiotic neomycin (Brown et al., 1993), polyamines such as spermine (Quinn et al., 1997), cationic polypeptides such as polyarginine and polylysine (Ray and Northup, 2002), and cationic proteins including beta amyloid (Ye et al., 1997). The cloning of the bovine parathyroid CaSR was followed subsequently by the cloning of its orthologs from human parathyroid (Garrett et al., 1995), rat kidney (Riccardi et al., 1995), and rat brain (Ruat et al., 1995).

The CaSR is known now to be expressed widely, with various $Ca^{2+}{}_o$-dependent and non-$Ca^{2+}{}_o$ dependent functions in cell and developmental biology as detailed elsewhere in this issue. It is also known to activate a large number of signaling pathways downstream of various G-proteins and multiple cell membrane-associated as well as cytoplasmic enzymes (review: Conigrave and Ward, 2013).

The CaSR mediates, for example, the activation of various protein kinases including protein kinase C isoforms, which negatively modulate CaSR function (Jiang et al., 2002; Davies et al., 2007; Lazarus et al., 2011; Young et al., 2014), and the mitogen activated protein (MAP) kinases $ERK_{1/2}$, p38 and JNK (Kifor et al., 2001; Tfelt-Hansen et al., 2003; review: Conigrave and Ward, 2013). The roles of protein kinases in CaSR-mediated inhibitory control of PTH secretion are not well-understood but $ERK_{1/2}$ appears to contribute (Corbetta et al., 2002) and could be activated downstream of either $G_{q/11}$ or G_i (review: Conigrave and Ward, 2013).

While the CaSR is expressed and trafficked to the plasma membrane as functional homodimers (Bai et al., 1998, 1999) that couple efficiently to $G_{q/11}$, it is also capable of forming heterodimers with other members of GPCR family C including metabotropic glutamate receptors (Gama et al., 2001) and $GABA_B$ receptors, especially $GABA_{B1}$ (Chang et al., 2007; Cheng et al., 2007). The consequences of heterodimerization for receptor localization to specific subdomains of the plasma membrane and for signaling pathway selection in different tissues and for the parathyroid, in particular, are not yet clear.

Physiological and Clinical Significance of the CaSR for Parathyroid Function

Parathyroid and Mineral Disorders Linked to CaSR Mutations (and Anti-CaSR Antibodies)

As the bovine parathyroid, rat kidney, and human parathyroid CaSR cDNAs were cloned (Brown et al., 1993; Garrett et al., 1995; Riccardi et al., 1995), it became possible to assess whether any recognized human disorders of calcium metabolism and/or parathyroid function arose from mutations of the CaSR. This was rapidly confirmed for two hypercalcemic disorders in which the CaSR is hypofunctional: the uncommon disorder known as familial hypocalciuric hypercalcemia (FHH); and the extremely rare disorder known as neonatal severe hyperparathyroidism (NSHPT; Pollak et al., 1993, 1994; reviews: Brown et al., 1995; Hendy et al., 2000). It was subsequently also confirmed for the hypocalcemic disorder known as autosomal dominant hypocalcemia (ADH; Pearce et al., 1996) in which the CaSR is hyperfunctional.

FHH

Deactivating, typically heterozygous, mutations of the CaSR gene in FHH result in impaired or disabled $Ca^{2+}{}_o$-dependent inhibition of renal Ca^{2+} reabsorption, leading to hypocalciuria, and as well as impaired $Ca^{2+}{}_o$-dependent feedback inhibition of PTH secretion, typically without frank elevations in the serum PTH level as a result of associated increases in $Ca^{2+}{}_o$

(Chu et al., 1995; review: Brown et al., 1995). Instead, the set-point for $Ca^{2+}{}_o$-dependent suppression of PTH secretion rises thereby increasing the value of the calciostat and the steady-state $Ca^{2+}{}_o$ adopts this new level. The primary driver for the increase in $Ca^{2+}{}_o$ appears to be impaired renal calcium excretion, resulting in characteristic hypocalciuria (uCa/Cr ratio < 0.04 mmol mmol^{-1}; uCa excretion < 1.5 mmol d^{-1}). With the identification of two variants of FHH arising from mutations of two other genes, Gα11 and AP2S (see below), the major form of FHH that arises from mutations of the CaSR has been recently renamed FHH1.

NSHPT

In contrast to FHH, homozygous or compound heterozygous deactivating mutations of the CaSR gene have been linked to a severe hypercalcemic disorder that presents in neonatal life with total plasma calcium concentrations that may exceed 4.0 mM (Ward et al., 2004). In addition, there are marked elevations in the serum PTH level, indicative of near-total failure of $Ca^{2+}{}_o$-mediated feedback control of PTH secretion along with skeletal demineralization and pathological fractures (Pollak et al., 1993; review: Brown et al., 1995). The disorder responds promptly to total parathyroidectomy i.e., excision of all four parathyroid glands (Marx et al., 1986) demonstrating that the bone disease is driven by severe primary hyperparathyroidism.

Whether still more severe disorders of skeletal development and metabolism might arise from other types of CaSR mutations is not yet clear. Recently developed mouse models, however, suggest that this is so (Chang et al., 2008; Richard et al., 2010; reviews: Goltzman and Hendy, 2015; Santa Maria et al., 2016). An authoritative database of CaSR mutations and their links to human disease is maintained at: http://www.casrdb.mcgill.ca/.

ADH

Two other rare mineral disorders affecting the parathyroid arise from activating mutations of the CaSR. In one, autosomal dominant hypocalcemia, there is hypocalcemia and inappropriately normal or frankly low serum PTH levels arising from a reduction in the set-point for extracellular Ca^{2+} (Pearce et al., 1996). One or more of the following may also be observed: hypercalciuria, consistent with enhanced inhibition of renal Ca^{2+} reabsorption; hypocalciuria (e.g., Tan et al., 2003), consistent with reduced glomerular filtration of Ca^{2+} ions and a largely intact renal Ca^{2+} reabsorption mechanism; hypomagnesemia; and hyperphosphatemia (reviews: Thakker, 2004; Egbuna and Brown, 2008). This is typically a chronic benign condition, often diagnosed as an incidental finding on plasma biochemical analysis, in which there may be a longstanding history of paresthesiae, intermittent fasciculations and/or contractions of isolated muscle groups. There may also be a history of one or more childhood seizures including febrile convulsions (reviews: Thakker, 2004; Egbuna and Brown, 2008).

In a second disorder, arising from more severe activating mutations of the CaSR, a form of renal salt wasting also occurs. This Bartter Syndrome (type-5) arises from unrestrained CaSR activation on the contraluminal membrane of the thick ascending limb, which disables NKCC2-dependent NaCl reabsorption (reviews: Gamba and Friedman, 2009; Riccardi and Brown, 2010).

The impact of gene dosage on the severity of autosomal dominant hypocalcemia has been evaluated in a mouse model, the *Nuf* mouse (L723Q, affecting a residue at the C-terminal end of iL-2), which exhibits hypocalcemia, suppressed serum PTH levels, hypocalciuria, hyperphosphatemia, and ectopic mineralization and cataracts (Hough et al., 2004). All aspects of the phenotype were more severe in homozygous when compared to heterozygous mice demonstrating that a gene dosage effect applies in the case of activating as well as inactivating mutations of the CaSR, and it is notable that renal hypophosphaturia occurred in homozygous but not heterozygous *Nuf* mice consistent with the idea that the CaSR normally suppresses renal phosphate excretion including PTH-induced inhibition of phosphate reabsorption (Riccardi et al., 2000; Ba et al., 2003; reviews: Riccardi and Valenti, 2016) and thus promotes phosphate retention. The disorder is amenable to treatment with negative modulators of the CaSR, also known as calcilytics (see below; Mayr et al., 2016; Nemeth and Goodman, 2016).

Disorders of Calcium Metabolism Arising from Autoantibodies that Target the CaSR

In addition to the impact of inactivating or activating CaSR mutations on calcium metabolism and parathyroid function as described above, several studies have drawn attention to the clinical impact of autoantibodies that target the CaSR with either inactivating (Kifor et al., 2003; Pallais et al., 2004) or activating (review: Brown, 2009) effects, presumably dependent on the peptide epitope that is recognized. These autoimmune disorders of calcium metabolism resemble other autoimmune endocrinopathies such as Grave's disease (review: Thakker, 2004). In one of these disorders associated with autoimmune polyendocrinopathy, autoantibodies to several CaSR epitopes have been identified corresponding to residues 41–69 at the receptor's N-terminus, 114–126 at the dimer interface, and 171–195 in the vicinity of the Venus FlyTrap (VFT) domain's binding cleft (Kemp et al., 2010).

Transgenic Mouse Models—Impact of Inactivating CaSR Mutations on Parathyroid Function

The first reported transgenic mouse in which the CaSR was "knocked out," was homozygous for a 20 bp insertion that disabled incorporation of CaSR exon-5 (referred to as CaSR exon-4 in the paper) into the mature, fully processed mRNA (Ho et al., 1995). CaSR exon-5 encodes residues 465–536 (http://www.casrdb.mcgill.ca) at the extreme C-terminal end of the VFT domain, immediately prior to the start of the Cysteine-rich domain. Mice with this genotype exhibited a condition comparable to NSHPT in which homozygotes were normal at birth but exhibited severe growth retardation and markedly reduced muscle power in the days after birth (Ho et al., 1995).

The results of biochemical analyses demonstrated the cardinal features of primary hyperparathyroidism including markedly elevated plasma Ca^{2+} concentration, suppressed plasma inorganic phosphate concentration, and markedly elevated serum PTH levels. In addition, the parathyroid glands

were enlarged with prominent chief cell hyperplasia (Ho et al., 1995). These findings are consistent with a severe resistance syndrome arising from markedly impaired Ca^{2+}-dependent feedback control of PTH secretion i.e., with loss of the parathyroid Ca^{2+} sensor.

Heterozygotes, unlike the homozygotes, were phenotypically normal in the weeks and months after birth but exhibited mild biochemical disturbances consistent with FHH in humans including mildly elevated plasma Ca^{2+} concentration, suppressed renal calcium excretion, and inappropriately normal plasma PTH levels. These findings suggest a mildly impaired but intact parathyroid Ca^{2+} sensing mechanism together with impaired extracellular Ca^{2+}-dependent inhibition of renal Ca^{2+} reabsorption resulting in an increase in the setpoint of the calciostat.

Is the Parathyroid Equipped with an Alternative Calcium-Sensing Receptor?

While other class C GPCRs, like the CaSR, exhibit Ca^{2+}-sensing properties (Kubo et al., 1998; Wise et al., 1999; Christiansen et al., 2007) it seems unlikely that the parathyroid is equipped with an alternative CaSR since, as described above, mice that are homozygous for either global (Ho et al., 1995) or tissue-selective (Chang et al., 2008) knockouts of the CaSR exhibit a severe, uncompensated form of primary hyperparathyroidism in which the plasma levels of both PTH and calcium are markedly elevated from birth. The phenotype suggests a marked impairment of Ca^{2+}_o-dependent negative feedback on PTH secretion with attendant hyperparathyroidism and PTH-dependent bone resorption. Thus, if the parathyroid expresses an alternative or supplementary calcium sensor, it is unable to compensate for loss of the CaSR. It is possible that under some circumstances Ca^{2+}-sensing is mediated not by CaSR homodimers but by CaSR heterodimers involving other members of GPCR family C including metabotropic glutamate receptors or $GABA_{B1}$ receptors as noted above (Gama et al., 2001; Chang et al., 2007; Cheng et al., 2007).

Previous work suggested a role for Ca^{2+}-permeable channels in the control of PTH secretion based on observations that stereoisomers of the Ca^{2+} channel modulator 202–791 either inhibited (+202 to 791) or stimulated (−202 to 791) PTH secretion (Fitzpatrick et al., 1986b), and antibodies that target skeletal muscle Ca^{2+} channels also modulated PTH secretion (Fitzpatrick et al., 1988). Other Ca^{2+} channel activators, including maitotoxin (Fitzpatrick et al., 1989), and the diltiazem analog TA-3090 (Chen and Brown, 1990) were also found to inhibit PTH secretion. This work was "turned on its head" by the successful development of "calcimimetics" by structural modification of an L-type Ca^{2+} channel blocker, fendiline (Nemeth et al., 1998), and the subsequent demonstration that modulation of PTH secretion by these agents arises not from actions on Ca^{2+} channels but rather the cloned CaSR (Nemeth et al., 2004; review: Nemeth, 2006). Thus, various agents that modulate Ca^{2+} channel activity can also interact with an allosteric site in the CaSR's heptahelical domain (Leach et al., 2016). Calcimimetics, positive modulators of the CaSR, and calcilytics, negative modulators of the CaSR, are discussed in greater detail below.

Nevertheless, more recent work raises the possibility that Ca^{2+}-permeable channels may indeed contribute to the control of PTH secretion. Thus, parathyroid cells express NMDA receptor subunits and NMDA inhibits PTH secretion (Parisi et al., 2009). While these receptors may contribute to the tonic control of PTH secretion, it is not known whether Ca^{2+} fluxes arising from the activation of NMDA receptors are sensitive to Ca^{2+}_o concentration in parathyroid cells. In addition, various amino acids and amino acid analogs are known to interact with the CaSR (Conigrave et al., 2000b, 2004; review: Conigrave and Hampson, 2010) and it is not yet clear whether the inhibitory effect of NMDA on PTH secretion is exerted by the activation of Ca^{2+}-permeable ion channels or via positive modulation of the CaSR.

THE PRESENT

Development of Calcimimetics and Their Utility in Several Forms of Hyperparathyroidism

As noted above, calcimimetics were developed from the Ca^{2+} channel blocker fendiline that induces Ca^{2+}_i mobilization and suppresses PTH secretion from bovine parathyroid cells (Nemeth et al., 1998; review: Nemeth, 2006). Drug development resulted in a new class of pharmaceuticals, the phenylalkylamine calcimimetics, which are positive allosteric modulators of the CaSR that markedly enhance the sensitivity of CaSR-mediated intracellular signaling pathways to Ca^{2+}_o (Nemeth et al., 1998). Early examples included NPS R467 and NPS R568, which together with their less potent S-isomers have been key agents for the analysis of CaSR-mediated effects in various cell and tissue systems. More recent examples include cinacalcet, an agent that is well-absorbed orally (Nemeth et al., 2004) and is effective clinically in the treatment of both secondary hyperparathyroidism due to chronic kidney disease (Moe et al., 2005; Messa et al., 2008) as well as primary hyperparathyroidism (Peacock et al., 2005, 2011; see also review: Nemeth and Shoback, 2013).

One key effect of calcimimetics is suppression of the serum PTH level. In primary hyperparathyroidism, for example, in which the plasma total calcium concentration is typically elevated from its normal upper limit of 2.6 mM to around 2.8–3.0 mM, oral therapy with cinacalcet suppressed serum PTH levels and restored the plasma calcium concentration into the normal range for up to 12 months or more (Peacock et al., 2005). Another key effect is suppression or even reversal of parathyroid hyperplasia. For example, cinacalcet suppresses parathyroid cell proliferation and reduces gland size in models of primary (Imanishi et al., 2011) and secondary (Colloton et al., 2005; Miller et al., 2012) hyperparathyroidism, and also induces apoptosis in second hyperparathyroidism (Tatsumi et al., 2013).

The demonstration that calcimimetics from the same class and across different classes exhibit different biased signaling profiles (Davey et al., 2012) is encouraging efforts to develop

new generation calcimimetics in support of tissue-specific CaSR-targeted pharmacotherapy e.g., parathyroid vs. kidney vs. thyroid C-cells (review: Leach et al., 2015). Recent modeling of calcimimetic binding in the CaSR's heptahelical domain suggests that agents such as A265347 with less pronounced biased signaling profiles may bind more deeply in the allosteric pocket (Leach et al., 2016).

More recently, a peptide activator of the CaSR (AMG-416; L-Cys-AcDCys-DAla-$(DArg)_2$-DAla-$DArgNH_2$) has entered clinical practice for the treatment of patients with secondary hyperparathyroidism on hemodialysis (Bell et al., 2015). Administered intravenously it has superior pharmacokinetics including effective suppression of PTH levels beyond 24 h (Walter et al., 2013) due, presumably, to its ability to form a di-sulfide with CaSR residue C482 in its extracellular domain (Alexander et al., 2015).

Calcilytics

Several classes of calcilytics (negative modulators of the CaSR) have been developed. These agents, in general, bind in the HH domain and suppress CaSR signaling. For this reason, they have proved useful in assessing the role of the CaSR in Ca^{2+}- or L-amino acid-induced cellular or tissue responses (e.g., Dvorak et al., 2004; Daly et al., 2013). In the parathyroid, calcilytics promote PTH secretion by reversing the inhibitory action of the CaSR (Nemeth et al., 2001). As a consequence, it was hoped that these agents might prove useful in the treatment of osteoporosis by elevating serum PTH levels to emulate the action of intermittent subcutaneous injections of PTH1–34 (teriparatide). However, none of the calcilytics that have entered human clinical trials, thus far, have been successful in significantly increasing bone density or reducing fracture risk (review: Nemeth and Goodman, 2016). Two main explanations seem reasonable: (i) the maximum increase in the serum level of endogenous PTH is significantly less than that achieved by subcutaneous injections of PTH1–34 (e.g., Kimura et al., 2011); or (ii) calcilytics suppress CaSRs in cells of the osteoblast lineage to interfere with PTH-induced cell maturation and key differentiated functions including matrix synthesis and mineralization (Dvorak et al., 2004).

Nutrient Activators of the CaSR

In addition to its regulation by Ca^{2+} ions, the CaSR also responds promiscuously to L-amino acids of various classes (Conigrave et al., 2000b), and one of the most potent, L-Trp, has been shown recently to bind in the receptor's VFT domain ligand-binding groove (Geng et al., 2016; see below). This behavior resembles that of several class C GPCRs (Conigrave and Hampson, 2006, 2010) and supports macronutrient sensing in various tissues including the gastrointestinal tract (review: Conigrave and Brown, 2006). Based on the signaling pathway analysis performed to date, however, Ca^{2+}_o and L-amino acids are not equivalent activators. In particular, L-amino acids preferentially activate a Ca^{2+}_i mobilizing pathway and have more limited actions on PI-PLC and $ERK_{1/2}$ (review: Conigrave and Ward, 2013). Nevertheless, L-amino acids are potent activators of Ca^{2+}_i mobilization in parathyroid cells and also suppress PTH secretion at physiologically relevant concentrations (Conigrave et al., 2004). Furthermore, glutathione and various analogs (e.g., S-methylglutathione) also activate Ca^{2+}_i mobilization and suppress PTH secretion, presumably by binding to the same VFT domain ligand-binding groove (Broadhead et al., 2011). These findings imply that protein nutritional state is negatively coupled to the control of PTH secretion and thus serum PTH levels. The full significance of these effects, however, is not yet known (see below).

Control of CaSR Gene Expression

Analysis of the promoter regions of the CaSR gene has led to the identification of two key positive modulators of expression: (i) inflammatory cytokines including IL-1β, IL-6 and TNFα (Canaff and Hendy, 2005); and (ii) hormonally active analogs of vitamin D including 1,25-dihydroxyvitamin D_3 (Canaff and Hendy, 2002), and possibly 25-hydroxyvitamin D_3, whose plasma levels are nearly 1000-fold higher. These results suggest that CaSR expression may be upregulated in the parathyroid and other CaSR-expressing tissues in response to various inflammatory conditions and in response to elevations in either serum 1,25-dihydroxyvitamin D_3 or 25-hydroxyvitamin D_3 levels.

RECENT DEVELOPMENTS AND THE FUTURE

G-Protein Coupling

The CaSR couples to various G-proteins (review: Conigrave and Ward, 2013). Notable from the perspective of parathyroid function are $G_{i,}$ which suppresses agonist-stimulated GPCR-mediated cAMP production and contributes to the activation of $ERK_{1/2}$ at least in part via β-arrestin, and $G_{q/11}$, which activates PI-PLC and induces Ca^{2+}_i mobilization, with attendant activation of several protein kinase C isoforms and $ERK_{1/2}$.

Both the G_i and $G_{q/11}$ pathways appear to be important for the inhibitory control of PTH secretion. With respect to G_q and G_{11}, it is now known that $G\alpha_q$ and $G\alpha_{11}$ are required for the normal control of PTH secretion. Thus, in a transgenic mouse in which parathyroid-specific ablation of $G\alpha_q$ was produced on a global $G\alpha_{11}$ null background, severe neonatal hyperparathyroidism was observed (Wettschureck et al., 2007) and resembled the phenotypes of both global (Ho et al., 1995) and parathyroid-specific (Chang et al., 2008) ablation of the CaSR. These findings demonstrate that G_q and G_{11} are required for CaSR-mediated control of PTH secretion and thus lie at the top of a key inhibitory signaling pathway(s). Consistent with these findings, inactivating and activating mutations of the human $G\alpha_{11}$ gene have been shown respectively to underlie variant forms of FHH (FHH2) and ADH (ADH2; Nesbit et al., 2013a; Gorvin et al., 2016; Piret et al., 2016).

Under certain circumstances, the CaSR also couples to G_s (review: Conigrave and Ward, 2013) but the significance of this pathway for the control of PTH secretion is unknown. It is interesting to speculate that the "inactive" form of the receptor, which is promoted under conditions of low Ca^{2+} and high phosphate concentrations (Geng et al., 2016) might preferentially couple to G_s in the parathyroid.

Receptor Trafficking

Receptor trafficking studies have largely focused on cell systems in which the CaSR is expressed heterologously (reviews: Breitwieser, 2013, 2014). These studies demonstrate that trafficking of the CaSR is modulated by various binding partner proteins (review: Huang and Miller, 2007), can be promoted by allosteric modulators such as cinacalcet and NPS-2143 acting as pharmaco-chaperones (Leach et al., 2013), and is sensitive to receptor-dependent signaling (Grant et al., 2011, 2012; review: Breitwieser, 2012). In the parathyroid, the CaSR interacts with caveolin and is thus likely to localize to sub-domains of the plasma membrane known as caveolae (Kifor et al., 1998). In addition, recent findings suggest that the CaSR is processed between the plasma membrane and intracellular endosomes via clathrin-coated vesicles since mutations of Arg15 of the sigma (σ) subunit of the clathrin-binding protein AP2 have been linked to a variant form of FHH, now known as FHH3 (Nesbit et al., 2013b). The findings suggest that the formation, and/or maintenance, of CaSR signaling complexes is impaired under conditions in which clathrin-coated vesicle-mediated processing of the CaSR is impaired.

X-ray Crystal Structures

While X-ray crystal structures of class C GPCR VFT domains (Kunishima et al., 2000; Tsuchiya et al., 2002), entire extracellular (VFT-plus-Cys-rich) domains (Muto et al., 2007), and even heptahelical domains (Doré et al., 2014) have been reported over the last 15 years, crystal structures for CaSR domains have only recently become available (Geng et al., 2016; Zhang et al., 2016).

These newly described CaSR structures provide information on the inactive and active forms of its VFT domain (Geng et al., 2016; Zhang et al., 2016) and entire extracellular domain (Geng et al., 2016). While the protein conformations of the active forms of the VFT domain structures were almost identical, the identification of divalent cation, and anion binding sites were quite different in the structures reported by the two groups. Zhang et al. (2016) identified just one Ca^{2+} site in the active form of the VFT domain and relied on modeling of electron densities to ascribe it to the ligand-binding cleft, where it was closely associated with an L-amino acid-binding site. Surprisingly, however, they identified a formaldehyde derivative rather than the native form of L-Trp in the site.

In the structures described by Geng et al. (2016), on the other hand, an anomalous mapping strategy was used to identify four, previously unrecognized, Ca^{2+} binding sites, one of which ("Site 2") was present in both the inactive and active structures and three of which were only identified in the active structure and, thus, may act to stabilize it. Interestingly, no Ca^{2+} binding site was located in the closed (active) form of the agonist-binding cleft in the structure reported by Geng et al., which was occupied instead by the amino acid L-Trp (Geng et al., 2016). In addition, Geng et al. identified several binding sites for inorganic phosphate in the inactive structure (Geng et al., 2016), raising the possibility that not only the $Ca^{2+}{}_o$ concentration but also the ratio of $Ca^{2+}{}_o$ to phosphate concentrations may control the receptor's transition between inactive and active states.

The findings that the receptor binds inorganic phosphate (P_i) as well as Ca^{2+} ions and that Ca^{2+} stabilizes the active state, whereas P_i stabilizes the inactive state have potentially important implications for understanding parathyroid function since elevated P_i concentrations stimulate PTH secretion (Slatopolsky et al., 1996) whereas elevated $Ca^{2+}{}_o$ inhibits it. Does the CaSR modulate its response to $Ca^{2+}{}_o$ according to the background level of inorganic phosphate? Does the Ca:P_i ratio determine PTH secretion rates by controlling the activation state of the CaSR? Does the CaSR act as a phosphate sensor in other tissues such as osteocytes or osteoblasts in bone?

Unresolved Problems

There are several unresolved problems. Four of them are considered below in the form of sets of questions.

Question-Set 1

What drives intrinsic PTH secretion and how does the CaSR suppress it in a G_i-independent manner? Is spontaneous PTH secretion truly constitutive, implying that the pathway by which PTH vesicles undergo exocytosis is unregulated? Alternatively, is it promoted by receptors expressed on the surface of parathyroid cells that are either constitutively active or exposed to locally released activators such as histamine from mast cells or prostanoids from chief or oxyphil cells?

Question-Set 2

What is the significance of amino acid-binding to the CaSR (Geng et al., 2016) for parathyroid function? Does the parathyroid CaSR read the local concentrations of L-amino acids arising from export of amino acids from the cytoplasm or are they determined by the amino acid concentrations in the bulk plasma. Does amino acid sensing by the CaSR primarily affect PTH secretion under conditions of protein deficiency and reductions in plasma amino acid levels as suggested by the phenomenon of secondary hyperparathyroidism in subjects on low protein diets (reviews: Conigrave et al., 2002, 2008) or does it act primarily to suppress PTH secretion under conditions of protein excess as suggested by parathyroid cell responses *in vitro* (Conigrave et al., 2004). Alternatively, might L-amino acid sensing by the CaSR provide a mechanism for adjusting the inhibitory gain on the receptor to the level of amino acid-dependent PTH synthesis?

Question-Set 3

What is the significance of CaSR heterodimerization for parathyroid function? Is the parathyroid subject solely to control by CaSR homodimers or are some Ca^{2+}-dependent signaling pathways (e.g., for the control of parathyroid chief cell number, or PreProPTH gene expression) subject to control by CaSR heterodimers with metabotropic glutamate receptors (Gama et al., 2001) or $GABA_{B1}$ receptors (Chang et al., 2007)?

Question-Set 4

Can CaSR expression be effectively upregulated in hypercalcemic conditions such as primary hyperparathyroidism or FHH to restore physiological control of plasma calcium levels and $Ca^{2+}{}_o$-dependent suppression of PTH secretion? Can CaSR expression be effectively downregulated in hypocalcemic conditions such as ADH to restore physiological control of plasma calcium and PTH levels? Can tissue-selective modulators of the vitamin D receptor

or cytokine receptors, or other strategies, be developed for the control of parathyroid CaSR expression?

CONCLUDING REMARKS

The role of the parathyroid in the whole body calcium economy is so important that the negative feedback loop by which PTH elevates plasma Ca^{2+} and Ca^{2+}, in turn, suppresses PTH secretion largely defines its place in human biology. Expression cloning of the CaSR, its identification as the key Ca^{2+} sensor of the parathyroid, and evaluation of its roles in normal tissue biology and in human disease have resolved key issues in calcium metabolism. New paradigms of Ca^{2+}-mediated control of tissue function and of the CaSR in macronutrient-sensing have followed. Incredibly, the molecular mechanism by which the CaSR suppresses PTH secretion is only partially solved: for the situation in which PTH secretion is stimulated by neurotransmitters or hormones that elevate cAMP levels. The mechanisms by which the CaSR suppresses intrinsic PTH secretion or the secretion of PTH downstream of hormones that activate PTH secretion by non-cAMP pathways remain undefined. Newly available X-ray crystal structures for the CaSR extracellular domain in its inactive and active conformations provide new opportunities to investigate the Ca^{2+} sensing mechanism.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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The Calcium-Sensing Receptor and Integrins in Cellular Differentiation and Migration

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The calcium-sensing receptor (CaSR) is a widely expressed homodimeric G-protein coupled receptor structurally related to the metabotropic glutamate receptors and GPRC6A. In addition to its well characterized role in maintaining calcium homeostasis and regulating parathyroid hormone release, evidence has accumulated linking the CaSR with cellular differentiation and migration, brain development, stem cell engraftment, wound healing, and tumor growth and metastasis. Elevated expression of the CaSR in aggressive metastatic tumors has been suggested as a potential novel prognostic marker for predicting metastasis, especially to bone tissue where extracellular calcium concentrations may be sufficiently high to activate the receptor. Recent evidence supports a model whereby CaSR-mediated activation of integrins promotes cellular migration. Integrins are single transmembrane spanning heterodimeric adhesion receptors that mediate cell migration by binding to extracellular matrix proteins. The CaSR has been shown to form signaling complexes with the integrins to facilitate both the movement and differentiation of cells, such as neurons during normal brain development and tumor cells under pathological circumstances. Thus, CaSR/integrin complexes may function as a universal cell migration or homing complex. Manipulation of this complex may be of potential interest for treating metastatic cancers, and for developmental disorders pertaining to aberrant neuronal migration.

Keywords: calcium, extracellular matrix, G-protein coupled receptor, GPRC6A, cerebellum, cerebellar granule cells, metastasis, medulloblastoma

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INTRODUCTION—GENERAL FEATURES OF CaSR STRUCTURE, ACTIVATION, AND EXPRESSION

The CaSR belongs to the metabotropic glutamate receptor subclass of G-protein coupled receptors (GPCRs). In addition to the eight metabotropic glutamate receptor subtypes, other members of this relatively small GPCR subfamily include the $GABA_B$ receptor, several taste receptors, and GPRC6A (reviewed in Conigrave and Hampson, 2010). Among these family members, the CaSR has particularly high homology, in terms of both protein sequence (31% amino acid identity) and gene structure, with GPRC6A (Kuang et al., 2005). Interestingly, GPRC6A is activated directly by several amino acids, the most potent of which are arginine and lysine, and is allosterically potentiated by extracellular Ca^{2+} (Kuang et al., 2005; Wellendorph et al., 2005), whereas the CaSR is activated directly by Ca^{2+} and allosterically modulated by amino acids (Conigrave et al., 2000). This similar but reciprocal pharmacological relationship is likely a reflection of the evolutionary

origins of the two receptors (Kuang et al., 2006). The close relationship between these two GPCRs is further illustrated by reports that both receptors are activated by multiple endogenous ligands (see Clemmensen et al., 2014 for review). For example, in addition to amino acids, GPRC6A is also activated by testosterone (Ko et al., 2014), and some groups have reported that osteocalcin, a bone-derived peptide that participates in pancreatic beta cell function, is also an endogenous ligand (Pi et al., 2011; De Toni et al., 2014; Wei et al., 2014); although this remains controversial (see Rueda et al., 2016).

The CaSR is a large heptahelical transmembrane glycoprotein consisting of 1078 amino acids with a molecular weight of approximately 120 kilodaltons. Like other members of the metabotropic glutamate receptor family, it likely exists in the plasma membrane in a dimeric configuration (Bai et al., 1999; Hu and Spiegel, 2007). In most cases the cellular event that initiates activation of the receptor is a rise in extracellular Ca^{2+}. However, in addition to Ca^{2+} ions the receptor can also be activated by a several other di- and trivalent cations and organic molecules such as the polyamines (Leach et al., 2014), some of which likely operate as additional or alternative endogenous ligands. A salient feature of this receptor is that it contains multiple Ca^{2+} binding sites within the active oligomeric complex and displays a high degree of positive cooperativity of activation (Breitwieser, 2013). In functional biochemical assays, the Hill coefficient of activation is typically 4–6 (Miedlich et al., 2002; Tharmalingam et al., 2011; Conigrave and Ward, 2013) and up to five Ca^{2+} binding sites have been predicted for the receptor (Zhang et al., 2014). The presence of multiple cation binding sites operating in a highly positive cooperative fashion translates into a state whereby small changes within a narrow concentration range of extracellular Ca^{2+} (1.1–1.3 mM) fully and rapidly activates the receptor.

The CaSR has been linked to a wide range of physiological processes. The first to be discovered and the most widely studied of these biological roles is its expression in the chief cells of the parathyroid gland and the regulation of parathyroid hormone release. The development of the drug Cinacalcet to treat hyperparathyroidism was based on the ability of the CaSR to suppress parathyroid hormone release from the parathyroid gland. Cinacalcet is a positive allosteric modulator of the CaSR. Its approval by the FDA in 2004 for the treatment of hyperparathyroidism represented the first commercial development of an allosteric modulator of a GPCR (Nemeth, 2013). The biochemical basis of Cinacalcet's efficacy in treating hyperparathyroidism with minimal side effects is likely achieved by the extremely high level of expression of the CaSR in the adult parathyroid gland. In addition to the parathyroid gland, the CaSR is also widely expressed in other tissues, and therefore its physiological functions extend beyond controlling parathyroid hormone release. These functions include suppression of vitamin D synthesis which lowers the drive for intestinal Ca^{2+} absorption, negative modulation of parathyroid hormone-induced phosphate excretion to raise the serum inorganic phosphate level, and decreased osteoclastic-dependent bone resorption (Leach et al., 2014). The CaSR's role in these processes are carried out by the activation of a variety of intracellular signaling pathways including stimulation of IP_3 turnover and the release of intracellular Ca^{2+}, the activation of ERK and Akt, and other signaling pathways. It has also become apparent that the CaSR is involved in additional processes including cellular differentiation and migration, brain development, stem cell engraftment, wound healing, and tumor growth and metastasis. This review will focus on the role of the CaSR in the differentiation and movement of cells, and the mechanisms underlying these processes in the developing CNS and in cancer. A summary of several key studies linking the CaSR with cell migration is shown in **Table 1**.

THE CaSR IN HEMATOPOIETIC CELL HOMING, AND BONE CELL DIFFERENTIATION, AND MIGRATION

During mammalian ontogeny, hematopoietic stem cells (HSCs) translocate from the fetal liver to the bone marrow where hematopoiesis occurs throughout adulthood. A characteristic of bone that likely contributes to an attractive microenvironment for stem cells is the high concentration of calcium ions at the HSC-enriched surface of the endosteum (the endosteum is the thin vascular membrane of connective tissue that lines the surface of the bony tissue that forms the medullary cavity of long bones). In fact, the bone endosteal surface has been shown to act as a niche for circulating CaSR-expressing HSCs to preferentially localize to the bone (Adams et al., 2006). HSCs prepared from mice lacking the CaSR were defective in localizing to the endosteal niche, and additionally, HSC maturation was altered as indicated by the observation that progenitor cells were present in an immature form in the circulation and spleen. Although $CaSR^{-/-}$ HSCs from fetal liver were normal in number and in proliferative capability, they were highly defective in localizing anatomically to the endosteal niche, a characteristic that correlated with defective adhesion of the HSCs to collagen I, an extracellular matrix protein (ECM) present in the endosteal surface (Adams et al., 2006). Together, these results indicate that the CaSR is responsible for retaining and adhering HSCs in close physical proximity to the bone endosteal surface.

Additional *in vitro* studies have shown that CaSR stimulation with Cinacalcet increased HSC growth in stromal cell co-cultures (determined using the "cobblestone area-forming cell assay" which measures progenitor cell-like and stem cell-like activities) by promoting HSC adhesion to ECM proteins such as collagen I and fibronectin (Lam et al., 2011). Moreover, co-stimulation of CXCR4 (a GPCR) and the CaSR resulted in augmented *in vivo* homing to the endosteal niche and engraftment capacity. This work suggested that modulation of the CaSR might be a viable strategy for enhancing HSC engraftment in bone marrow (Lam et al., 2011).

The role of the CaSR in HSC homing has been further established using a biodegradable composite biomaterial composed of Ca^{2+} phosphate glass/polylactic acid which was developed to mimic elevated Ca^{2+} levels surrounding the bone microenvironment (Aguirre et al., 2012). Using this biomaterial, Aguirre et al. (2012) demonstrated that bone marrow-derived HSC mobilization, differentiation, and

TABLE 1 | Summary of studies linking the CaSR and cellular migration.

Cells implicated in CaSR mediated cell migration	Intracellular signaling pathways involved in CaSR mediated cell migration	References
Cerebellar granule cell precursors (GCP)	ERK2 and AKT mediated increase in plasma membrane expression of β1 integrins	Tharmalingam et al., 2016
Renal carcinoma cells	AKT, PLC, JNK, p38	Joeckel et al., 2014
Cardiac fibroblasts	PLC, IP3	Zhang et al., 2014
Rat medullary thyroid carcinoma cells	PLC, $[Ca^{2+}]_i$, ERK1/2	Tharmalingam et al., 2011
Human bronchial epithelial cells	PLC, ERK1/2	Milara et al., 2010
Monocyte-macrophage lineage cells (RAW 264.7)	(PI3K), Akt, PLC	Boudot et al., 2010
Breast cancer cells (MDA-MB-231)	ERK1/2, PLC	Saidak et al., 2009
Gonadotropin-releasing hormone neurons (GnRH)	CaSR activation promotes monocyte chemoattractant protein-1 (MCP-1) secretion which binds CC motif receptor-2	Chattopadhyay et al., 2007
Haematopoietic stem cells (HSC)	Intracellular signaling mechanism was not determined	Adams et al., 2006
Murine osteoblastic cell line (MC3T3-E1)	Intracellular signaling mechanism was not determined	Yamaguchi et al., 1998
Murine bone marrow-derived stromal cell line (ST2)	Intracellular signaling mechanism was not determined	Yamaguchi et al., 1998

angiogenesis occurs via CaSR activation in the presence of elevated extracellular Ca^{2+}. One mechanism in which the CaSR promotes HSC homing to the bone environment is by increasing the expression of CXCR4 in the presence of elevated extracellular Ca^{2+} (Wu et al., 2009). CXCR4 is involved in leukocyte trafficking and antagonists of this receptor are being developed for the treatment of inflammatory diseases, cancer, and HIV. CXCR4 regulates homing of leucocytes, endothelial progenitors, and bone marrow cells in response to SDF-1 present in the bone endosteal niche; here, extracellular Ca^{2+} acting through CaSR activation augments SDF-1 signaling by serving as a positive regulator of CXCR4 expression to promote stem cell mobilization and homing (Wu et al., 2009).

The CaSR is expressed in both osteoclasts and osteoblasts, the cells involved with resorption of the mineralized bone matrix and cells that replace the resorbed bone, respectively (Sugimoto et al., 1993; Marie, 2010). The dynamic balance between osteoclasts and osteoblasts determines bone remodeling and serum Ca^{2+} concentrations. Bone tissue likely has elevated Ca^{2+} levels compared to other tissues. However, studies reporting actual measurements of Ca^{2+} in bone are sparse, typically use microelectrode-based measurements, and differ widely in the estimates of Ca^{2+} concentrations. An early study reported the extracellular level of Ca^{2+} in bone to be about 27 mM, and at sites of osteoclastic bone resorption the local Ca^{2+} concentration was estimated to be as high as 40 mM (Silver et al., 1988). In another analysis performed using microelectrode measurements in bone slice cultures, the extracellular Ca^{2+} level was estimated to be 2 mM at sites of osteoclast mediated bone turnover (Berger et al., 2001). However, the fact that the latter estimate was derived from tissue slices *in vitro* leaves open the question of its applicability in the intact bone. In any case, since maximum CaSR responses are typically achieved at 2–4 mM extracellular free calcium (Tharmalingam, 2014), even the lower of the two estimates for bone cited above is within the range of CaSR activation.

CaSR-expressing osteoblasts appear to utilize the CaSR as a chemoattractant receptor to sense elevated extracellular Ca^{2+} at osteoclast mediated bone resorption sites. Migration of CaSR-expressing osteoblasts to bone remodeling sites allows replacement of the missing bone during the osteoblastic phase of bone turnover (Sugimoto et al., 1993; Theman and Collins, 2009). Signaling studies demonstrate that CaSR stimulation in osteoblasts results in activation of phospholipase C (PLC), extracellular signal-regulated kinase (ERK1/2), and JNK signaling cascades. These CaSR-stimulated signaling pathways contribute to osteoblast migration, differentiation and bone remodeling (Sharan et al., 2008; Yamaguchi, 2008; Marie, 2010).

Similar to osteoblast migration, localization and homing of CaSR-expressing osteoclast precursor cells to the bone environment is important for initiating bone remodeling. Using RAW 264.7 cell line derived from murine osteoclast precursor cells, Boudot et al. (2010) demonstrated that extracellular Ca^{2+} mediated activation of the CaSR was crucial for migration of these cells in a directional manner. The phosphoinositide 3-kinase (PI3K)/Akt and PLCβ signaling pathways were identified as mediators in the migratory effect. These results suggest that the presumed extracellular Ca^{2+} gradient present in bone is an initiating factor for the homing of osteoclast precursors, and may play a role in the initiation of bone remodeling (Boudot et al., 2010).

A final example of CaSR-mediated cellular migration is illustrated by the migration of gonadotropin-releasing hormone neurons in the hypothalamus. The CaSR is expressed in primary cultures of gonadotropin-releasing hormone neurons from murine basal forebrain, and in two different gonadotropin-releasing hormone neuronal cell lines (Chattopadhyay et al., 2007). Activation of the CaSR with elevated extracellular Ca^{2+} promoted gonadotropin-releasing hormone neurons to engage in directional chemotaxis. CaSR stimulation resulted in Ca^{2+} influx through N-type calcium channels and subsequent secretion of monocyte chemoattractant protein which synergistically promoted gonadotropin-releasing hormone neuron migration

toward extracellular Ca^{2+} (Chattopadhyay et al., 2007). These *in vitro* experiments were supported by studies in CaSR knockout mice where CaSR null gonadotropin-releasing hormone neurons showed approximately 27% fewer neurons in the final resting position in the preoptic area of the anterior hypothalamus compared to wild-type littermates (Chattopadhyay et al., 2007).

THE CaSR IN CANCER

An *in silico* analysis of GPCR gene expression profiling gleaned from microarray data sets of non-small cell lung cancer, breast cancer, prostate cancer, melanoma, gastric cancer and diffuse large B cell lymphoma, indicated that the CaSR is up-regulated in primary and metastatic cancer cells compared to normal tissue (Li et al., 2005). Over the past 15 years many studies have reported that the CaSR can act to either promote or suppress tumorgenesis and metastasis depending on the cancer type (Brennan et al., 2013). For example, in parathyroid and colon cancers, CaSR expression is significantly reduced leading to loss of the growth suppression when exposed to elevated extracellular Ca^{2+}. Activation of the CaSR in these tumors has been reported to decrease cell proliferation and tumor progression (Singh et al., 2013). Thus, CaSR expression decreases the tumorigenicity of parathyroid and colon cancers. Conversely, activation of the CaSR facilitates metastasis to bone in breast, prostate, renal and various other invasive cancers (Brennan et al., 2013). In addition, increased bone resorption induced by dietary Ca^{2+} deficiency promotes breast cancer bone metastasis, while increased dietary Ca^{2+} intake has preventative effects in colon cancer and parathyroid tumor progression (Lipkin, 1999; Butler et al., 2010; Macleod, 2013).

Similar to HSC homing, several types of primary tumor cells migrate to bone tissue. Metastatic cells preferentially attach to regions of active bone turnover and remodeling (e.g., blood, femur, pelvis, ribcage, and skull). The CaSR is expressed in both normal breast ductal epithelial cells and in primary breast cancer cells. Stimulation of the CaSR has been shown to promote the migration of several metastatic bone-preferring breast cancer cell lines. Highly bone metastatic MDA-MB-231 breast cancer cells display more robust migration compared to the MCF7 and T47D breast cancer cells, which have a much lower metastatic potential *in vivo* (Liu et al., 2009). MDA-MB-231 cells are estrogen receptor-positive while MCF-7 cells are estrogen receptor-negative suggesting the role of estrogen responsive factors in the control of CaSR function. Moreover, the BT474 breast cancer cells, which do not metastasize to the bone, did not respond to CaSR stimulation. Thus, extracellular Ca^{2+} appears to be a chemoattractant for bone preferring metastatic breast cancer cells toward a Ca^{2+} rich environment, and importantly, the level of CaSR expression has been shown to correlate positively with the magnitude of breast cancer metastasis potential (Saidak et al., 2009).

Breast cancer metastasis to bone results in increased osteolytic activity at the site of invasion. In highly invasive MDA-MB-231 cells, stimulation of the CaSR induces secretion of multiple cytokines and growth factors that result in endothelial cell migration and *in vitro* angiogenesis (Hernandez-Bedolla et al., 2015). These effects were inhibited by anti-CaSR blocking monoclonal antibodies and the calcilytic (CaSR antagonist) NPS-2143. The CaSR was also shown to mediate a pro-angiogenic environment at the site of invasion by promoting the secretion of pleiotropic molecules such as GM-CSF, EGF, MDC/CCL22, FGF-4, and IGFBP2, which are characteristic chemotactic and angiogenic factors. Conversely, constitutive secretion of IL-6 and β-NGF was partially inhibited by CaSR stimulation in MDA-MB-231 cells. However, in normal mammary cells, constitutive secretion of RANTES, angiogenin, and oncostatin M was attenuated by CaSR activation, whereas secretion of IL-6 was increased. Thus, altered secretion of chemotactic and pro-angiogenic cytokines in breast cancer cells is modulated by the CaSR (Hernandez-Bedolla et al., 2015).

The CaSR also modulates breast cancer metastasis by mediating the secretion of parathyroid hormone-related peptide (PTHrP). During lactation, activation of the CaSR in normal mammary epithelial cells down-regulates PTHrP in milk and in the circulation, and increases Ca^{2+} transport into milk. However, a switch in CaSR G-protein usage during malignant transformation appears to convert this feedback loop into a PTHrP feed-forward cycle in breast cancer cells that exacerbates the growth of osteolytic skeletal metastases (Vanhouten and Wysolmerski, 2013). CaSR stimulated secretion of PTHrP from breast cancer cells promotes osteolytic activity and is increased by transforming growth factor-β released from resorbed bone. Hence, in PTHrP-secreting breast cancers that metastasize to bone, the CaSR initiates a vicious cycle in which PTHrP-induced bone resorption raises the concentration of extracellular Ca^{2+} and transforming growth factor-β within the bone microenvironment, which then act in concert to evoke further PTHrP release and worsening osteolysis (Sanders et al., 2001). In some clinical cases, humoral hypercalcemia of malignancy occurs when secretion of PTHrP by cancer cells causes hypercalcemia in the absence of skeletal metastases (Sanders et al., 2000).

In contradiction to the studies described above demonstrating CaSR expression up-regulation in breast cancer progression, another study of CaSR protein expression in 148 cases of breast cancer using tissue microarray by immunohistochemistry reported lower CaSR expression in breast cancer lesions compared with paired non-tumor tissues (Li et al., 2014). A multivariate analysis showed that CaSR was of independent prognostic significance for both overall survival and cause-specific survival of breast cancer patients; the patients with lower expression of CaSR were also significantly associated with distant metastasis-free survival. Therefore, this dataset suggested a tumor suppressor role of the CaSR in breast cancer (Li et al., 2014). In summary, most but not all of the evidence favors a role of the CaSR in facilitating breast cancer metastasis; additional confirmatory studies need to be conducted looking at levels of the CaSR in human breast cancer biopsy samples in order to validate the findings of immortalized breast cancer cell models described above.

Similar to metastatic breast cancer cells, prostate cancers also metastasize primarily to skeletal regions suggesting that the elevated extracellular Ca^{2+} environment of the bone may

provide a favorable niche for its localization and progression. In two highly metastatic prostate cell lines (PC-3 and C4-2B cells), elevated CaSR expression and increased proliferation was demonstrated compared to a non-skeletal metastatic, epithelial-derived prostate cell line (Liao et al., 2006). CaSR knockdown resulted in reduced PC-3 cell proliferation *in vitro* and reduced metastatic progression *in vivo*. The positive effect of CaSR activation on PC-3 cell proliferation was due to increased inhibition of cyclic AMP accumulation and elevated expression of cyclin D1, a protein required for cell cycle transition. Additionally, the effects of CaSR activation of PC-3 cell attachment and migration were attributed to activation of the Akt signaling pathway (Liao et al., 2006). Moreover, in human prostate cancer tissue sections and microarrays, CaSR expression was reported in both normal prostate and in primary prostate cancer samples as assessed by immunohistochemistry—but metastatic prostate cancer tissue obtained from bone had higher CaSR expression than primary prostate cancer tissue (Feng et al., 2014). Importantly, CaSR expression in primary prostate cancers of patients with metastases to tissues other than bone was not different from that in primary prostate cancer of patients with or without bony metastases. CaSR expression in cancer tissue was not associated with the stage or status of differentiation of the cancer (Feng et al., 2014). Taken together, these results suggest that the CaSR may have a role in promoting metastasis of prostate cancer to bone.

A link has also been indicated for the CaSR in renal carcinoma metastasis. To assess the role of the CaSR in kidney cancer, (Joeckel et al., 2014) distributed 33 matched specimens of normal and tumor tissue and 9 primary cells derived from renal cell carcinoma patients into three categories: non-metastasized, metastasized into the lung, and metastasized into bones during a 5-year period after nephrectomy (Joeckel et al., 2014). Thirty percent of renal cell carcinomas resulted in bone metastasis. Transcript and protein expression analysis showed the highest CaSR expression in samples obtained from patients with bone metastases. Ca^{2+}-induced enhancement of renal cell carcinoma cell migration and proliferation were only identified in cells obtained from patients with bone metastases and absent in the other two groups. Analysis of intracellular signaling mechanisms of metastasizing carcinoma cells revealed that CaSR activation resulted in enhanced activity of Akt, PLCγ-1, p38α, and JNK signaling pathways, whereas PTEN expression was abolished. Based on these intriguing findings, it was suggested that CaSR expression levels could be utilized as a novel prognostic marker for predicting renal carcinoma cell bone metastasis (Joeckel et al., 2014).

THE CaSR AND CELL ADHESION PROTEINS—THE INTEGRINS

Integrin Expression, Activation, and Bi-Directional Signaling

Our previous work delineated a novel interaction between the CaSR and a class of cellular adhesion proteins known as the integrins (Tharmalingam et al., 2011). Identification of the integrins as CaSR interacting proteins was initially determined in rat thyroid carcinoma cells from CaSR immuno-affinity pull-down experiments combined with mass spectrometry. Pharmacological stimulation of the CaSR using the positive allosteric modulator NPS R-568 potently enhanced thyroid carcinoma cell adhesion and migration. Evidence that these effects occurred via the integrins was indicated by the demonstration that the enhancement of cell-ECM adhesion by NPS R-568 could be largely blocked by the addition an integrin blocking peptide (GRGDSP) that competes with fibronectin for β1-integrin binding. Additional confirmation was obtained whereby shRNA-mediated knock down of β1-containing integrins reduced CaSR-mediated cell adhesion and migration. We concluded that (a) the CaSR is associated with β1-containing integrins in a macromolecular protein complex and (b) that stimulation of the CaSR promotes cell adhesion and migration via integrin activation (Tharmalingam et al., 2011).

The integrins are a family of proteins that mediate cell-to-cell contact by binding to the ECM. Integrins are cell adhesion molecules that interact with the ECM to provide a dynamic link between the extracellular adhesion molecules and the intracellular actin cytoskeleton. These proteins are unique in that they are able to bind to ECM proteins and induce intracellular signaling cascades, and are also capable of conveying intracellular signaling messages to the ECM by modulating their binding affinity for the ECM. By mediating cell-ECM interactions integrins play an essential role in cellular migration. They are also involved in other cellular processes including cellular differentiation, proliferation, embryogenesis, CNS development, inflammation and cancer (Iwamoto and Calderwood, 2015; Maartens and Brown, 2015; Wang et al., 2015).

Integrins are heterodimers of type 1 membrane-spanning glycoproteins composed of an α and a β subunit (**Figure 1**). In mammals, there are 18 α and 8 β subunits that form 24 heterodimeric pairs (see Campbell and Humphries, 2011 and Maartens and Brown, 2015 for reviews). Both α and β integrin subunits are characterized by a very large extracellular domain of 700–1100 residues, a single transmembrane domain, and a short cytoplasmic domain of 30–50 residues, with both subunits held together via non-covalent interactions. The outermost N-terminal region of the α integrin subunit that faces the extracellular side of the cell is composed of a head structure with seven-bladed β-propellers, which connects to the leg structure composed of a thigh, calf-1 and calf-2 domains (Barczyk et al., 2010). A region between the thigh and calf-1 domain known as the "α subunit genu" acts as a pivot point for integrin domain extension.

In the resting state integrins adopt a conformation where the integrin leg structures are severely bent, generating a closed topology where the head domain are juxtaposed to the leg regions close to the cell membrane (**Figure 1**). In this conformation the integrin ligand binding sites are not optimal for binding ECM proteins, and therefore the bent conformation represents a low-affinity state. The presence of ECM ligands induces an intermediate-affinity conformation in which a switch-blade like extension of the head region shifts away from the legs (Luo and Springer, 2006; Barczyk et al., 2010). Destabilization of the

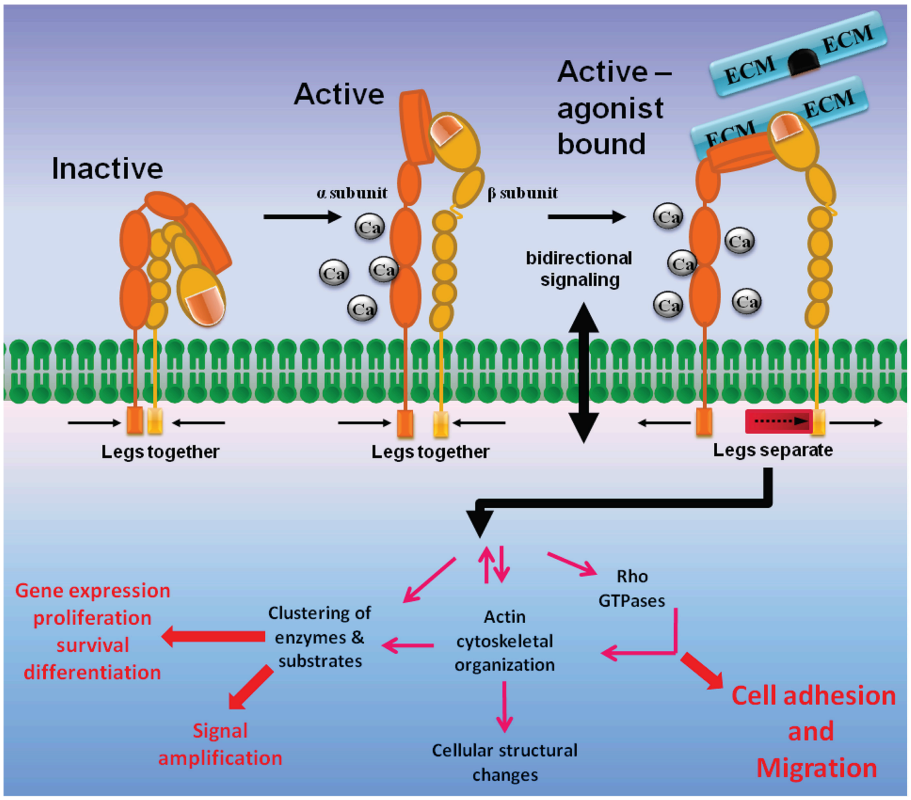

FIGURE 1 | Integrin structure and bidirectional signaling. Integrins are membrane-spanning glycoprotein heterodimers that mediate cell adhesion and migration. The large extracellular domains contain sites for ligand and cation binding, while the small intracellular region senses changes in intracellular signaling pathways that convey information to the ECM via conformational modifications. Inactive integrins are present in a bent conformation (low affinity, left). Intracellular activation signals induce integrins to attain an upright position with a dual extended head-piece arrangement (middle). The upright conformation is characterized by an intermediate affinity state and a high affinity, ligand-bound state where the integrin α and β subunit tails are separated. Integrin inside-out signaling is achieved when intracellular signaling proteins push the β integrin leg away from the α integrin subunit, allowing for formation of an integrin high affinity activation state (right). Stabilization of integrin binding to ECM initiates outside-in signaling, where the integrins mediate downstream signaling that includes focal adhesion and actin stress fiber formations, activation of Rho GTPases, and cellular structural changes, etc., which ultimately leads to promotion of cell adhesion and migration.

α/β integrin association fully activates the integrins into a high-affinity conformation (O'Toole et al., 1991; Lu et al., 2001), and transmembrane domain separation is necessary for integrin signaling (Wang and Luo, 2010).

From the intracellular side, integrins do not bind actin directly, but rather indirectly by proteins they recruit with their cytoplasmic tails. The dynamic complex of proteins at cell–ECM adhesions has been termed the integrin adhesome. A crucial adhesome component is the cytoskeletal-associated protein talin, which can simultaneously bind the cytoplasmic tail of integrin β subunits and actin, as well as coordinate the solicitation of other proteins to the adhesome (Critchley, 2009). Integrin inside-out signaling is mediated by the binding of talin to the β integrin C-terminal tail, a process that is controlled by the small GTPase Rap1 and its effectors such as RapL and RIAM (Mehrbod et al., 2013). Rap1 is activated by various GPCRs that are activated by chemoattractant stimulation. Stimulation of membrane spanning receptors mediates cell adhesion and migration by activating signaling cascades that lead to integrin inside-out signaling (Mehrbod et al., 2013). Upon separation of the integrin α and β subunits, the integrins adopt a high affinity active conformation that allows binding of ECM proteins. Ligand (ECM) occupancy and integrin clustering in-turn activates intracellular signaling cascades that mediate cell cytoskeletal changes, a process known as integrin outside-in signaling (**Figure 1**).

Upon binding to ECM proteins, integrin dimers cluster together laterally on the membrane via cell surface insertion of integrins localized in caveolae-rich microdomains (Maheshwari et al., 2000). Integrin clustering induces actin filament assembly and reorganization of actin filaments into larger stress fibers in turn causes further integrin clustering, providing a positive feedback loop that maintains enhanced matrix binding. The aggregation of ECM proteins, integrins, cytoskeletal proteins, and signaling kinases forms unique structures known as focal adhesion complexes (Parsons et al., 2010). In particular, integrin ligation and clustering activates focal adhesion kinase (FAK), which in-turn activates Src-family kinases (Mitra and Schlaepfer, 2006; Hu and Luo, 2013). Activated Src phosphorylates cytoskeletal proteins such as paxillin, actin bundling protein α-actinin and vinculin to form nascent adhesion structures.

Binding of these initial cytoskeletal proteins recruits further actin nucleating proteins that result in focal adhesion complex and actin stress-fiber formation. These cytoskeletal changes mediated by integrin outside-in signaling ultimately allow cells to adhere and spread over the ECM proteins.

Intriguingly, and also similar to the CaSR, the integrins contain multiple divalent cation binding sites in the extracellular domain (Xiong et al., 2002). The integrin cation binding sites can be occupied by Ca^{2+} or by Mn^{2+} ions. However, the integrins differ from the CaSR in that the cations are necessary but not sufficient for integrins to convert from the inactive bent conformation into the active extended conformation. Both the presence of cations bound to the multiple cation binding sites is required, along with the direct physical association with ECM ligands for integrins to attain the extended structure and concomitant activation (Dai et al., 2015). Based on these features, *we hypothesize that a rise in extracellular Ca^{2+} ions would serve to both prime the integrin heterodimer and activate the CaSR (see further discussion below).*

Integrins are crucial players in several aspects of bone tissue biology (see Marie et al., 2014 for review). Several integrin heterodimers including α5β1 integrin, α2β1 integrin, and α4β1 integrin have been shown to play important roles in bone formation and maintenance, and in bone cell differentiation (Hamidouche et al., 2009; Guan et al., 2012; Marie, 2012). In general, activation of integrins on osteoblasts leads to cell differentiation. In bone (and other tissues) ECM–integrin binding induces the recruitment and phosphorylation of focal adhesion kinase and activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) ERK1/2, leading to activation of the transcription factor RUNX2 and osteoblast differentiation.

Of special interest here in light of the identification of the CaSR- β1 integrin complexes, is that β1-containing integrins play an important role in osteoblast differentiation. Direct evidence supporting this concept comes from mice expressing a dominant-negative β1 integrin subunit in mature osteoblasts. These mutant mice show reduced bone mass, defective bone formation and loss of ICAP-1, an important modulator of β1 integrin activation resulting in defective osteoblast differentiation and delayed bone formation (Globus et al., 2005; Brunner et al., 2011). The a5β1 integrin heterodimer which binds to the ECM protein fibronectin, is essential for osteoblast formation, and α5 integrin suppression reduces osteoblast differentiation, whereas its overexpression facilitates osteoblast differentiation in cultured mesenchymal skeletal cells. These effects are mediated via FAK/MAPK ERK1/2 signaling (Hamidouche et al., 2009). It has also been shown that integrin αvβ3 is critical for CYR61-mediated BMP-2 expression and subsequent osteoblastic differentiation through activation of the ILK/MAPK ERK1/2 signaling pathway in osteoblasts (Su et al., 2010). Similarly, α2β1, a major receptor for collagen type 1, was reported to control collagen synthesis in osteoblasts in mice (Stange et al., 2013). This integrin heterodimer plays a role in mesenchymal skeletal cells osteogenic differentiation and survival through activation of ROCK, FAK, and MAPK ERK1/2 signaling. Together, these findings provide insight into the role of integrin-mediated signaling pathways in the control of osteoblast differentiation, fate, and function (Marie et al., 2014).

Integrins and Metastasis

Integrins have been intensively studied in the context of cancer with hundreds of literature citations on the topic; therefore, we will restrict our discussion here to the most pertinent findings on the association of β1-containing integrins and metastasis to bone.

β1 integrins are activated in metastatic prostate cancer cells and mediate increased prostate cancer cell metastasis to lymph nodes and bone. Dissemination of prostate cancer cells to the bone marrow occurs early in the disease. Cancer cell proliferation and active metastases after a period of undetectable disease is known as tumor dormancy. Dormancy-reactivation has been studied in patient-derived xenograft lines where the propensity to proliferate through tumor cell contact with each other, and with bone marrow stroma was demonstrated (Ruppender et al., 2015). Proliferating prostate cancer cells displayed tumor cell-cell contact and integrin clustering after ECM contact (e.g., fibronectin) in bone marrow stroma. Activation of myosin light chain kinase, a downstream effector of integrin-β1 and TGF-β2, in non-proliferating cells promoted cell proliferation. This activity leads to the deactivation of growth suppressor like E2F4 and activation of cell cycle regulator CDK6 thereby promoting cell proliferation (Ruppender et al., 2015). These data illustrate the involvement of integrins in the escape of prostate cancer cells from dormancy.

Similar to CaSR expression in metastatic prostate cancers, β1-containing integrins have been shown to be constitutively activated in prostate cancer cell lines with high metastatic potential, but not in cancer cell lines with low metastatic potential (Lee et al., 2013).Activation of β1 integrins promotes resistance to anoikis. Anoikis is a form of programmed cell death that is induced by anchorage-dependent cells detaching from the surrounding ECM. Usually cells stay close to the tissue to which they belong since the communication between proximal cells as well as between cells and ECM provide essential signals for growth or survival. When cells are detached from the ECM, there is a loss of normal cell–matrix interactions, and they may undergo anoikis. However, metastatic tumor cells may escape from anoikis and invade other organs. Resistance to anoikis occurs via stimulation of FAK/Akt-mediated survival pathway and neutralizing β1 integrin activation using an anti-β1 integrin antibody reduced prostate cancer metastasis *in vivo* (Lee et al., 2013). In addition, talins which are adaptor proteins that regulate focal adhesion signaling by conjugating integrins to the cytoskeleton, bind integrins and are essential for integrin activation. In this context, talin has been shown to be important for β1 integrin activation, and Cdk5-mediated phosphorylation of talin leading to β1 integrin activation was shown to increase the metastatic potential of prostate cancer cells (Jin et al., 2015).

Integrins are also involved in breast cancer metastasis. Bone metastasis affects more than 70% of advanced breast cancer patients, but the underlying molecular mechanisms remain unclear. Although some evidence suggests that integrins mediate adhesion of malignant cells to bone ECM, their role during bone colonization is also not fully understood. The role of β1 integrins

in bone colonization has been investigated *in vitro* and *in vivo* in tissue-engineered humanized bone models (Thibaudeau et al., 2015). *In vitro*, bone- metastatic breast cancer cells with suppressed integrin β1 expression showed reduced attachment, spreading, and migration within human bone matrix compared to control cells. Cell proliferation *in vitro* was not affected by β1 integrin knockdown, but tumor growth *in vivo* within humanized bone microenvironments was significantly inhibited upon β1 integrin suppression. Tumor cells invaded bone marrow spaces in the humanized bone and formed osteolytic lesions whereas osteoclastic bone resorption was, however, not reduced by β1 integrin knockdown (Thibaudeau et al., 2015). These observations indicate that β1-containing integrins likely play a prominent role in bone colonization.

Some data are also available linking integrins and bone metastasis in renal carcinomas. Renal carcinoma cells that form bone metastasis constitutively activate osteoclasts, which then induce bone resorption and release pro-proliferative factors. As noted above, this phenomenon also occurs in bone metastasis of other types of tumors including breast and prostate cancer. Constitutive activation of osteoclasts leads to the release of fibronectin and collagen which can facilitate cancer cell attachment via integrins to ECM and vascular endothelial cells. Cell adhesion of bone metastasizing cells to fibronectin and to collagen I (but not adhesion to collagen IV) is high and bone has a high concentration of these two ECM proteins (Ode et al., 2010). In one study, 9 renal cell carcinoma primary cell lines and 30 renal cell carcinoma tissue specimens (normal and tumor tissue) collected from 3 patients with no metastasis, and 10 samples with lung or bone metastasis within 5 years after nephrectomy were collected and analyzed (Haber et al., 2015). Compared to renal cell carcinoma cells from patients without metastasis, the migration of cells from patients with bone metastasis was enhanced 14-fold, and adhesion to fibronectin and collagen I were both enhanced 6-fold. Notably, proliferation was decreased in metastasizing cells. In addition, elevated activity of Akt and FAK and integrin α5 expression, and reduced PTEN expression was observed in primary cells from patients with bone metastasis compared to non-metastasizing cells. Thus, (Haber et al., 2015) concluded that increased expression of integrin α5 and downstream signaling via Akt could help tumor cells to adhere to ECM proteins and facilitate migration to bone tissue.

In summary, three lines of evidence lend support to the hypothesis that CaSR-integrin complexes may be a factor in some forms of cancer metastasis: (1) the findings outlined above and others indicating a role for β1-containing integrins in cancer metastasis to bone, (2) the considerable body of evidence demonstrating elevated CaSR expression in prostate, breast, and kidney cancers that metastasize to bone, and (3) the presence of CaSR-β1 integrin macromolecular complexes in thyroid carcinoma cells (Tharmalingam et al., 2011). We postulate that in circulating tumor cells, CaSR-integrin protein complexes could function together as a cell surface detection mechanism for attaching to the ECM components of the bone microenvironment (**Figure 2**). The CaSR could act as the calcium sensor and become activated by the (presumed) high extracellular Ca^{2+}; in bone tissue this would be followed in turn by activation of the associated integrins on the tumor cell to signal attachment to bone ECM proteins such as fibronectin and collagen. It should be emphasized that this proposed sequence of events is speculative and further work is needed to rigorously test the idea.

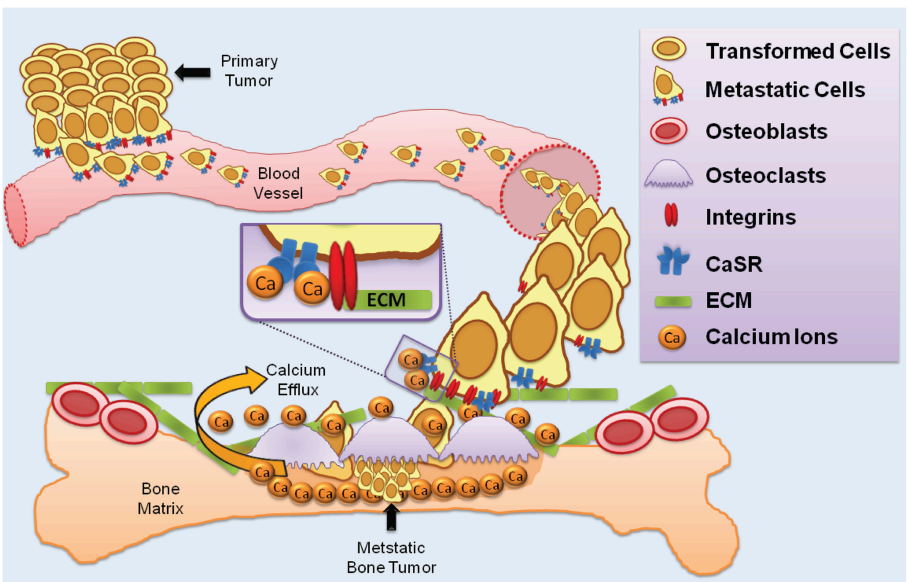

FIGURE 2 | CaSR and integrins in cancer metastasis to bone. The diagram illustrates a proposed multi-step process whereby primary tumor cells enter the vasculature and colonize resorbing regions in bone. Tumor cells with elevated CaSR and integrin expression intravastates blood vessels and circulate in the bloodstream. Resorbing bone houses osteoclasts which release Ca^{2+}; elevated extracellular Ca^{2+} is detected by the CaSR which activates integrin heterodimers to promote binding to ECM proteins such as collagen and laminin.

THE CaSR AND INTEGRINS IN THE DEVELOPING CNS

Developmental Expression of the CaSR and Integrins in the CNS

CaSR expression in the central nervous system (CNS) was first demonstrated using a rat striatal cDNA library where it was determined that the receptor is expressed in both neurons and glia (Ruat et al., 1995). In adults, the CaSR is localized to nerve terminals with discrete punctate expression. Upon depolarization, the synaptic space contains elevated Ca^{2+}. Due it is localization within nerve terminals, the CaSR may regulate neurotransmitter disposition in response to synaptic Ca^{2+} changes (Ruat et al., 1995; Ruat and Traiffort, 2013). In the immature brain the receptor is expressed in several regions including the orbital cortex, cerebellum (see below), and hippocampus (Ferry et al., 2000; Tharmalingam et al., 2016). Analysis of rat hippocampal mRNA and protein expression showed that peak CaSR expression occurs from postnatal day 12 to day 24, followed by a 3-fold decrease to adult levels (Chattopadhyay et al., 1997). Cellular analysis demonstrated CaSR expression in pyramidal cells of all the layers of hippocampus and in granule cells of the dentate gyrus.

Studies on of CaSR knockout mice during postnatal brain development have indicated that the CaSR plays an important role in the differentiation of numerous brain cell types. For example, CaSR null mice exhibited developmental delay due to impaired differentiation as assessed by reduced expression of proliferating cell nuclear antigen, neuronal specific nuclear protein (neuronal differentiation marker), glial fibrillary acidic protein (glial differentiation marker), and myelin basic protein (oligodendrocyte differentiation marker) when compared to age-matched wild-type littermates (Liu et al., 2013). Brain weight and size were drastically reduced in the CaSR null mice and cell proliferation was also decreased in the CaSR knockout animals. Concurrent deletion of the CaSR with parathyroid hormone gene corrected for the effects of hyperparathyroidism, hypercalcemia, hypophosphatemia, and whole-body growth retardation (Liu et al., 2013). However, further analysis of these animals established that brain cell proliferation was normal whereas differentiation was greatly reduced in the CaSR-PTH double knockout animals. The role of the CaSR in differentiation vs. proliferation was also investigated using cultured neural stem cells derived from the subventricular zones of CaSR null neonatal mice, which exhibited normal proliferation capacity but decreased differentiation capacity when compared with wild-type animals (Liu et al., 2013). Taken together, the CaSR is crucial for differentiation of neurons, glia and oligodendrocytes. Delayed brain development in CaSR null newborn mice is therefore the result of direct effects of the CaSR on cell differentiation, and indirect CaSR effects on cell proliferation via dysregulation of parathyroid hormone secretion and serum Ca^{2+} (Liu et al., 2013).

The CaSR expression pattern in adult rodent brain differs substantially from the immature brain. The regions of moderate to high expression during development do not express the receptor, or express it at very low levels, in the adult rodent brain. However, the CaSR is expressed in a few regions of the adult CNS including the olfactory bulbs, area postrema, and the subfornical organ (Ferry et al., 2000; Yano et al., 2004). The subfornical organ is a hypothalamic thirst center; its neurons project onto supraoptic and paraventricular nuclei where they control the activity of vasopressin-secreting neurons (Ruat and Traiffort, 2013). Expression in the subfornical organ suggests that the receptor is also involved in central fluid and electrolyte balance (Yano et al., 2004). The adult cerebral arteries also display an intense network of CaSR immunoreactive fibers demonstrating possible functional role in vessel innervation. Studies on adult CaSR neuronal expression have determined that the CaSR controls neuronal excitability by regulating ion channel function (Phillips et al., 2008; Vyleta and Smith, 2011). These findings reinforce the idea that the CaSR modulates Ca^{2+}-dependent neuronal functions in both the immature and in the adult brain.

β1 integrins are expressed in all cell types in the CNS and integrin heterodimers containing the β1 integrin are indispensable for many functions in the brain where the β1 subunit forms obligate heterodimers with numerous α integrin subunits to create functional receptor complexes. Each brain region and cell type expresses its own repertoire of α integrin subunits resulting in different integrin receptor expression profile and function (reviewed by Pinkstaff et al., 1999). In the developing nervous system, spatial and temporal changes in integrin expression parallel changes in neurogenesis, differentiation, and migration (Schmid and Anton, 2003). In early CNS development, neuronal migration during establishment of the cerebral and cerebellar cortex requires β1 integrin-mediated adhesion and migration (Hatten, 1999). Neurons originating from the ventricular zone migrate radially using glial cells and detach from the glial fibers at the cortical plate to establish connections at their final resting position. Loss of β1 integrins in neurons results in grossly disorganized structures in the most superficial layer of the cortex (Milner and Campbell, 2002), and selective deletion of β1 integrins in glia resulted in failure of glial cell contact formation with the meningeal basement membrane and blocked neuronal radial migration (Blaess et al., 2004; Frick et al., 2012). Therefore, normal assembly of the cerebral and cerebellar cortex requires β1 integrin mediated neuronal migration and glial adhesion (Milner and Campbell, 2002).

CaSR-Integrin Complexes in Cerebellar Development

The well-established migrational patterns of immature cerebellar granule-cell precursor neurons (GCPs) in the early postnatal cerebellum provides an excellent model system to delineate the molecular properties of neuronal migration. GCPs originate in the rhombic lip during late embryonic stages and then migrate rostrally to a secondary germinal layer, known as the external granule-cell layer of the cerebellum, where they continue to proliferate after birth. In rodents GCPs proliferate in the periphery of the external granule layer-cerebellar basement membrane interface over postnatal days 5–20 (**Figure 3**).

Various factors such as laminin (expressed on the basement membrane), sonic hedge-hog (released from Purkinje cells), brain-derived neurotropic factor (released from Bergmann glia), and insulin-like growth factors contribute to GCP proliferation. GCPs that have proliferated and exited the cell-cycle undergo tangential migration within the external granule layer (**Figure 3**). Tangentially migrating GCPs attach to the Bergmann glial processes and undergo radial migration whereby the GCPs migrate radially along the Bergmann glia fibers through the cerebellar molecular layer and the Purkinje cell layer, and into their final resting position in the internal granule-cell layer (Chedotal, 2010). By 3 weeks after birth GCP proliferation ceases and the remaining GCPs complete migration into the internal granule layer, which is retained in the mature brain while the external granule layer disappears. In the adult CNS cerebellar granule cells are the most numerous class of neurons in the vertebrate brain.

The role of β1 integrins in cerebellar GCP proliferation and migration has been well documented. Potent stimulants of GCP proliferation such as sonic hedgehog and SDF-1 have been shown to induce GCP proliferation only in the presence of β1 integrin mediated adhesion to laminin (Klein et al., 2001; Blaess et al., 2004). β1 integrins control GCP migration by binding to ECM proteins such as laminin, vitronectin, reelin, etc., which aid in the homing of GCPs from the external granule layer to the internal granule layer (Pons et al., 2001; Borghesani et al., 2002; Porcionatto, 2006). Thus, GCP migration relies heavily on integrin activation. Notably, defects in GCP migration result in medulloblastoma, the most common cause of malignant brain tumors in young children (see below).

We have recently demonstrated that the CaSR, together with integrins, functions as a cell-surface chemoattractant receptor expressed on the surface of cerebellar GCPs to facilitate GCP differentiation into mature granule cells, and to simultaneously guide their movement during cerebellar maturation (Tharmalingam et al., 2016). A robust but transient increase in CaSR expression in GPCs was observed that peaked during the second postnatal week, the period of maximal GPC migration. Our results support a model whereby the CaSR acts as a chemoattractant sensor to detect a gradient of extracellular Ca^{2+}, while integrin heterodimers in conjunction with ECM proteins function as the driving force to mediate cell movement (Tharmalingam et al., 2016). The data also indicated that CaSR-mediated stimulation of ERK2 and Akt phosphorylation provides a biochemical link between Ca^{2+}-sensing and the integrin-mediated movement of GCPs (**Figure 5**; also see further discussion below).

A key question to be addressed is, what is the source of the CaSR stimulus in the immature cerebellum? One possibility is that activation of the CaSR may be mediated by local

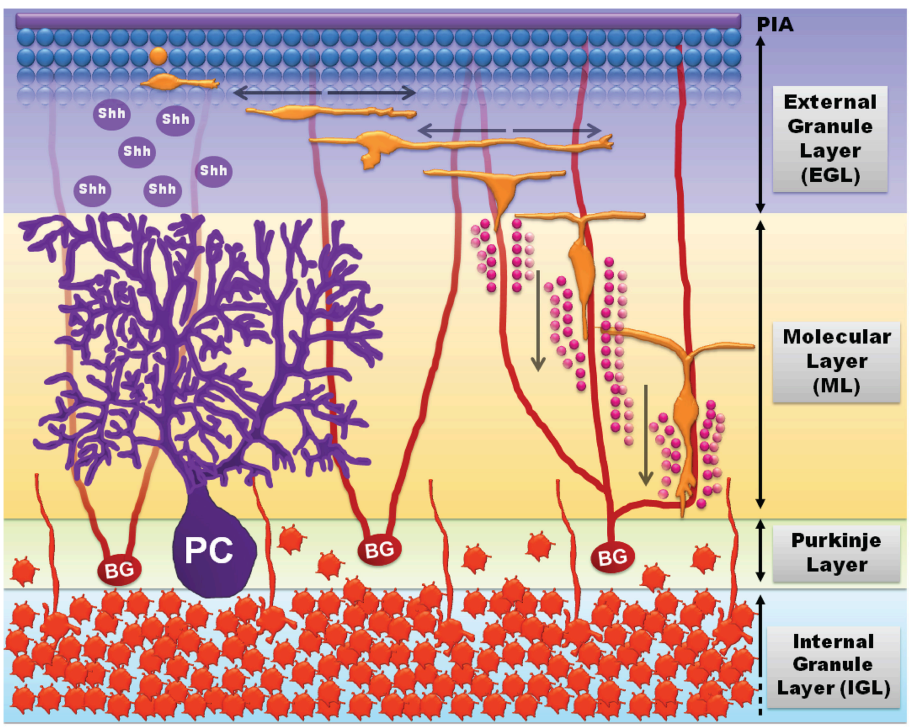

FIGURE 3 | Cerebellar development and GCP migration (adapted from Tharmalingam, 2014). GCPs (blue spheres, top) proliferate in response to growth signals including sonic hedgehog (shh) released by Purkinje cells (PC, purple). Mitotic GCPs exit the cell cycle to become migratory post-mitotic bipolar GCPs (orange) that undergo tangential migration in the external granule-cell layer (EGL). The bipolar migratory GCPs sprout a third process and attach to Bergmann glia (BG) fibers (red) to undergo radial migration through the molecular layer (ML) and the Purkinje layer (PL), and into the internal granule-cell layer (IGL; mature granule neurons, dark orange). GCPs undergo radial migration by sensing extracellular matrix cues (laminin, vitronectin, etc.) and radially expanding transglial Ca^{2+} waves (pink circles) released by Bergmann glial processes.

extracellular Ca^{2+} released by the Bergmann glia. Tangentially migrating unipolar GCPs in the external granule layer sprout a third axon that allows the GCPs to attach to Bergmann glial fiber tracts to undergo radial migration into the internal granule layer. Bergmann glial fibers have been shown to generate radially expanding transglial Ca^{2+} waves that initiate at a central point and expand radially to encompass multiple Bergmann glial processes (Hoogland et al., 2009). The Ca^{2+} waves generated by Bergmann glia could locally activate the CaSR expressed on GCPs to promote radial migration. Another potential source of CaSR activation may be from polyamines. Polyamines such as spermine and spermidine are potent agonists of the CaSR and polyamine concentrations are elevated during postnatal cerebellar development and then subsequently decline to adult levels (Jasper et al., 1982). The period of increased polyamines corresponds to the peak period of CaSR expression and GCP migration. Moreover, the initial step in the synthesis of the polyamines is mediated by the enzyme ornithine decarboxylase. In the cerebellum of rats injected with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase, granule cells fail to migrate properly and the development of the cerebellar cortex is stalled in an immature state (Bartolome et al., 1985). Taken together these findings indicate that polyamines might act alone, or in conjunction with Ca^{2+} ions, to stimulate the CaSR and guide GCP migration in the developing brain.

Cerebellar Granule Cells and Medullablastoma

Defects in GCP migration result in medulloblastoma, the most common cause of malignant brain tumors in children under four (Hatten and Roussel, 2011; Remke et al., 2013; Macdonald et al., 2014). Medulloblastoma is a highly heterogeneous group of tumors characterized by aberrant GCP proliferation and migration. Unlike most neuronal populations, GCPs remain mitotically active after birth (Roussel and Hatten, 2011). Impaired migration causes GCPs to remain longer in the proliferative-niche of the external granule layer where they are more prone to transformational insults due to increased exposure to proliferation-inducing molecules. There are currently no effective treatments for medulloblastoma. Although a role for the CaSR in medulloblastoma has not been established, the identification of key proteins regulating GCP migration and progression is a major goal in medulloblastoma research, and could lead to novel approaches for the development of effective therapeutics.

Medulloblastoma has been modeled *in vitro* using DAOY cells which are transformed human cerebellar granule cells. Wei et al. (2015) studied the function of the CaSR in these cells and compared them to normal cerebellar granule cells in culture (Wei et al., 2015). They examined the CaSR in conjunction with OGR1 (also known as GPR68), an extracellular proton-sensing GPCR coupled to Gq and the release intracellular Ca^{2+}. In addition to protons, this receptor is also activated by the benzodiazepine anxiolytic drug lorazepam (Huang et al., 2015). Previous work had established that OGR1 activation in DAOY cells leads to complex intracellular Ca^{2+} signals and activation of the ERK signaling pathway, thereby providing a mechanistic explanation of how the acidic environment may influence transformed cell function and/or survival. This action is lost on differentiation, suggesting a link between OGR1 activity and proliferative behavior of the transformed neurons (Huang et al., 2009). Curiously, Ca^{2+} signaling through OGR1 and CaSR appears to be opposite in the two cell types. In normal granule cells, OGR1-dependent Ca^{2+} signaling is slow and small while CaSR-dependent intracellular Ca^{2+} signaling is fast and large, whereas the opposite occurs in DAOY cells (Wei et al., 2015). It was suggested that the lack of inhibition of OGR1 signaling by CaSR in DAOY cells is likely due, at least in part, to low expression of functional CaSR in these cells, given that knockdown of CaSR in granule cells also leads to disinhibition of OGR1 signaling. Moreover, CaSR-dependent inhibition of OGR1 activity is absent in DAOY cells and intracellular acidification, which may accompany extracellular acidosis, inhibited CaSR responses but potentiated OGR1 responses (Wei et al., 2015).

OGR1 is functionally expressed in brain tumor cells and changes in its activity may be relevant to acidosis in tumors. Both OGR1 and CaSR have been implicated in several brain disorders that are exacerbated by changes in the concentrations of extracellular H^+ and Ca^{2+} (Kingsley et al., 2007). The CaSR and OGR1 are also co-expressed in other tissues that experience extracellular acidification under physiological conditions including kidney, bone, and lung. Therefore, in addition to cerebellar neurons, a perturbation in the balance between OGR1 and CaSR signaling may contribute to the development and progression of other pathological conditions.

POTENTIAL MOLECULAR MECHANISMS UNDERLYING CaSR-INTEGRIN INTERACTIONS AND CELLULAR MIGRATION

Cell migration is a multistep process that requires continuous coordinated formation and disassembly of cytoskeletal proteins (Webb et al., 2002). The migratory cycle progresses from activation of a chemoattractant receptor, protrusion extension toward a chemoattractant gradient, formation of stable adhesions at the leading edge of the protrusions via integrin engagement of ECM proteins, release of adhesions and retraction at the cell rear, and the translocation of cell body forward (**Figure 4**). Increased integrin-ECM ligation promotes outside-in integrin signaling characterized by focal adhesion complex formation and actin cytoskeletal polymerization (Vicente-Manzanares et al., 2009). These cytoskeletal changes drive the initial protrusion extension of the plasma membrane allowing the cell to spread. Cell polarity is achieved when the leading edge forms stable adhesion contacts via actin polymerization, while the trailing edge disassembles actin formation, and stable adhesion contacts serve as traction points for the propulsive forces that move the cell body forward (Webb et al., 2002). Release of integrin mediated adhesions at the rear allows translocation

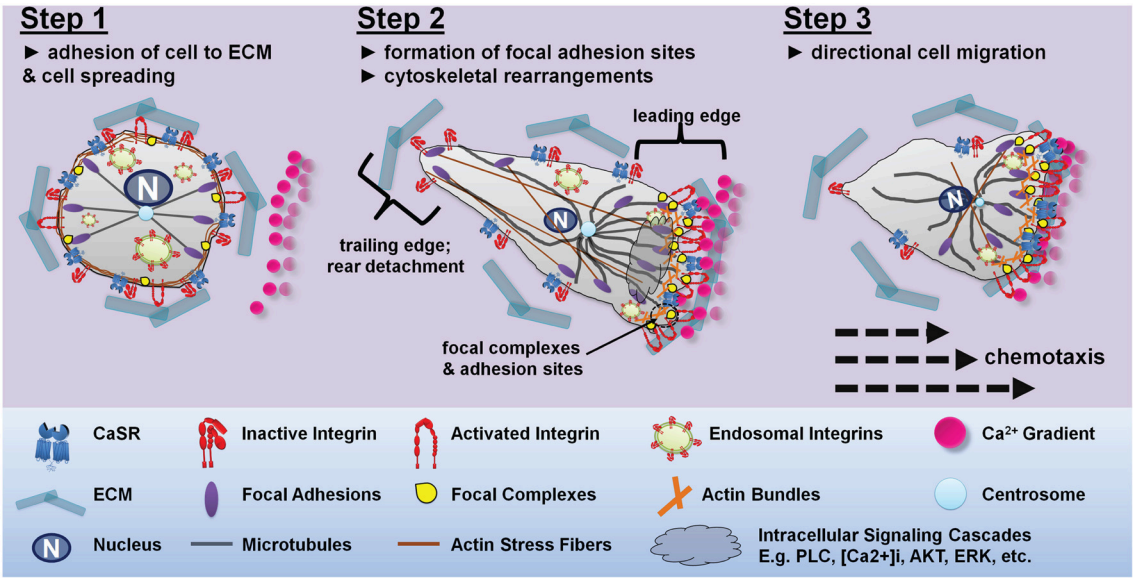

FIGURE 4 | Proposed mechanism for CaSR-integrin mediated directional cell migration toward a CaSR-agonist gradient. Step 1: under resting conditions there is a homogenous distribution of integrins along the plasma membrane of the cell. Integrins present in the active confirmation are bound to the ECM proteins allowing the cell to spread evenly on the ECM substratum. In the absence of CaSR agonists, the integrins present in the CaSR-integrin complex adopt an inactive bent conformation. Step 2: exposure of the cell to a Ca^{2+} gradient results in CaSR-integrin mediated cell polarization. The CaSR-integrin complexes present proximal to the Ca^{2+} gradient uses the CaSR to sense the stimulus allowing for localized activation of intracellular signaling pathways. Activation of CaSR triggers PLC-IP_3 mediated rise in intracellular Ca^{2+} causing integrin inside-out activation and elevated activated integrins at the site of CaSR stimulation. CaSR activation also promotes ERK2 and Akt-mediated trafficking and insertion of cytoplasmic endosomal integrins into the plasma membrane. Increased integrin-ECM interactions at the leading edge establishes cell polarity by allowing the formation of adhesion contacts and actin polymerization at the migrating front, while the tailing edge disassembles actin formation. Step 3: CaSR stimulation dependent redistribution of integrins and actin stress formation from the trailing edges to the migrating front is important for guided cell migration. The transfer of actin fibers to the leading edge of the cell, while limiting the level of integrins, actin and microtubules available in the retracting edge prompts directed cell migration toward the calcium gradient. The dynamic assembly and disassembly, together with actomyosin contractility moves the cell toward the Ca^{2+} gradient.

of the cell in the direction of the chemoattractant gradient (**Figure 4**).

The CaSR and integrins are both abundantly localized in caveolae-rich microdomains (Breitwieser, 2013; Iwamoto and Calderwood, 2015; Maartens and Brown, 2015). In cerebellar GCPs, this microdomain-type expression pattern is revealed in discrete puncta present throughout the cell cytosol. In GCPs, *activation of the CaSR resulted in increased cell migration by promoting cell surface expression of β1 integrins, but surprisingly, stimulation of the CaSR did not increase the cell surface expression of the CaSR itself* (Tharmalingam et al., 2016). This observation might be attributed to trafficking mechanisms which control receptor cell surface expression. CaSR and integrin cell surface expression is driven by agonist stimulation, in that activation of the surface receptor activates signaling cascades which allows further insertion of caveolae-rich microdomain containing receptors to the surface, thus allowing persistent signaling in the presence of chronic agonist stimulation (Grant et al., 2011; also see discussion below).

At the molecular level, one potential mechanism for functional cooperation between the CaSR and integrins is via a protein-protein interaction between the CaSR homodimer and the integrin heterodimers within a tetrameric complex, or as part of a larger complex containing additional proteins. A direct protein-protein interaction would require that the CaSR homodimer and integrin heterodimers are either physically in contact with each other, which has not yet been directly demonstrated, or present with additional proteins that influence each other by conformational changes within a macromolecular complex. In this scenario, we propose a working model whereby an agonist-induced conformational change in the CaSR induces flipping of the associated integrin heterodimers into an active conformation. Both the integrins and the CaSR possess large extracellular domains. Although no studies directly examining conformational changes in the CaSR have been reported, other members of this GPCR subfamily such as the $GABA_B$ receptor and the metabotropic glutamate receptors, are known to undergo closure of their large extracellular Venus flytrap domains upon agonist binding (Tsuchiya et al., 2002; Pin et al., 2009). Activation of the CaSR could directly trigger integrin activation thereby inducing cellular adhesion, differentiation, or migration depending on the cellular environment.

An alternative mechanism to consider is a series of biochemical events that entail stimulation of CaSR/integrin complexes followed by the activation of intracellular signaling cascades. Activation of the CaSR is linked to several intracellular signaling pathways which could modify integrin conformation from within the cell. Our previous work in thyroid carcinoma

cells provided evidence for an indirect mechanism in which stimulation of the CaSR induces activation of PLC and release of intracellular Ca^{2+}, which in turn promotes cell adhesion and migration (Tharmalingam et al., 2011; see **Figure 5A**). Stimulation-dependent redistribution of integrins and actin stress formation from the trailing edges to the migrating front is important for chemoattractant-guided cell migration, and PLC activation and the release of intracellular Ca^{2+} have been shown to be important for integrin inside-out activation (Rowin et al., 1998). Specifically, a rise in intracellular Ca^{2+} increases cell adhesion and migration by specifying the location and timing of cell process retraction (Sjaastad et al., 1996; Valeyev et al., 2006); migrating cells display increased intracellular Ca^{2+} gradients which increase toward the rear of cells (Brundage et al., 1991; Brust-Mascher and Webb, 1998). Elevated intracellular Ca^{2+} has also been linked to increased contractility and adhesion disassembly mediated by calpain, a Ca^{2+} dependent protease (Doyle and Lee, 2002; Robles et al., 2003; Valeyev et al., 2006). Thus, a CaSR-induced rise in intracellular Ca^{2+} may promote integrin inside-out activation at the migrating front while simultaneously inducing adhesion disassembly at the rear of the cell, which then translates into cellular migration.

In contrast to thyroid carcinoma cells, CaSR-integrin actions mediating migration of GCPs during cerebellar development appears to be mediated not by phospholipase C, but rather by the ERK and Akt signaling (Tharmalingam et al., 2016). ERK2 and Akt signaling is known to promote plasma membrane insertion of β1 integrins localized in caveolae-rich endosomal microdomains (Maheshwari et al., 2000; Kinashi, 2005). Phosphorylation of ERK2 supports cell polarization by controlling the proper orientation of centrosomes, an important step for the accumulation of proteins involved in cell movement at the leading edge of the migrating cell (Bisel et al., 2008; Imamura et al., 2010).

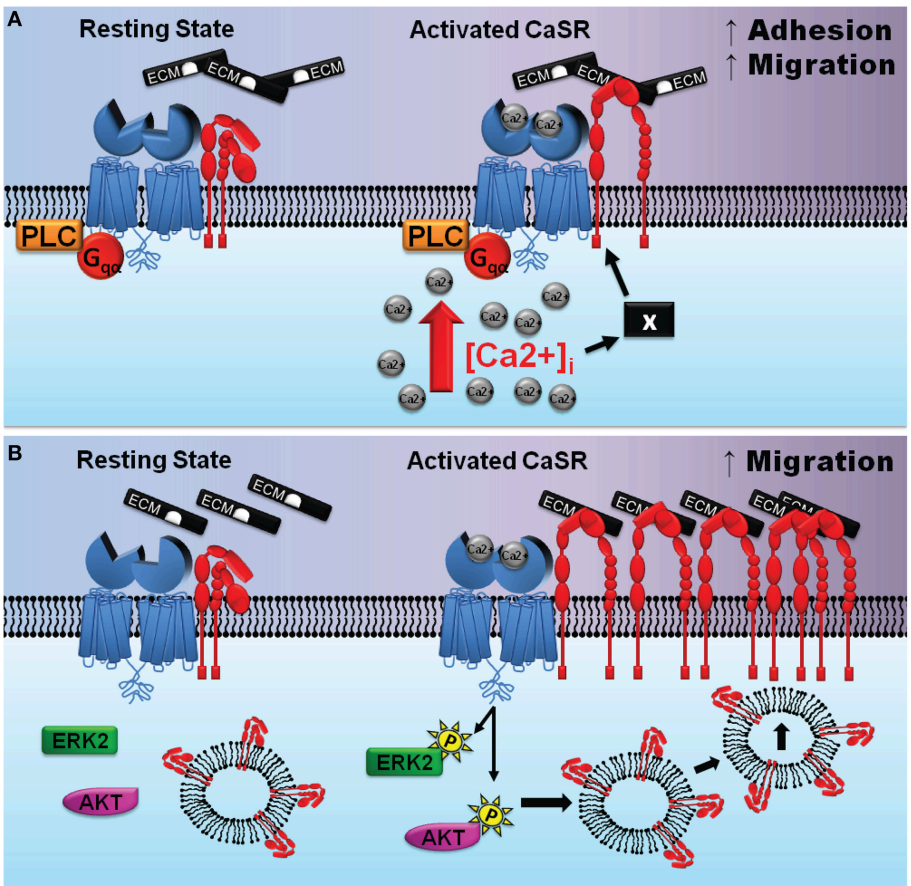

FIGURE 5 | Potential mechanisms of CaSR-integrin actions in cancer cells and the developing CNS. (A) (adapted from Tharmalingam et al., 2011) depicts one possible scenario where the CaSR is associated with integrin heterodimers in a signaling complex in thyroid carcinoma cells. Left, the complex prior to CaSR stimulation; right, elevated extracellular calcium activate the CaSR causing its Venus flytrap domain to undergo a conformational change. This protein domain movement may induce integrins to flip it into an activated state thereby promoting binding to the ECM and enhanced cellular migration. The "X" denotes a hypothetical protein that may participate in facilitating the CaSR-mediated activation of integrins subsequent to increased $[Ca^{2+}]_i$. **(B)** (adapted from Tharmalingam et al., 2016) depicts cross-talk between CaSR (blue), integrin heterodimers (red), and intracellular protein kinases in the developing cerebellum. Left, the CaSR/integrin complex prior to stimulation; right, elevated extracellular Ca^{2+} (and/or polyamines) activate the CaSR which then increases phosphorylation of ERK2 and Akt promoting β1 integrin cell surface expression. The accumulation of integrins at the leading edge of GCPs facilitates stable attachments between the GCPs and the ECM and enhances cellular migration.

We propose that stimulation of the CaSR activates phosphorylation of ERK2 and Akt which then signals the insertion of microdomains containing co-localized CaSR-integrin complexes (**Figure 5B**). Upon entering the plasma membrane, the CaSR agonists activate the newly inserted receptors, which then transactivates the integrins which causes engagement of ECM ligation. The ligated integrins may remain on the plasma membrane to mediate cell adhesion and migration, whereas the endocytotic mechanisms remove the CaSR from the cell surface and degrade the receptor via lysosomal enzymes. In this scenario, accumulation and clustering of integrins would take place at the cell surface, whereas the CaSR would serve as both the signal-sensing partner and as an integrin trafficking partner in the protein complex whose net cell surface expression may not change. In summary, in cerebellar neurons, CaSR-mediated activation of ERK and Akt may mediate trafficking of β1 integrins to the leading edge of the cell and facilitate the formation of a physical link between the ECM and focal adhesion complexes which act to promote neuronal migration.

CONCLUSIONS, IMPLICATIONS, AND OUTSTANDING QUESTIONS

1. The CaSR and GPRC6A are close structural paralogs that are widely expressed in many organs. Both receptors are activated and/or modulated by cations and amino acids.
2. The CaSR and integrins share no sequence homology but both are membrane proteins that function as dimers and possess multiple cation binding sites. Both proteins also undergo large conformational movements into an activated state after agonist stimulation. CaSR/integrin protein complexes have been identified in thyroid carcinoma cells and in cerebellar granule cells, and may exist in other tissues and cells.
3. The CaSR has been shown to play an essential role in cellular differentiation and migration in several cells and tissues, and CaSR-mediated cellular migration likely occurs, at least in some cells, via the activation of CaSR/integrin protein complexes. In thyroid carcinoma cells, the CaSR together with integrins facilitate cellular migration. In the developing cerebellum, activation of the CaSR induces neuronal differentiation and migration. Notably, stimulation of the CaSR in cerebellar neurons did not change the level of plasma membrane expression of the CaSR itself, but instead promoted the trafficking of integrins to the cell surface. Thus, it appears that cerebellar granule cell migration is mediated by a CaSR-induced increase of integrins to the neuronal plasma membrane.
4. In addition to thyroid carcinoma cells and cerebellar granule neurons, the question arises as to what other cells and tissues might utilize the CaSR/integrin system? In light of the widely distributed expression of the CaSR in many organs and cell types, together with the ubiquitous expression of the integrins, could CaSR/integrin protein complexes function as a universal cell migration/homing complex?
5. In a given tissue or cell type the physiological role of the CaSR may change under pathological conditions. For example, upon transformation from normal to cancerous cells, some evidence suggests that the CaSR is associated with different or opposite effects on cell proliferation vs. metastasis. This phenomenon might be compatible with, or linked to, the evolving concept that rapid cell division and proliferation must be arrested prior to metastasis and tumor cell tissue invasion, and that factors operating to promote either cell division or metastatic tissue invasion might be mutually exclusive (e.g., see Matus et al., 2015). In this context, could stimulating CaSR activity via positive allosteric modulators or calcimimetics hinder tumor cell proliferation, and conversely, could inhibiting CaSR activity via calcilytics or receptor knockdown, block or stymie cancer metastasis? This raises the issue that the development of CaSR therapeutics as applied to oncology could be complicated by the possibility of a CaSR drug promoting tumor cell proliferation while inhibiting metastasis, or vice versa. In any case, a more thorough analysis of CaSR pharmacology, expression levels, and signaling mechanisms in tumor cell biology could ultimately provide solutions that translate into novel therapeutic applications.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the text; ST created and prepared the figures.

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REVIEW
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Molecular Basis of the Extracellular Ligands Mediated Signaling by the Calcium Sensing Receptor

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Ca^{2+}-sensing receptors (CaSRs) play a central role in regulating extracellular calcium concentration ($[Ca^{2+}]_o$) homeostasis and many (patho)physiological processes in multiple organs. This regulation is orchestrated by a cooperative response to extracellular stimuli such as small changes in Ca^{2+}, Mg^{2+}, amino acids, and other ligands. In addition, CaSR is a pleiotropic receptor regulating several intracellular signaling pathways, including calcium mobilization and intracellular calcium oscillation. Nearly 200 mutations and polymorphisms have been found in CaSR in relation to a variety of human disorders associated with abnormal Ca^{2+} homeostasis. In this review, we summarize efforts directed at identifying binding sites for calcium and amino acids. Both homotropic cooperativity among multiple calcium binding sites and heterotropic cooperativity between calcium and amino acid were revealed using computational modeling, predictions, and site-directed mutagenesis coupled with functional assays. The hinge region of the bilobed Venus flytrap (VFT) domain of CaSR plays a pivotal role in coordinating multiple extracellular stimuli, leading to cooperative responses from the receptor. We further highlight the extensive number of disease-associated mutations that have also been shown to affect CaSR's cooperative action via several types of mechanisms. These results provide insights into the molecular bases of the structure and functional cooperativity of this receptor and other members of family C of the G protein-coupled receptors (cGPCRs) in health and disease states, and may assist in the prospective development of novel receptor-based therapeutics.

Keywords: calcium sensing receptor, cooperativity, amino acids, structure, trafficking, disease mutations

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INTRODUCTION

In 1883, Sydney Ringer serendipitously discovered Ca^{2+} to be essential for the contraction of isolated hearts (Ringer, 1883). After the mid-twentieth century, research on Ca^{2+} has grown at an exponential rate, and Ca^{2+} is now considered as a universal signal carrier for biological information (Krebs and Michalak, 2007). Ca^{2+} controls life and death, as it modulates the process of fertilization as well as apoptosis. Ca^{2+} was primarily considered as a crucial secondary messenger via rapidly and efficiently regulated changes in intracellular calcium levels, and modulated extensive molecular signaling components through calcium channels, exchangers along with pumps. The discovery

of the calcium sensing receptor (CaSR) defined an additional role of extracellular Ca^{2+} as a first messenger (Smajilovic and Tfelt-Hansen, 2007). It is known that serum Ca^{2+} concentration can regulate the secretion of parathyroid hormone (PTH) and, therefore, research was pursued to investigate how this process is accomplished. In 1993, Dr. Edward M. Brown cloned CaSR, which is primarily responsible for this type of regulation, from bovine parathyroid gland (Brown et al., 1993). The previously observed cytosolic Ca^{2+} changes in parathyroid cells, as well as in other *in vitro* expression systems, were triggered by changes in serum Ca^{2+} concentration and have been largely proven to be mediated by the CaSR (Nemeth and Scarpa, 1987; Muff et al., 1988). In addition to the key tissues involved in extracellular Ca^{2+} and Mg^{2+} homeostasis (e.g., parathyroid, thyroid, kidney, bone), CaSR has also been reported to be present in diverse other, non-homeostatic tissues (e.g., brain, skin, etc.) (Lundgren et al., 1994; Hebert, 1996; Cima et al., 1997; Hinson et al., 1997; Cheng et al., 1998; Kovacs et al., 1998; Chattopadhyay et al., 1999; Brown and MacLeod, 2001; Buchan et al., 2001; Mathias et al., 2001; Hofer and Brown, 2003; Chang and Shoback, 2004; Fudge and Kovacs, 2004; Hofer et al., 2004). To date, extracellular Ca^{2+} has been shown to be a first messenger via CaSR and 14 other family cGPCRs, including metabotropic glutamate receptors (mGluRs) and γ-aminobutyric acid $(GABA)_B$ receptors. As shown in **Figure 1**, high $[Ca^{2+}]_o$ triggers multiple CaSR-regulated intracellular signaling pathways, including $G_{q/11}$ signaling, $G_{i/o}$signaling, G_s signaling, extracellular signal-regulated kinases 1 and 2 ($ERK_{1/2}$) signaling, and intracellular calcium mobilization.

CaSR is comprised of 1078 amino acids, and is encoded by exons 2–7 to encompass a 19 amino acids (AA) N-terminal signal peptide, a ∼600 AA extracellular domain (ECD), a cysteine rich domain (CRD), a ∼250 AA 7-transmembrane (7TM) domain, and the ∼216 AA intracellular domain (ICD) (Pollak et al., 1993; Chikatsu et al., 2000; Hendy et al., 2013). The ECD of CaSR not only plays crucial roles in the sensing of nutrients, such as Ca^{2+}, L-Phe and polypeptides, allowing ligands to modulate CaSR cooperatively, but is also essential for the dimerization of the receptor (Ray et al., 1999; Zhang et al., 2014b). Binding of Ca^{2+} and other CaSR ligands to the ECD is thought to produce conformational changes in the 7TM domain, introducing alterations in intracellular loops and the C-terminal domain, which further trigger the downstream signaling pathways (Brown and MacLeod, 2001). The intracellular C-terminal domain (residues 863–1078) is quite diverse among species. It participates in controlling the CaSR signaling and its cooperativity, modulating receptor trafficking, expression and desensitization (Gama and Breitwieser, 1998; Bai, 2004; Ward, 2004; Huang et al., 2006).

A unique characteristic of CaSR is the high cooperativity of Ca^{2+} dependent activation, which tightly controls the secretion of parathyroid hormone when the receptor is exposed to serum Ca^{2+} concentrations within its responsive range and also regulates the delicate Ca^{2+} homeostasis within the body as a whole (Brown et al., 1993). Functional cooperativity of CaSR (i.e., based on biological activity determined using functional assays), particularly the functional positive homotropic cooperative response to extracellular calcium, is essential for the receptor's ability to respond over a narrow physiological range of $[Ca^{2+}]_o$ (1.1–1.3 mM) (Breitwieser, 2006). CaSR has an estimated Hill coefficient of 3–4 for its regulation of biological processes, such as activating intracellular Ca^{2+} signaling, inhibiting PTH release in parathyroid cells (**Figure 1**) and stimulating calcitonin secretion in C-cells (Walter et al., 2013). Other extracellular mineral cations, such as Mg^{2+}, and amino acids, are able to function as agonists and co-agonists to regulate/potentiate the Ca^{2+}-induced activation of the CaSR. L-amino acids, especially aromatics, at physiological conditions potentiate the Ca^{2+}–elicited activation of the CaSR by altering the EC_{50} values for extracellular calcium evoked intracellular calcium responses via positive heterotropic functional cooperativity (Conigrave et al., 2000; Francesconi and Duvoisin, 2004; Wang et al., 2006). This capacity of CaSRs to integrate both divalent cations and other extracellular stimuli, such as amino acids (Vetter and Lohse, 2002), is a feature shared by other cGPCRs (Wise et al., 1999; Galvez et al., 2000; Gether, 2000; Oldham and Hamm, 2008; Rosenbaum et al., 2009). In this paper, we will review the major discoveries and approaches that have been taken in uncovering the molecular basis of the cooperative responses of CaSR and its impact in understanding molecular bases of diseases (Zhang et al., 2014b). Key determinants contributing to the functional cooperativity of CaSR including the calcium and ligand binding sites, and the connectivity between distant sites on the ECD domain as well as protein expression at the membrane surface and intracellular domain will be discussed.

CHALLENGES

To date, how Ca^{2+}, Mg^{2+}, and amino acids cooperatively modulate intracellular Ca^{2+} signaling is a long-standing unanswered question mainly due to the lack of a determined CaSR structure. Like other cGPCRs, CaSR functions as a dimer (Bai et al., 1998a, 1999; Pace et al., 1999; Kunishima et al., 2000; Zhang et al., 2001; Bai, 2004; Suzuki et al., 2004) with a very long N-terminus that is predicted to fold into a bilobed ECD (Brown et al., 1993; Hebert and Brown, 1995; Pearce et al., 1996; Hinson et al., 1997; Hofer and Brown, 2003; Hu and Spiegel, 2003; Jingami et al., 2003; Quinn et al., 2004). The ECD has been shown to play an important role in the cooperative responses of the receptor to changes of $[Ca^{2+}]_o$, amino acids, metabolites, and neurotransmitters (Bai, 1999, 2004; Conigrave et al., 2002; Zhang et al., 2002; Chang and Shoback, 2004; Conigrave and Lok, 2004; Mun et al., 2004). Determination of the X-ray structure of the ECD of CaSR is largely hampered by difficulty in crystallization due to heterogeneous and extensive glycosylation (11 potential N-glycosylation sites) and challenges associated with membrane proteins (Yang et al., 2005). Further, Ca^{2+} and ligand-binding sites with weak binding affinities and rapid off rates are often not occupied in a determined X-ray structure. For example, no bound Ca^{2+} has been observed in over 30 X-ray structures of the ECD of mGluRs (Kunishima et al., 2000; Tsuchiya et al., 2002; Jiang et al., 2010; Zhang et al., 2014a),

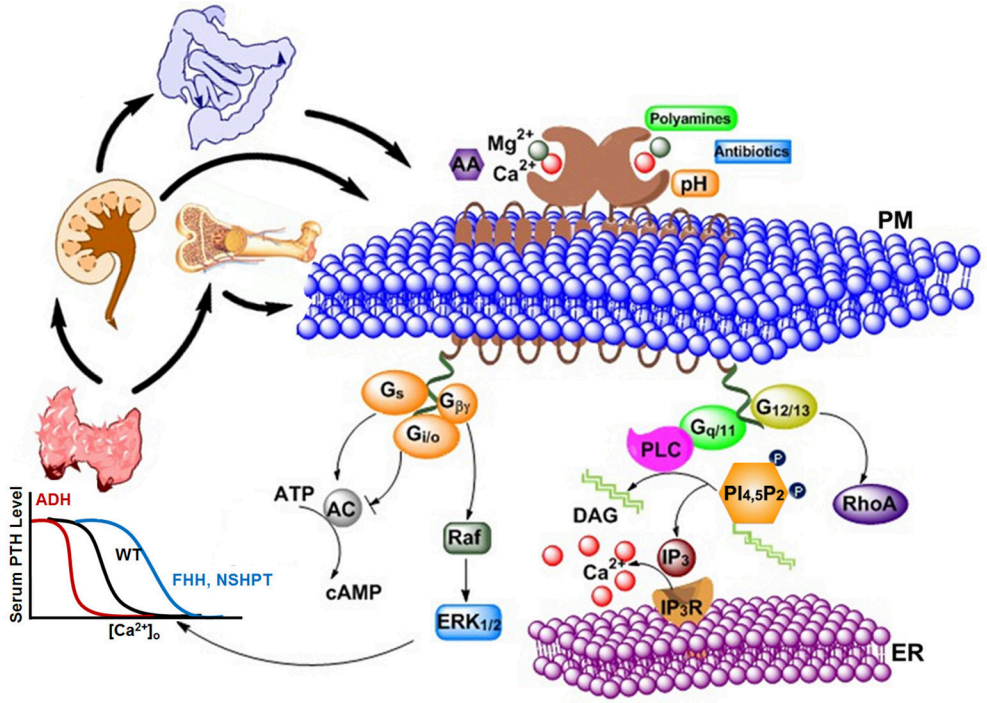

FIGURE 1 | Various types of agonists, including cations, peptides, amino acids, antibiotics, etc., can act on the extracellular calcium-sensing receptor (CaSR) to generate a complex intracellular signaling network. CaSR is also a pleiotropic receptor in its regulation of four G protein-mediated intracellular signaling pathways ($G_{q/11}$, $G_{i/o}$, G_s, and $G_{12/13}$). The correlations and crosstalk among different signaling cascades contribute the cooperative responses of intracellular calcium responses as well as parathyroid hormone (PTH) secretion (left) and intracellular calcium responses (right) to extracellular calcium. Bottom left: The sigmoidal relationship between calcium concentration in blood and PTH level in serum is demonstrated. Higher Ca^{2+} concentration is required for normal level of PTH in patients with familial hypocalciuric hypercalcemia (FHH) or patients with Neonatal Severe Primary Hyperparathyroidism (NSHPT), as the response curve shifts to the right; on the other hand, lower Ca^{2+} concentration than normal is enough to trigger PTH secretion in patients with autosomal dominant hypocalcemia (ADH). PM, Plasma membrane; ER, Endoplasmic reticulum; AA, arachidonic acid; AC, adenylate cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; $ERK_{1/2}$, extracellular-signal-regulated kinase; G_s, $G_{i/o}$, $G_{12/13}$, and $G_{q/11}$, subunits of the s-, i-, 12/13, and q-type alpha subunit of heterotrimeric G proteins, respectively; $G_{\beta\gamma}$, G beta and gamma complex; IP3, inositol-1,4,5-trisphosphate; IP_3R, inositol-1,4,5-trisphosphate receptor; PLC, phospholipase C; $PI(4,5)P_2$, phoshatidylinositol-4,5-bisphosphate; RhoA, Ras homolog gene family, member A.

despite the clear modulatory effect of $[Ca^{2+}]_o$ on this receptor. Furthermore, additional challenges result from the lack of direct binding assays for weak Ca^{2+}-binding, amino acid-binding (K_d ~mM) and limitations in obtaining purified membrane proteins with native conformations (Nagar et al., 1996; Hu and Spiegel, 2003; Magno et al., 2011). The quantification of functional cooperativity with binding cooperativity and visualization of the molecular connectivity in tuning cooperativity requires innovative approaches.

MODELING STRUCTURES OF THE CaSR

Several regions of the CaSR and mGluRs are highly homologous, providing the basis for molecular modeling of the structure of CaSR under various conditions based on the determined crystal structures of mGluRs with and without ligands or agonists/antagonists (Bai, 2004; Hu and Spiegel, 2007; Huang Y. et al., 2007). Various mGluR structures have indicated that agonist binding induces a rearrangement of the dimeric VFT by bringing the two monomers closer together owing to pulling the lower lobes inwards upon the change toward the closed state. This is further evidenced by the addition of gadolinium, which is found to likely stabilize the active form of mGluR1 (Tsuchiya et al., 2002). Recent crystal structures of various mGluRs have been solved that shed light on several possible sequential activation events. Orthosteric ligands bind to the VFT in the dimer, stabilizing the closed conformation to induce an activation signal to the transmembrane domain by the CRD, which then activates G protein-regulated signaling pathways (Kunishima et al., 2000; Jingami et al., 2003; Muto et al., 2007; Wu et al., 2014).

Figure 2 shows three modeled structures of the ECD of CaSR in different conformational states based on the crystal structure of mGluRs. The ECD of CaSR possesses a featured bilobed VFT structure similar to mGluRs and bacterial periplasmic binding proteins (O'Hara et al., 1993; Ray et al., 1999; Reyes-Cruz et al., 2001). These modeled structures of CaSR derived from several crystal structures of mGluR1 provide molecular views of large conformational variances among different states and therefore provide a potential functional mechanism of CaSR and by extension, of other cGPCRs. The VFT region alternates between closed and open states, determined by the presence of

a ligand (e.g., glutamate for mGluR1 or Ca^{2+} for CaSR), which then causes the signal to be relayed to the 7TM domain. As cGPCRs are only functional as dimers, the dimerization of CaSR is likely to be driven by the hydrophobic interactions between the monomers along the dimer interface. CaSR and mGluR protomers are also covalently linked by several disulfide bridges between the two monomers. A ligand induced rearrangement of the dimeric VFT brings the two monomers closer together by pulling the lower lobes inwards upon the change toward the closed state. Similar to mGluR, the ligand was proposed to interact with both lobes of the VFT to stabilize the closed form by providing additional points of contact between the lobes (Kunishima et al., 2000).

The homology models of the CaSR 7TM domain was built based on the crystal structure of bovine rhodopsin by several independent research groups (Petrel et al., 2003; Miedlich et al., 2004; Hu et al., 2006; Bu et al., 2008). Miedlich et al. docked the CaSR with the antagonist, NPS 2143, and the calcimimetic, NPS R-568, to the derived 7TM domain model structure and reported a shared pocket composed of residues F668, F684 and E837 (Miedlich et al., 2004). The evidence that there are additional Ca^{2+}-binding sites located at the 7TM domain of the CaSR (7TM) have been provided by different independent groups (Hauache et al., 2000; Hu et al., 2002, 2005). The ECD portion of CaSR, including the CRD linker, has a 30–35% sequence similarity across all mGluRs, while the linker alone has 65–70% similarity (Bai, 2004). Together with a recent determination of structure of the ECD domain of mGluR3 with the cysteine-rich domain and the transmembrane domain of mGluR1 makes it possible to model the full length CaSR shown in **Figures 2C,D**. Due to high sequence homology, the modeled transmembrane structure provides more structural information than previous modeled 7TM domains based on the crystal structure of bovine rhodopsin (Petrel et al., 2003; Miedlich et al., 2004; Hu et al., 2006; Bu et al., 2008). These modeling and structural studies, therefore, provide important insights into the large conformational changes induced by agonist binding to both mGluR and CaSR. For instance, the ECD is changed from an open status to a closed form, although the calcium binding sites and amino acid binding sites remain elusive due to their weak binding affinities.

IDENTIFICATION OF CA^{2+} BINDING SITES IN THE ECD AND THEIR HOMOTROPIC COOPERATIVITY

Since the ECD is responsible for the main binding activity, researchers turned to identifying the binding regions for Ca^{2+} and amino acids based on studies from homology modeling, mutational studies and the effect of disease related mutations (Silve et al., 2005). To overcome the challenges involved in identifications of calcium binding sites with weak binding affinity of membrane proteins, Huang et al. developed a computational algorithm to be used for the prediction of potential Ca^{2+} binding pockets based on a statistical analysis of multiple calcium binding proteins (Huang Y. et al., 2007; Kirberger et al., 2008; Huang et al., 2009; Wang et al., 2009, 2010; Zhao et al., 2012). Shown in **Figure 3**, five potential Ca^{2+}-binding sites were predicted in each monomer of the ECD of the homologous model of CaSR. Site 1 (S147, S170, D190, Y218, and E297) is located within the hinge region between the two lobes of the ECD (Kubo et al., 1998; Silve et al., 2005; Huang Y. et al., 2007). It is interesting to note that E297, S147, and S170 were reported to be important for sensing Ca^{2+} and/or amino acids (Bai et al., 1999; Mun et al., 2005; Silve et al., 2005). Recently, a novel Ca^{2+}-binding site in the similar hinge region adjacent to the reported L-Glu binding site in mGluR1 was found to synergistically activate the receptor with L-Glu, and enhance the activity of the mGluR1 orthosteric and allosteric ligands (Jiang et al., 2010, 2014). This same phenomenon may also be applicable to other type of cGPCRs.

Since the initial prediction, several complementary approaches have been used to provide important insights into how CaSR functions and the behavior of the receptor at the molecular level. First, functional cooperativity contributed by each Ca^{2+}-binding site was determined by site-directed mutagenesis and monitoring intracellular Ca^{2+} oscillations, IP production and $ERK_{1/2}$ activation in living cells. We reported that the predicted Ca^{2+}-binding site 1 within the hinge region of the ECD of CaSR and its interaction with other Ca^{2+}-binding sites within the ECD is essential in tuning functional positive homotropic cooperativity of CaSR caused by changes in $[Ca^{2+}]_o$. Furthermore, molecular dynamic simulations indicated that there is molecular connectivity among the other predicted Ca^{2+}-binding sites with site 1 and that the VFT hinge domain plays a central role in functional cooperativity (Zhang et al., 2014a). These results from various functional studies suggest that cooperative binding of Ca^{2+} at multiple predicted Ca^{2+}-binding sites of the CaSR likely maximizes its capacity to respond over a narrow physiological range of $[Ca^{2+}]_o$ independent of amino acids or other agonists (Bai et al., 1998a; Zhang et al., 2001; Bai, 2004; **Figure 2**)

The direct binding of Ca^{2+} to the other predicted Ca^{2+}-binding sites in the ECD of CaSR and related binding cooperativity has been probed by using several methods. Ca^{2+} binding capabilities of predicted Ca^{2+}-binding Site 3 (residues E224, E228, E229, E 231 E232), and Site 5, which is formed by contiguous Ca^{2+} binding residues (residues E378, E379, T396, D398, E399), were verified using a grafting approach (Huang Y. et al., 2007). By grafting peptide sequences composed of key predicted Ca^{2+} ligand binding residues separated by flexible linkers into a non-Ca^{2+}-binding protein, site specific Ca^{2+} binding capability was determined using a Tb^{3+}-sensitized FRET assay. Huang et al. also applied this approach to probe Ca^{2+} binding at three additional Ca^{2+} binding sites and potential binding cooperativity. Three major subdomains of the CaSR containing various numbers of predicted Ca^{2+} binding sites were expressed and purified. Their Ca^{2+}-binding capabilities were examined using mutagenesis studies combined with various spectroscopic studies utilizing 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence, intrinsic tryptophan fluorescence spectra and nuclear magnetic resonance (NMR) (Huang et al., 2009). It was striking to observe that subdomain 1 with three binding sites (including sites 1, 2, and 3) exhibited a large

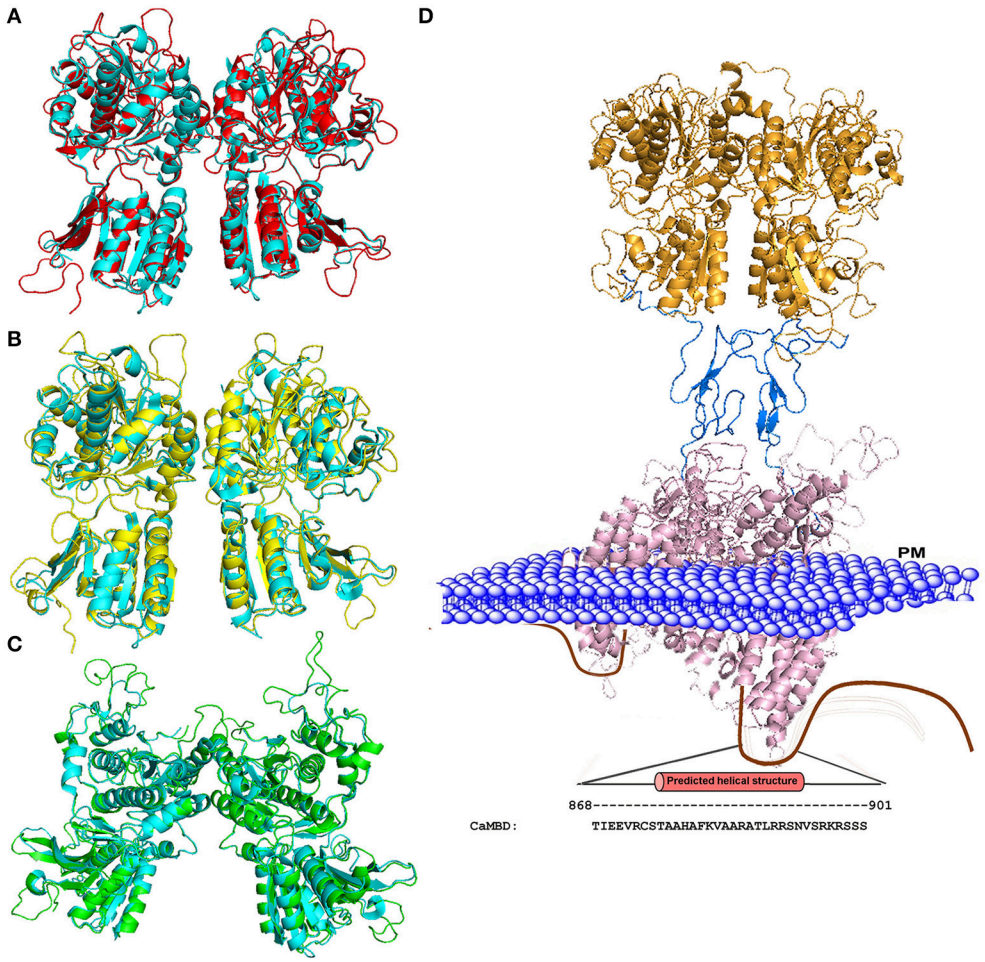

FIGURE 2 | Homology models of the CaSR ECD. (A) Structural alignment of the mGluR1 holo form (cyan; PDB ID 1EWK, mGluR1 with glutamate) with the modeled structure of CaSR (red). **(B)** Structural alignment of mGluR1 apo form I (cyan, PDB ID 1EWV) with the modeled structure of CaSR (yellow). **(C)** Structural alignment of mGluR1 apo form II (cyan, PDB ID 1EWT) with the modeled structure of CaSR (green). **(D)** Homology model of the full CaSR structure. The ECD is based on the crystal structure of CaSR, the cysteine rich domain modeled from mGluR3 (PDB ID 2E4U), and the 7TM domain is modeled from mGluR1 with a ligand (PDB ID 4OR2). The C-terminal of CaSR can interact with protein kinases, ubiquitin ligase, CaM, etc. The region including residues from 868 to 901 is predicted to be CaM binding site. Mutations on the CaM binding site compromise the stability of surface expressed CaSR (Huang et al., 2010). PM: Plasma Membrane.

Ca^{2+} dependent conformational change. A binding process with strong and weak Ca^{2+}-binding affinities has been clearly unveiled for subdomain 1 by both fluorescence and NMR studies. These studies not only confirmed the existence of multiple Ca^{2+} binding sites, but also revealed that the molecular connectivity among multiple metal binding sites is essential for a highly cooperative functional activity (**Figure 2**).

The complex glycosylated and high mannose CaSR ECD (residues 20–612) were purified from HEK293S and its mutant cell line (Zhang et al., 2014c). Using various spectroscopic methods, it was shown that both form the ECD bound to Ca^{2+} with a K_d of 3.0–5.0 mM. The local conformational changes of the proteins induced by their interactions with Ca^{2+} were visualized by NMR with specific ^{15}N Phe-labeled forms of the ECD. These studies also suggest that glycosylation does not affect calcium binding properties.

IDENTIFICATION OF AMINO ACID BINDING SITE AND HETEROTROPIC FUNCTIONAL ACTIVITY

The extracellular calcium induced activation of CaSR is potentiated by L-amino acids to maintain the whole body Ca^{2+} homeostasis, making the receptor a multimodal and multimetabolic sensor (Francesconi and Duvoisin, 2004; Breitwieser, 2006; Conigrave et al., 2007a). Under physiological conditions, L-amino acids, especially aromatic amino acids (e.g., L-Phe, L-Trp), as well as short aliphatic and small polar amino acids, potentiate the extracellular calcium triggered CaSR activity by altering the EC_{50} values required for Ca^{2+}-evoked intracellular calcium responses and its functional cooperativity. For example, the EC_{50} for Ca^{2+} decreased from 4.2 ± 0.2 to 2.5 ± 0.1 mM in the presence of L-Phe (Conigrave et al., 2000,

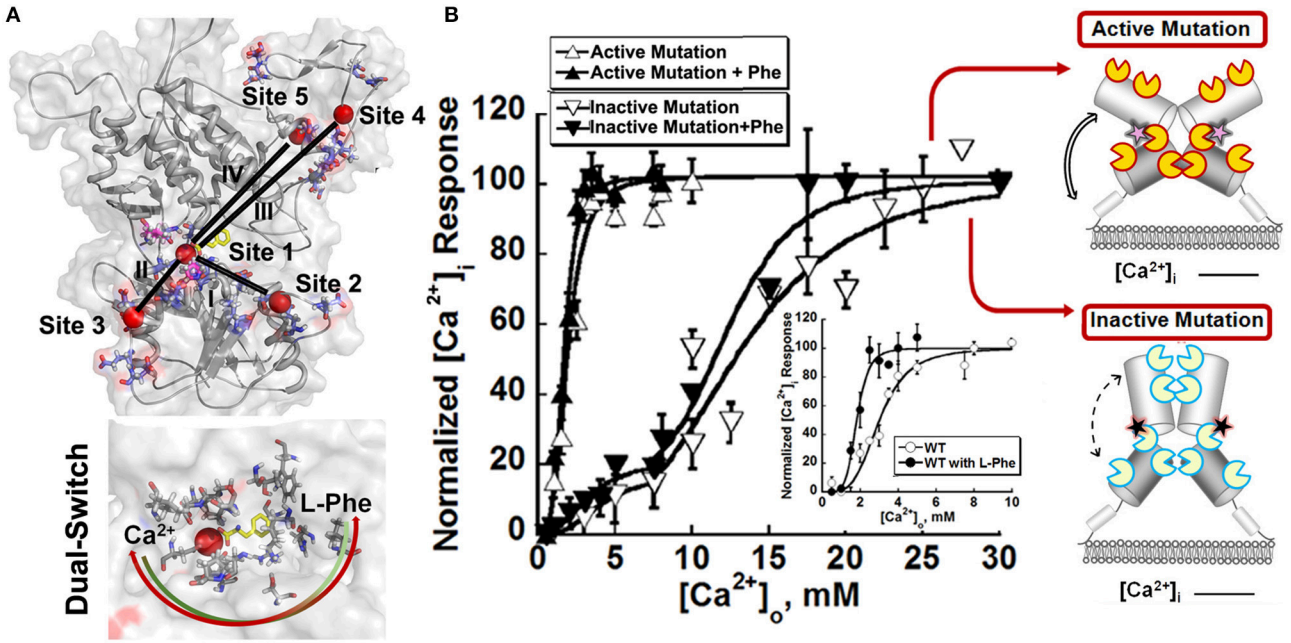

FIGURE 3 | Molecular connectivity and heterotrophic cooperativity. (A) The correlated motions between calcium binding site 1 and the other calcium binding sites, designated by roman numbers (I~V), are mapped onto the CaSR ECD models. The correlation map and parameters for molecular dynamics simulation can be found in the paper (Zhang et al., 2014a). Bottom: The hetero-communication between Ca^{2+} and an amino acid functions as a dual switch that enhances the function of CaSR by positively impacting multiple Ca^{2+}-binding sites within the ECD. Red sphere: Ca^{2+}. Two headed arrow: both ligands can affect the binding of the other, therefore, the downstream signaling pathways. **(B)** The intracellular Ca^{2+} responses of HEK293 cells transiently overexpressing an active mutation or an inactive mutation. Inset: Representative response of WT CaSR to the changes of extracellular Ca^{2+} in the absence or presence of L-Phe, showing homotropic, and heterotropic cooperativity. Some disease mutations can interrupt the homotropic cooperativity shown as intracellular calcium responses to changes in the extracellular calcium concentration as well as the heterotropic cooperativity in the presence of allosteric modulators (e.g., L-Phe). Open circles: in the absence of L-Phe; Closed circles: in the presence of 5 mM L-Phe. Yellow "pacman": Ca^{2+} binding sites in an active receptor; Blue "pacman": Ca^{2+} binding sites in an inactive receptor. Pink stars: gain-of-function mutations in the hinge region. Black stars: loss-of-function mutations in the hinge region. Two headed arrows with solid lines: enhanced correlation motions between different Ca^{2+} binding sites. Two headed arrows with dash lines: impaired correlation motions.

2007a; Wang et al., 2006). At the threshold level of extracellular Ca^{2+} concentration, L-amino acids induced slow intracellular Ca^{2+} oscillations with a frequency at around 1–2 peaks/min (Breitwieser, 2006). In aggregate, the levels of amino acids in human serum in the fed state are close to those activating the CaSR *in vitro* (Conigrave et al., 2000, 2007b) and can further enhance functional cooperativity via positive heterotropic cooperativity. The CaSR in cells within the lumen of the gastrointestinal (GI) tract are also activated by L-Phe and other amino acids, which have been long recognized as activators of key digestive processes. Thus, the CaSR enables the GI tract to monitor events relevant to both mineral ion and protein/amino acid metabolism, in addition to its sensing capability in blood and related extracellular fluids (Conigrave et al., 2000; Wang et al., 2006; Broadhead et al., 2011; Liou et al., 2011). Distinctive signaling pathways elicited by amino acids compared with Ca^{2+} have been demonstrated in quite a few studies (Breitwieser, 2006; Broadhead et al., 2011). Ca^{2+} activates CaSR though activation of the heterotrimeric GTP binding protein G_q, causing the generation of Ins(1,4,5)P3 (IP3), which binds to the IP3 receptor on the ER, releasing stored Ca^{2+}. On the other hand, L-Phe in the presence of Ca^{2+} induces coupling to heterotrimeric GTP binding protein $G_{12/13}$, leading to the activation of RhoA via filamin A.

Some potential L-Phe binding site residues in the CaSR were identified based on the conserved amino acid residues involved in the binding of glutamate to mGluRs (Zhang et al., 2002). Their studies demonstrated that mutating three adjacent Ser (S169, S170, S171) to Ala eliminated L-Phe potentiated receptor activity measured by $[Ca^{2+}]_i$ mobilization using a fluorescence-based cell population assay. Meanwhile, studies from Mun et al. showed the binding site for amino acids was within the VFT domain of CaSR by utilizing CaSR-mGluR chimeric receptor constructs. The receptor lacking the ECD exhibited impaired response to L-amino acids (Mun et al., 2004). The same group showed that two residues, Thr145 and Ser170, could be crucial for sensing L-amino acids, using mutagenesis and fluorimetry studies (Mun et al., 2005).

Recently, an L-Phe binding site adjacent to the previously predicted calcium-binding site 1 based on computational docking results was reported. The potential L-Phe-binding site is composed of residues Leu51, Thr145, Ser170, Tyr218, and Ser272. The residue Tyr218 is involved in both Ca^{2+} and Phe binding. Extensive mutational studies using both single

cell imaging and fluorimetric assays identified the importance of L-Phe-binding pocket for positive heterotropic cooperativity between extracellular Ca^{2+} and L-Phe in eliciting CaSR-mediated Ca^{2+} signaling (Zhang et al., 2014a). The frequency of sinusoidal intracellular Ca^{2+} oscillations in CaSR transfected HEK293 cells not only depend on the extracellular Ca^{2+}, but also affected by the binding of allosteric modulators. For example, the binding of CaSR with Ca^{2+} alone produces a intracellular Ca^{2+} oscillations frequency (1.5/min at room temperature) which is different than those produced by Ca^{2+} with L-amino acid (e.g., L-Phe) (2.2/min) (Breitwieser, 2006; Zhang et al., 2014b). This change in frequency is extremely important for the role of CaSR signal transduction in the many different tissue and organ environments in which it is present. For instance, the Ca^{2+} oscillations in parathyroid, kidney, intestine and bone are tightly related to maintenance of Ca^{2+} homeostasis while the Ca^{2+} signals are processed differently in many epithelial cells and the nervous system (Riccardi, 2002; Breitwieser, 2006).

The hetero-communication between Ca^{2+} and an amino acid functions as a dual switch that globally enhances functional positive homotropic cooperative activation of CaSR in response to Ca^{2+} signaling by positively impacting multiple Ca^{2+}-binding sites within the ECD (**Figure 3**; Zhang et al., 2014a). A direct interaction between the CaSR ECD and L-Phe was finally reported using saturation transfer difference NMR approaches with a determined binding affinity of ~10 mM in the absence of Ca^{2+} (Zhang et al., 2014c). The study further demonstrated that L-Phe increases the binding affinity of the CaSR ECD for Ca^{2+}. Thus, dual binding of calcium and amino acids at the hinge regions of the bilobed VFTD of the CaSR leads to activation of the receptor with highly cooperative responses to the changes in the extracellular concentrations of these agonists (**Figure 4**).

MOLECULAR BASIS OF DISEASE RELATED CASR MUTATIONS

More than 200 mutations of the CaSR that lead to the disorders of Ca^{2+} homeostasis have been identified (http://www.casrdb.mcgill.ca/) (Hendy et al., 2009). Mutations of CaSR may inhibit receptor activity, lead to an over activation of the receptor, or markedly impair the receptor's structure. The majority of these disease-associated mutations are missense mutations, with a single amino acid substituted, often from one base pair change. Amino acids insertion, deletion, open reading frame shift, and splice-site mutations have also been reported (Hendy et al., 2009). The diseases associated with inactivate receptors include cases of Familial Hypocalciuric Hypercalcemia (FHH) (Ward et al., 2006) and Neonatal Severe Hyperparathyroidism (NSHPT) (Thakker, 2004) while the disorders associated with the CaSR activating mutations include Autosomal Dominant Hypocalcemia (ADH) (Hendy et al., 2000) and Bartter syndrome type V (Watanabe et al., 2002).

Below is the summarized four types of disease mutations based on their distinct mechanisms of action. Type I and type II mutations alter the CaSR's function, especially EC_{50} or Hill

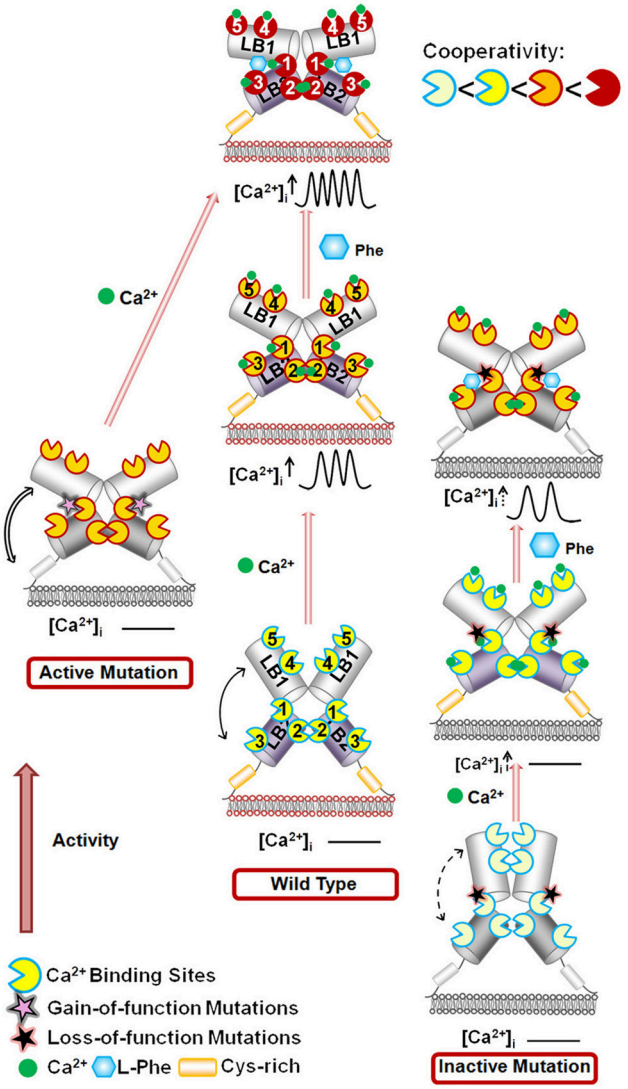

FIGURE 4 | Schematic representation of the mechanisms underlying the effects of the mutations on the CaSR and the modulation of receptor activity by extracellular Ca^{2+} and L-Phe. Ca^{2+} and L-Phe modulate the activity as well as the cooperativity of CaSR (the color changes of the receptor from ivory to red indicate an increase in functional activity). Elevating $[Ca^{2+}]_o$, e.g., to 3.0 mM, is proposed to change the basal WT CaSR status into an active form in a positive homotropic cooperative manner and further trigger intracellular Ca^{2+} oscillations. L-Phe binds to the hinge region between lobe 1 and lobe 2, modulating the receptor together with Ca^{2+} in a positive heterotropic cooperative way. This could potentiate conversion of the receptor to a "fully active" form associated with a higher frequency of intracellular Ca^{2+} oscillations and a left-shifted EC_{50}. Loss-of-function CaSR mutants (indicated by ivory color) could cause a disruption of the cooperativity among the various Ca^{2+}-binding sites as shown by impaired correlation motions (dashed arrows). The impaired receptor function and the cross-talk between Ca^{2+}-binding sites can be at least be partially rescued for some mutants by L-Phe (e.g., P221Q). However, if the mutation interferes with the interaction between CaSR and L-Phe, the function of the receptor may not be fully recovered (e.g., L173P). CaSR gain-of-function mutants (left) exhibit enhanced correlated motions (double line arrows) and their activity is not further potentiated by L-Phe, potentially due to a ceiling effect. LB1(2): Lobe 1 or lobe 2 in the VFT domain of CaSR.

number without altering surface expression or trafficking. Type III and type IV mutations largely affect degree of cooperativity via altering surface expression and trafficking of the protein to the plasma surface (**Figure 5**, **Table 1**).

The first type of mutational effect, type I, is via direct alterations in key calcium and ligand binding capability at or in close proximity to the predicted ligand binding sites in the ECD (**Figure 5**). Hannan et al. found that more than 50% of their newly identified CaSR mutations in patients with FHH, NSHPT, and ADH are within the ECD of the receptor (Hannan et al., 2012). Analysis using the homology modeled structure of the CaSR further revealed that >50% of these missense substitutions are located within 10 Å of one or more proposed calcium-binding sites, indicating that the bilobed VFT domain plays a pivotal role in interacting with Ca^{2+} and regulating the function of CaSR (Hannan et al., 2012). Intriguingly, more than half of the mutations near Ca^{2+}-binding sites are situated close to Site 1, suggesting the importance of the VFT domain cleft. The molecular connectivity between Site 1 and rest of the calcium-binding sites can possibly facilitate the positive cooperativity of the CaSR (Zhang et al., 2014d).

Through Ca^{2+} binding site modeling and mutational studies, several key residues for the predicted Ca^{2+} binding sites in the hinge region of the VFT domain have been identified (Deng et al., 2006; Huang Y. et al., 2007; Huang et al., 2009). Amongst them, more than half of the ECD mutations are near the proposed Ca^{2+} binding sites, and a few of them are found directly in the potential binding site residues. One ADH mutation (E297D) and several FHH mutations (Y218S, Y218C, and E297K) are within Ca^{2+} binding site 1. The FHH mutation D215G is located in Ca^{2+} binding site 2 while two ADH mutations E228Q and E228K are in Site 3. The Ca^{2+} binding Site 4 embraces residues involved in a carcinoma-associated mutation E350V and an ADH mutation E354A. Mutations on these key binding residues leads to a decrease of the EC_{50} of Ca^{2+} in ADH in order to produce PTH, and an increase of EC_{50} in FHH or NSHPT. This can also lead to an alteration of binding cooperativity between the other Ca^{2+} binding sites, allowing them to bind more or less effectively as indicated by changes in the Hill number. Several studies have

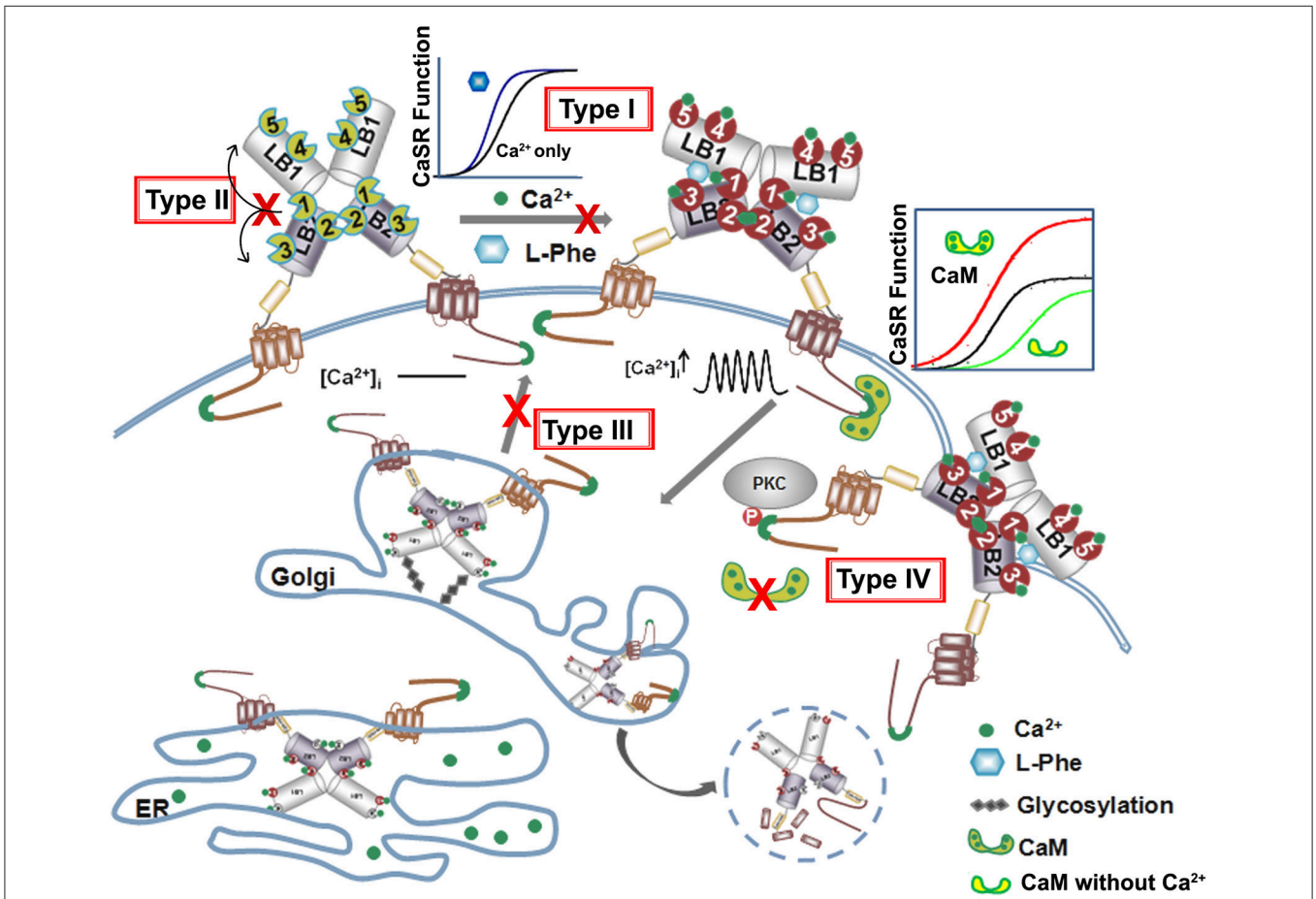

FIGURE 5 | Summary of four types of disease mutations. The CaSR trafficking, expression and surface stabilization contribute to its functional cooperativity. Type I disease associated mutations directly alter key calcium and ligand binding capability at or in close proximity of the predicted ligand binding sites in the ECD. Type II mutations alter CaSR function especially EC_{50} or Hill number without altering surface expression or trafficking, but affect the molecular connectivity between different ligand binding sites. Type III mutations disrupt the cooperativity via interfering with the receptor cell surface expression and Type IV mutations largely affect potency of cooperativity by altering trafficking and protein stability via interaction with proteins binding to the CaSR intracellular C-tail.

TABLE 1 | Summarized molecular basis of disease related CaSR mutations.

Mutation types	Function on CaSR	Examples	References
Type I	Mutations directly affecting key Ca^{2+} and ligand binding residues or in close proximity of binding pocket. Leads to a loss-of- or gain-of-function of CaSR.	E297K, E297D, Y218S, Y218C, R227L, E191K, E228Q, E228K, E350V, E354A, D215G, etc.	Pearce et al., 1996; Silve et al., 2005; Deng et al., 2006; Huang Y. et al., 2007; Huang et al., 2009; Hannan et al., 2012; Zhang et al., 2014d
Type II	Mutations disrupting the molecular connectivity embedded into the hinge region of the ECD between other proposed Ca^{2+} sites, without effect on surface expression or intracellular trafficking.	L173F, L173P, P221Q, and P221L.	Felderbauer et al., 2006; Zhang et al., 2014b
Type III	Mutations leading to a decreased or increased potency of cooperativity by altering its cell surface expression. Have lower expression or overexpression on the cell surface of CaSR leads to a change in Ca^{2+}-sensing capabilities.	P39A, G143E, L174R, C582Y, R638L, S657Y, P748R, G778D, E297K, C395R, A804D, P55L, S137P, R185Q, D215R, G553R, R795W, V817I, P798T, S657Y, G670E, R680H, M734R, etc.	Bai et al., 1998a; Fan et al., 1998; Zhang et al., 2001; Christopoulos and Kenakin, 2002; Casadó et al., 2007; Huang and Breitwieser, 2007; White et al., 2009; Leach et al., 2013
Type IV	Mutations which effect cooperativity of CaSR by hampering its intracellular trafficking capabilities and protein stability via the intracellular C-terminal tail and its binding partners.	T876ins, F881L, R886W, and R886P for FHH/NSHPT and S895del, del920–970, mutations on partner proteins, etc.	Thakker, 2004; Kemp et al., 2009; Stepanchick et al., 2010; Grant et al., 2011; Arulpragasam et al., 2012; Chakravarti et al., 2012; Davey et al., 2012; Breitwieser, 2013; Nesbit et al., 2013

investigated the effect of FHH Ca^{2+} binding site 1 mutations, such as Y218S (Pearce et al., 1996) and E297K (Bai et al., 1999), both of which led to a loss-of-function with an increase in EC_{50} for Ca^{2+} in terms of intracellular Ca^{2+} mobilization. Additionally, E297K also decreases inositol-1-phosphate (IP1) accumulation, but does not change CaSR cell expression levels as compared to the wild-type (WT) (Bai et al., 1999). ADH mutation E297D, as well as FHH mutation E297K, were analyzed by Silve et al. for IP1 accumulation and expression levels (Silve et al., 2005). Again, they saw no change in cell expression compared to the WT, but they did see an activation shift for E297D, as the EC_{50} for Ca^{2+} induced IP stimulation changed from the WT EC_{50} of 4.30 ± 0.20 to 2.70 ± 0.30 mM, and a loss-of-function shift in EC_{50} for E297K (Silve et al., 2005).

Additionally, mutations in residues neighboring the binding site also show the same trend in an altered EC_{50} and cooperativity but with no apparent effect on cell surface expression or trafficking, such as the FHH/NSHPT mutations S171N, R227Q, R227L, S296N, F351V, W352X, C395R, G397R, the ADH mutations E191K, E241K, Q245R, and cancer-associated mutations, S169F and S171G. Mutations on these binding site residues or their neighboring residues would have major effects on either direct binding or conformational direction of the binding site residues, respectively. Pearce et al. looked at two such mutants R227L (NSHPT) and E191K (ADH) (Pearce et al., 1996). R227L increased EC_{50} for extracellular Ca^{2+} induced intracellular Ca^{2+} response to 9.3 mM compared to the WT 4.0 mM, while the E191K mutant decreased EC_{50} for $[Ca^{2+}]_o$ from the WT 3.7 to 2.8 mM. Western blot analysis showed that compared to WT there was no increase or loss in expression (Pearce et al., 1996). Thus, concluding that binding site residues, and their immediate bordering residues, significantly alter the charge or conformation of the binding pocket therefore altering functionality into a gain-of or loss-of-function mutation with a major effect on protein expression, translation, and/or trafficking.

The second type of disease mutations, type II, disrupts molecular connectivity embedded into the hinge region of the ECD (**Figures 3**, **5**). Mutations at the particular region of the N-terminal VFT domain produce either receptor inactivation (L173P, P221Q) or activation (L173F, P221L) related to hypercalcemic or hypocalcemic disorders. We have shown that both L173P and P221Q markedly impair the functional positive cooperativity of the CaSR as reflected by Ca^{2+}–induced intracellular Ca^{2+} oscillations, IP_1 accumulation and extracellular signal-regulated kinases ($ERK_{1/2}$) activity. In contrast, L173F and P221L show enhanced responsiveness of these three functional readouts to $[Ca^{2+}]_o$ change. Further analysis of the dynamics of the VFT domain mutants using computational simulation studies supports disruption in the correlated motions in the loss-of-function CaSR mutants, while these motions are enhanced in the gain-of-function mutants. WT CaSR was modulated by L-Phe in a heterotropic positively cooperative way, achieving an EC_{50} for Ca^{2+} induced intracellular responses similar to those of the two activating mutations. The response of the inactivating P221Q mutant to Ca^{2+} was partially rescued by L-Phe, illustrating the capacity of the L-Phe binding site to enhance the positive homotropic cooperativity of CaSR. L-Phe had no effect on the other inactivating mutant. Moreover, our results carried out both *in silico* and within intact cells indicate that residue Leu173, which is adjacent to residues that are part of the L-Phe-binding pocket, exhibited impaired heterotropic cooperativity in the presence of L-Phe. Thus, P221 and L173 are important for the positive homo—and heterotropic cooperative regulation elicited by agonist binding.

The third type of mutations, type III, directly affect CaSR protein surface expression level which, in turn, alters potency or cooperativity. Apparent functional cooperativity and the EC_{50} for extracellular Ca^{2+} elicited intracellular response of the CaSR are also determined by the receptor expression level at the plasma membrane (Christopoulos and Kenakin, 2002; Casadó et al., 2007; Huang Y. et al., 2007). Like many other membrane proteins, CaSR's surface expression level is determined by the rate of internalization and folding in the endoplasmic reticulum

(ER) and Golgi, and membrane insertion. The CaSR protein is synthesized in the ER and is trafficked intracellularly in the ER and Golgi complex as it undergoes translations in the ER and post-translational modifications before shuttling to the cell surface. Interruptions in this trafficking from translation to cell surface expression, such as those from disease mutations, can lead to loss-of or gain-of-function CaSR on the cell surface, CaSR that is retained in the ER or Golgi, or degraded altogether. Breitwieser's group investigated how these disease mutations affect trafficking as a whole. They identified several classes of mutations, spanning the whole CaSR length, which they categorized based on their ability to traffic to the cell surface and to have their function rescued by the positive allosteric modulator NPS R-568. Class Ib mutations (Q27R, P39A, R66C, G143E, L174R, G549R, C582Y, R638L, S657Y, G670E, P748R, G778D, L849P) interrupt trafficking so substantially that the CaSR is not able to leave the ER, while Class Ia mutations (E297K, C395R, and A804D) similarly remain in the Golgi complex. All Class I mutations fail to traffic to the cell surface. Class II mutations still allows CaSR to reach the cell surface and their function either can be enhanced by NPS R-568 (Class IIa: P55L, R62M, S137P, R185Q, D215R, R227Q, G553R, L650P, R680C, V689M, R795W, V817I) or not (Class IIb: L159P and P798T) (Huang and Breitwieser, 2007; White et al., 2009). Consistent with these observations, Leach et al. reported that FHH mutations S657Y, G670R, G670E, R680H, R680C, M734R, G778D, and V817I significantly decrease cell surface expression compared to the WT; while ADH mutations F821L, V836L, and A843E increase cell surface expression, although the ADH mutation F832S did lead to a 45% decrease in surface expression (Leach et al., 2013).

The 14 cysteines found in the ECD and the cys-rich domain were also found to affect CaSR function and cell surface expression (Bai et al., 1998a; Fan et al., 1998; Zhang et al., 2001). Of these 14 cysteines, 9 have disease mutations associated with them producing ADH, FHH, and various carcinomas. C129S and C131S, the cysteines thought to participate in the homodimerization of CaSR through disulfide bond formation, only led to a decrease of expression to 88 and 97%, respectively, compared to WT CaSR. Whereas, C60S led to a decrease in expression by half, and all other Cys mutations linked to disease led to a decrease to lower than 28% or undetectable levels (Fan et al., 1998). It is clear that the disruption of disulfide formation as well as trafficking have profound effects on functional cooperativity via alterations in both the Hill number and the concentration of active form on the plasma surface.

The fourth mutational effect, type IV, alters CaSR interactions with trafficking and stabilization partners leading to a change in surface expression that further influences CaSR signaling as well as functional cooperativity. Breitwieser et al. have concluded that agonist-driven insertional signaling (ADIS) contributed to the hallmarks of CaSR signaling, including the high degree of cooperativity and the lack of functional desensitization. Additionally, they have shown that the life cycle of the CaSR controls the cellular abundance of CaSR, suggesting an intimate link between trafficking and signaling (Breitwieser, 2012, 2013). As shown in **Figure 5** and **Table 2**, a large number of proteins have been reported to interact at the C-terminal tail, the protein interaction sensitive zone, of the CaSR (Hebert and Brown, 1995; Chakravarti et al., 2012; Brown, 2013). Interactions of these proteins may be important for both CaSR signaling and functional cooperativity. Among these proteins, E3 ubiquitin ligase, dorfin is linked to ubiquitination and degradation of CaSR via binding to C-terminal amino acids 880–968 (Huang et al., 2006) and/or the C-terminal amino acids 920–970 (Zhuang et al., 2012). Zhang et al. investigated the effect of lysosomal mediated degradation of CaSR C-terminus and found that loss of the region 920–970 led to the highest cell expression level and decrease in degradation (Zhuang et al., 2012). Several insertion and deletion mutations in these regions of the C-terminus of CaSR lead to ADH, resulting in reduced degradation and, therefore, over-expression at the cell surface. Additionally, we have reported that the surface expression of CaSR is largely influenced by interaction of calmodulin (CaM) at residues 871–898 at the C-terminal tail of CaSR. Interestingly, multiple disease mutations, such as T876ins, F881L, R886W, and R886P for FHH/NSHPT and S895del for ADH reside at this CaM binding region (Huang et al., 2010). CaM has been reported to bind both the immature and mature forms of CaSR, which suggests its role in modulating anterograde trafficking (Bai et al., 1998b). We have shown that deletion of the CaM binding region significantly abolishes/reduces functional cooperativity of calcium oscillations. Therefore, CaM binding stabilizes the receptor on the cell membrane and thus increases potency of its functional activity (Huang et al., 2010). It is worth pointing out that this region also is involved in phosphorylation and biased signaling (Davey et al., 2012; Leach et al., 2012). Moreover, protein 14-3-3 was reported to interact with the arginine-rich domain of CaSR (^{890}RRxxxxRKR^{898}), which may lead to the retention of CaSR in the ER (Stepanchick et al., 2010; Grant et al., 2011; Arulpragasam et al., 2012). CaSR associated disorders can also be caused by different mechanisms other than direct mutations on the receptor itself. Recent studies by Nesbit et al. and Rogers et al. revealed that disorders with FHH or ADH phenotype can be associated with mutations on partner proteins associated with CaSR-mediated signaling, for instance the loss-of-function mutations of the sigma subunit of adaptor protein-2 (AP2) and mutations on G-protein subunit-α11 (GNA11), (Nesbit et al., 2013; Rogers et al., 2014). Moreover, antibodies against the CaSR can also regulate the function of the receptor (Thakker, 2004; Kemp et al., 2009, 2010).

ADDITIONAL MECHANISMS TO ALTER THE FUNCTIONAL ACTIVITY OF CASR

There are several possible reasons why a single CaSR could play a major role in various organisms, organs and tissue types at different physiological environments. First, besides Ca^{2+}, other divalent cations such as Mg^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, Sr^{2+} (Thomsen et al., 2012), and trivalent cations La^{3+} and Gd^{3+} can also interact at similar reported calcium binding sites, and/or in their local vicinity, via altered electrostatic interactions of the protein. In general, the higher the positive charge density, the higher the potency as CaSR agonists. The CaSR has a relatively low affinity for Mg^{2+} with an EC_{50} at about 10 mM as measured

TABLE 2 | The calcium-sensing receptor interacting proteins.

Protein	Assay used	Function	CaSR domain	References
AMSH	Y2H, GST	Trafficking/de-Ub enzyme	C-terminal	Herrera-Vigenor et al., 2006
β-Arrestin	Functional	Trafficking/signaling	Unknown	Lorenz et al., 2007
Caveolin	Co-IP	Structural/scaffolding/ trafficking/signaling	Intracellular loop 1 and 3	Kifor et al., 1998, 2003; Sun and Murphy, 2010; Breitwieser, 2013
E3 Ub ligase	Y2H, IP, functional	Trafficking	C-terminal	Huang et al., 2006
Filamin	Y2H, Co-IP, GST, functional	Structural/scaffolding/trafficking	C-terminal	Awata et al., 2001; Hjälm et al., 2001; Pi et al., 2002
GRK-2	Functional	Signaling	ICD	Lorenz et al., 2007
GRK-4	Functional	Signaling	ICD	Lorenz et al., 2007
Kir4.1	Y2H, Co-IP, functional	K channel	C-terminal	Huang C. et al., 2007
Kir4.2	Y2H, Co-IP, functional	K channel	C-terminal	Huang C. et al., 2007
PI-4-Kinase	Co-IP,	Signaling	Unknown	Huang et al., 2002
PKC	Functional	Signaling	C-terminal	Lorenz et al., 2007
RAMP1	Co-IP, functional	Structural/trafficking	ECD and 7TM	Bouschet et al., 2005
RAMP3	Co-IP, functional	Structural/trafficking	ECD and 7TM	Bouschet et al., 2005
RGS proteins	Functional	Signaling	Unknown	Huang et al., 2002, 2004
Rho	Co-IP	Signaling	Unknown	Huang et al., 2002
14-3-3	Yeast two-hybrid screen, CaSR tail pull-down studies, Co-IP	Trafficking, expression, signaling	Proximal membrane region, CaSR C-terminal tail	Grant et al., 2011; Arulpragasam et al., 2012
CaM	GST, Co-IP, functional	Signaling/stabilize	C-terminal	Huang et al., 2010
P24A	Co-IP	Stabilize/ trafficking	C-terminal	Stepanchick and Breitwieser, 2010
Sar1	Functional	Trafficking	ECD	Zhuang et al., 2010
Rab1, Rab7, Rab 11a	Functional, RNAi	Trafficking/signaling	Possible ECD	Zerial and McBride, 2001; Grosshans et al., 2006
Dorfin	Co-IP, functional	Trafficking	Intracellular loop, C-terminal	Huang et al., 2006
Integrins	Co-IP, proteomic analysis, co-localization, functional	Cell migration and adhesion in cancer cells, trafficking, signaling	Unknown	Tharmalingam et al., 2011

for ligand induced current in oocytes, whereas there has been a reported EC_{50} of 3 mM for Ca^{2+} in the same assay (Brown et al., 1993). Mg^{2+} also has only half the maximal capacity for the production of inositol phosphate and arachidonic acid when stimulated by Ca^{2+} in CHO cells transiently expressing rat CaSR (Ruat et al., 1996). Consistently, CaSR transiently expressed in HEK293 cells has EC_{50}s for intracellular calcium responses of 15–20 and 3–5 mM for Mg^{2+} and Ca^{2+} under physiological conditions, respectively (Bai et al., 1996; Nearing et al., 2002). CaSR ligands are also important for therapeutics, for example, Sr^{2+} (as a ranelate salt) has been shown to be effective for the treatment of osteoporosis (Kendler et al., 2009). CaSR has also been seen to respond to binding ligands (e.g., Ca^{2+}, Mg^{2+}, L-Phe, etc.) on a pH dependent manner where slightly lower extracellular pH of 7.2 inhibits the binding of the ligand to CaSR, and a higher pH of 7.6 favors the binding process (Campion et al., 2015).

Second, various classes of agonists, antagonists and drugs can also regulate the activation of CaSR via the ECD. The established homotropic cooperativity and heterotropic co-activation model of CaSR can also explain the regulatory action on CaSR. Three basic peptides including poly-arginine, protamine, and poly-lysine exhibited a dose-dependent inhibition of dopamine-stimulated cAMP accumulation in dispersed bovine parathyroid cells with EC_{50} values at micromolar or sub-micromolar concentrations (Brown et al., 1991). Studies from Quinn et al. demonstrated alteration of several cellular parameters including $[Ca^{2+}]_i$ change, IP production, and the activity of a nonselective cation channel via polyamines. The potency of polyamines was directly proportional to number of positive charges with the order of spermine > spermidine > > putrescine (Quinn et al., 1997). Glutathione and its γ-glutamyl peptides also allosterically modulate the CaSR at a site that appears to be similar to the L-amino acids binding site but with >100-fold higher apparent affinity (Conigrave et al., 2000; Wang et al., 2006; Broadhead et al., 2011). The EC_{50} values for GSH and the oxidized form glutathione disulfide (GSSG), to induce intracellular Ca^{2+} mobilization, were in the range of sub-micromolar (0.08 and 0.33 μM, respectively) (Wang et al., 2006). In 2006, using $[Ca^{2+}]_i$ mobilization, PTH secretion, as well as intracellular cAMP inhibition, Broadhead et al. showed γ-glutamyl-tripeptides (γ-Glu-Cys-Gly, S-methylglutathione and S-propylglutathione, and dipeptides γ-Glu-Ala and γ-Glu-Cys) to be positive allosteric modulators of CaSR. They also found that the double mutant T145A/S170T had an impaired response to these peptides, indicating a potential peptide binding site at the extracellular domain of CaSR (Broadhead et al., 2011). Recently, a peptide drug AMG 416 has been shown to act as an agonist of the

CaSR and is undergoing clinical trials (Walter et al., 2013). Interestingly, the action of AMG 416 is also modeled to be at the hinge region of the Phe-Site1 calcium binding site (Alexander et al., 2015). Mutations on Cys482 at the hinge region lead to impaired peptide activity in both pig and human CaSR (Alexander et al., 2015).

Third, calcium sensitivity could be related with the difference in CaSR sequence observed in various species. In mammals, CaSRs were detected in multiple organ systems besides parathyroid glands (e.g., kidneys, colon, skin, parathyroid, stomach, vascular, bone, etc.), participating in various functions, such as Ca^{2+} and fluid reabsorption, acid secretion, osteoblast and keratinocyte differentiation, etc. (Tfelt-Hansen and Brown, 2005; Alfadda et al., 2014). The CaSR sequences among major clades exhibit few differences both at the ECD and ICD. The length of the ICD varies greatly among the clades (Herberger and Loretz, 2013). Intriguingly, the sequence of the putative calcium binding sites, especially the Site 1 within the ECD thought to be the main Ca^{2+}-binding domain, is relatively conserved among vertebrates (Huang et al., 2009). On the other hand, residues in the other predicted Ca^{2+}-binding sites differ substantially, which may be an effective way to adjust CaSR sensitivity to a specific physiological environment, i.e., with varying Ca^{2+}, Mg^{2+}, or pH, and also for evolution. For example, CaSR is present in different organs of fish, suggesting it might play a crucial role in sensing the changes in the salinity in surrounding water with calcium concentrations changes from 10 mM in sea water to 0.07–2.0 mM in freshwater. The presence of many alternatively spliced forms of the human CaSR could imply an impact of these altered sequences on the CaSR's Ca^{2+} sensitivity (Oda et al., 1998, 2000).

Fourth, Ca^{2+} signaling could be modulated through the formation of CaSR hetero-dimers with other GPCRs or higher order oligomers with non GPCR chaperones in tissues other than parathyroid. Gama et al. showed co-localization of the CaSR and mGluR1α in hippocampal and cerebellar neurons by immunoprecipitation of CaSR from bovine brain. The CaSR became sensitive to glutamate triggering internalization and exhibited altered trafficking via reported hetero-dimerization with $GABA_B$ receptors (Gama et al., 2001). Thus, it was speculated that the CaSR would respond to Ca^{2+} concentration within the synaptic cleft at various levels of synaptic activity. Recent work by Kim et al. revealed that CaSR forms a heteromeric complex with the inhibitory type B γ-aminobutyric acid receptor 1 ($GABA_BR1$) in hippocampal neurons (Kim et al., 2011). This study demonstrates a novel receptor interaction, which contributes to ischemic neuron death through CaSR upregulation and $GABA_BR1$ downregulation, and feasibility of neuroprotection by concurrently targeting these two receptors. It is also a new way to tailor functional cooperativity and specificity.

CONCLUSION AND PERSPECTIVE

Recently, the crystal structure of extracellular domain has been first solved by our group. We reported the first CaSR crystal structure with multiple Mg^{2+} binding sites (Zhang et al., 2016). Consistent to our modeled structure reported earlier, the determined X-ray structure of the ECD shares the same fold of the mGluRs with the VFT motif. Unexpectedly, we also identified a tryptophan derivative L-1,2,3,4-tetrahydronorharman-3-carboxylic acid (TNCA) at the hinge region between the two subdomains where orthosteric ligand binding is thought to occur. We further demonstrated that TNCA binds to CaSR at unusually high affinity and potentiates CaSR activity with Ca^{2+} and Mg^{2+} (Zhang et al., 2016). Geng and colleagues subsequently reported X-ray structures of CaSR ECD in both apo form and the Ca^{2+} binding form. Similar as proposed in earlier studies, CaSR active form contained multiple binding sites for Ca^{2+}. Additional Ca^{2+} bound sites and PO_4^{3-} binding sites were reported. They also showed that L-Trp bound to the orthosteric agonist-binding site in CaSR active form (Geng et al., 2016). These advances and various pioneer studies since the discovery of CaSR in understanding the cooperative extracellular Ca^{2+} signaling mediated through CaSR have uncovered important structural and functional characteristics about this receptor. Family cGPCRs all sense extracellular calcium with differing degrees and also exhibit sensitivity to either amino acids, neurotransmitters, or related ligands. The discovered co-activation by calcium and amino acids of CaSR is likely to have a broad impact for the regulation of cGPCRs. However, many questions about CaSR are still unanswered. Though accelerated elucidation of cGPCR structures have been accomplished, the unavailability of the full CaSR structure and largely "invisible loop residues" in the determined structure of cGPCRs limit the next step forward on understanding of molecular mechanism of CaSR. Thus, high resolution structures of CaSR with various forms of agonists and antagonists are essential to gain a better understanding of the underlying mechanism in CaSR regulated physiological functions as well as pathological activities. Equal importance should be directed at studies designed to probe the functional cooperativity of CaSR as it plays a pivotal role in controlling the receptor response within a narrow fluctuation of metal concentration. Family cGPCRs are highly relevant for drug design and have tremendous potential as therapeutic targets because cGPCRs play vital roles in neurotransmitter release and Ca^{2+} homeostasis. The aforementioned results, along with further determination of the structure of CaSR, will provide great insights into the molecular basis of the structure and function of CaSR and shed new light on other cGPCRs in health and disease states. Thus, further uncovering of the structural and functional mysteries of CaSR could aid the development of novel receptor-based therapeutics to use in the treatment of many different diseases.

AUTHOR CONTRIBUTIONS

JY, CZ, CM, JZ, and RG contributed to the manuscript's conception and wrote the manuscript. KH performed computational analysis. EB made searches and helped with the manuscript.

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Autosomal Dominant Hypocalcemia (Hypoparathyroidism) Types 1 and 2

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Extracellular calcium is essential for life and its concentration in the blood is maintained within a narrow range. This is achieved by a feedback loop that receives input from the calcium-sensing receptor (CASR), expressed on the surface of parathyroid cells. In response to low ionized calcium, the parathyroids increase secretion of parathyroid hormone (PTH) which increases serum calcium. The CASR is also highly expressed in the kidneys, where it regulates the reabsorption of calcium from the primary filtrate. Autosomal dominant hypocalcemia (ADH) type 1 is caused by heterozygous activating mutations in the CASR which increase the sensitivity of the CASR to extracellular ionized calcium. Consequently, PTH synthesis and secretion are suppressed at normal ionized calcium concentrations. Patients present with hypocalcemia, hyperphosphatemia, low magnesium levels, and low or low-normal levels of PTH. Urinary calcium excretion is typically increased due to the decrease in circulating PTH concentrations and by the activation of the renal tubular CASR. Therapeutic attempts using CASR antagonists (calcilytics) to treat ADH are currently under investigation. Recently, heterozygous mutations in the alpha subunit of the G protein G11 (Gα11) have been identified in patients with ADH, and this has been classified as ADH type 2. ADH2 mutations lead to a gain-of-function of Gα11, a key mediator of CASR signaling. Therefore, the mechanism of hypocalcemia appears similar to that of activating mutations in the CASR, namely an increase in the sensitivity of parathyroid cells to extracellular ionized calcium. Studies of activating mutations in the CASR and gain-of-function mutations in Gα11 can help define new drug targets and improve medical management of patients with ADH types 1 and 2.

Keywords: autosomal-dominant hypocalcemia, CASR, calcium metabolism, GNA11, G11, calcilytics, YM254890, hypocalcemia

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PHYSIOLOGY OF EXTRACELLULAR CALCIUM HOMEOSTASIS

Calcium is vital for many functions of the body and blood calcium concentrations must be maintained within a narrow physiological range of 8.5–10.5 mg/dl (2.12–2.62 mmol/L). The calcium-sensing receptor (CASR), a class C G-protein coupled receptor comprised of 1078 amino acids, is an integral component of the homeostatic system that controls blood calcium concentrations (Hofer and Brown, 2003). In humans, the two major calcium-controlling hormones that maintain serum calcium within the normal range are parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D. PTH is produced and secreted by the parathyroid glands, which sense extracellular calcium through the calcium sensing receptor, located on the surface of the parathyroid chief cells (Brown et al., 1993).

When serum ionized calcium is low, increased amounts of PTH are secreted into the circulation; PTH acts mainly on kidney and bone, through binding and activating the PTH/PTHrP receptor, to increase serum ionized calcium. In bone, PTH induces osteoblasts to trigger osteoclasts to release calcium and phosphorous into the circulation, and it stimulates osteocytic osteolysis (Tazawa et al., 2004). In addition, PTH acts at the distal tubules of the kidneys to induce calcium reabsorption. In the proximal tubule, PTH inhibits phosphate reabsorption from the urine resulting in a decrease in serum phosphate levels. PTH also activates the 1α-hydroxylase enzyme (CYP27B1) in the renal proximal tubule, which converts 25-hydroxyvitamin D to the active form, 1,25-dihydroxyvitamin D, or calcitriol. This active vitamin D functions in the intestine to increase the absorption of calcium (and phosphate) from the gut. Taken together, these concerted actions of PTH mobilize calcium in an effort to restore the serum calcium to the above-mentioned normal range.

CASR AND PARATHYROID CELLS

The calcium sensing receptor is expressed in most tissues (Brown et al., 1993). However, the highest levels of its expression are found in the parathyroid glands and in the kidneys. The minute-to-minute tight regulation of calcium is achieved through the ability of the CASR to sense minor changes in extracellular calcium. The relationship between PTH secretion and the extracellular calcium concentration has been described as a steep sigmoidal curve. In the parathyroid cell, activation of the CASR by extracellular ionized calcium stimulates intracellular signal transduction pathways, resulting in an increase in intracellular calcium (nM range), that ultimately leads to an inhibition of PTH production and secretion. This is in stark contrast to the usual signaling pattern of intracellular calcium, which is characterized by the stimulation of hormone production and secretion in response to an increase in intracellular calcium. The mechanism for this peculiar opposite effect in the parathyroids is unknown.

Several signaling pathways downstream of the CASR are engaged in the parathyroid cell. In the presence of ligand, the CASR activates Gq and G11(Hofer and Brown, 2003), leading to a stimulation of phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol and inositol 1,4,5-trisphosphate (IP3), which in turn causes activation of protein kinase C (PKC) and release of intracellular calcium, respectively. The CASR also couples to the G-protein Gi, which inhibits adenylate cyclase and leads to a reduction in intracellular cAMP.

In addition to extracellular calcium, the parathyroids also respond to phosphate, 1,25-dihydroxyvitamin D, and FGF23, both directly and indirectly, to modulate PTH secretion (Naveh-Many et al., 2002; Silver and Naveh-Many, 2009).

CASR AND THE KIDNEYS

In the kidney, the CASR is highly expressed in the basolateral membrane of the thick ascending limb (TAL; Loupy et al., 2012; Toka et al., 2012; Crisi et al., 2013). While there is conflicting evidence for the expression of CASR mRNA and protein in other parts of the nephron, functional studies suggest that it is more widely expressed. For example, in the collecting duct, CASR activation leads to urine acidification and a reduction in water reabsorption, which could reduce the risk of kidney stone formation (Renkema et al., 2009).

In the TAL, calcium is reabsorbed passively along the paracellular pathway, driven by luminal electropositivity, which is dependent on the rate and extent of NaCl reabsorption. In the apical membrane of the TAL, Na^+, $2Cl^-$, and K^+ are electroneutrally transported into the cell. K^+ is recycled back into the lumen through the apical ROMK channel leading to lumen positivity, the main driver of paracellular calcium transport from the lumen into the TAL. In the distal convoluted tubule (DCT), calcium is reabsorbed through an active transcellular transport. Calcium enters the DCT through TRPV5 and exits the cell via the sodium-calcium exchanger 1 (Riccardi and Valenti, 2016).

Activation of the CASR in the kidney leads to a decrease in calcium (and water and sodium) reabsorption. While these exact pathways are not entirely elucidated, two potential mechanisms have emerged. One involves the paracellular transport of calcium from the lumen into the TAL, which is dependent on several claudins, a family of proteins that establish barriers in tight junctions. The CASR was discovered to control claudin-14 expression and therefore absorption of calcium through the paracellular tight junctions (Gong and Hou, 2014). The second possible mechanism is a reduction of the activity of the apical K^+ channel, which generates the lumen positivity. Patch clamp studies showed that both calcium and neomycin are able to reduce the activity of this potassium channel (Wang et al., 1996, 1997).

Investigators studying CASR knockout mouse models demonstrated that the CASR also defends against hypercalcemia by increasing calcium excretion by the kidney, independent of its role in the parathyroid glands (Kantham et al., 2009).

AUTOSOMAL-DOMINANT HYPOCALCEMIA TYPE 1

Key to the understanding of the role of the CASR in the parathyroids was the discovery of mutations in the *CASR* gene leading to human disease, and analysis of these mutations in mouse models (Hannan and Thakker, 2013). Patients with activating or inactivating germline mutations in the CASR present with hypocalcemia or hypercalcemia, respectively. Inactivating mutations of the CASR lead to familial hypocalciuric hypercalcemia (FHH). The mirror image of FHH, autosomal-dominant hypocalcemia (ADH) type 1, is caused by activating mutations in the CASR and is the most common genetic form of isolated hypoparathyroidism. These activating CASR mutations lead to a leftward shift in the calcium-PTH curve and therefore suppression of PTH secretion at physiological levels of extracellular calcium. Biochemical hallmarks of AHD1 are hypocalcemia, which is typically mild to moderate, hyperphosphatemia, hypercalciuria, and inappropriately low

but detectable PTH levels. Symptoms of ADH1 are caused by hypocalcemia (mainly neuromuscular irritability) and are typically mild.

In addition to this functional defect in the parathyroids, activating CASR mutations have independent effects in the kidneys. Therefore, patients with ADH1 have two mechanisms contributing to hypercalciuria. First, low concentrations of PTH, which normally induce reabsorption of calcium from the primary filtrate, result in relative hypercalciuria. Second, increased activation of the mutated CASR through extracellular calcium in the distal renal tubules leads to even more pronounced hypercalciuria for any given blood calcium level.

The presentation of the index case of kindred G (D'Souza-Li et al., 2002) is typical for ADH1. Blood chemistries of this 21-year old asymptomatic woman were tested because her three sisters and her mother all had hypocalcemia. Her laboratory results showed mild hypocalcemia (Ca = 7.5 mg/dl, normal 8.5–10.5), mild hyperphosphatemia (P = 4.8 mg/dl, normal 2.6–4.5) and hypomagnesemia (Mg = 1.4 mg/dl, normal 1.8–2.5), low but detectable PTH (PTH = 16 pg/ml, normal 10–60) and an elevated calcium/creatinine clearance ratio (0.088, normal < 0.02). Sanger sequencing revealed a heterozygous missense mutation leading to the substitution of alanine to threonine in position 835, located in the third extracellular loop of the CASR. *In vitro* studies using HEK cells transfected with wildtype and mutant CASR cDNA revealed the expected leftward shift in the calcium-response curve (D'Souza-Li et al., 2002).

Diagnostic sequencing of the CASR gene is used to confirm ADH1. More than 200 mutations of the CASR have been reported, of which more than 70 are associated with ADH1, the vast majority are heterozygous missense mutations (www.casrdb.mcgill.ca). The CASR consists of three major domains: the large extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular C-terminus. Many mutations associated with ADH1 are located in the second peptide loop of the ECD, which is predicted to be important for dimer formation, as well as in the TMD 5 and 6 and in the region of the third extracellular loop.

Clinical management of ADH1 is guided by the known high risk for renal calcifications, kidney stones and kidney failure. In asymptomatic patients, treatment should be avoided. When hypocalcemic symptoms occur frequently enough to warrant treatment, careful therapy with the lowest amount of calcium and activated vitamin D is initiated. Goal calcium levels should be as low as possible to alleviate symptoms. Thiazide diuretics, often used in hypoparathyroidism because of their urinary calcium lowering effect, have also been shown to be beneficial in ADH1 (Sato et al., 2002). In clinical studies in patients with ADH1, once daily dosing of PTH(1–34) only corrected serum calcium for a part of the day, and twice daily sc administration of PTH(1-34) led to better control of blood calcium (Winer et al., 1998, 2008). However, PTH does not correct the effects of the activated CASR in the kidneys, and indeed, urinary calcium excretion was not normalized by PTH(1–34) injections. Studies of continuous PTH(1–34) administration via pump therapy did normalize urinary calcium in cases of postsurgical hypoparathyroidism (Winer et al., 2012). Trials with PTH(1–84), which is an approved treatment for other forms of hypoparathyroidism, excluded patients with ADH1.

Small molecule allosteric modulators of the CASR have been identified (Nemeth and Goodman, 2016). Positive modulators (type II calcimimetics), such as cinacalcet, increase activation of the CASR. Cinacalcet is primarily used for treatment of patients with secondary hyperparathyroidism on dialysis. Calcilytics, which are negative allosteric CASR modulators, inhibit the activation of the CASR and therefore are of potential interest for the treatment of ADH1. In cell culture experiments studying activating CASR mutants, calcilytics have been shown to normalize the leftward shift of the calcium response curve (Dong et al., 2015; Hannan et al., 2015). The utility of calcilytics was further demonstrated in two studies in mice harboring activating CASR mutations. In one study, two knockin mouse models of ADH1 with activating mutations in the CASR were generated. Daily oral administration of the calcilytic JTT-305/MK-5442 to these mice was shown to increase PTH and calcium, and to reduce urinary calcium excretion (Dong et al., 2015). A second study using the Nuf mouse, another model of ADH1, showed that intraperitoneal injection of the calcilytic NPS2143 transiently stimulated serum PTH secretion and increased serum calcium without increasing urinary calcium (Hannan et al., 2015). In a phase 2 clinical trial, IV administration of the structurally closely related small molecule NPSP795 increased plasma PTH levels and decreased the urinary fractional excretion of calcium in five patients with ADH1 (Ramnitz et al., 2015). In addition to a potential treatment for ADH1, calcilytics could also have a role in reducing urinary excretion of calcium in other patients with hypoparathyroidism, through blocking the renal CASR.

AUTOSOMAL-DOMINANT HYPOCALCEMIA TYPE 2

With the discovery that mutations in the gene encoding the alpha subunit of the G protein G11 (*GNA11*) cause hypocalcemia, ADH was divided into type 1, reviewed above, and type 2, caused by activating mutations in *GNA11*. Patients with ADH2 present with a wide range of hypocalcemic symptoms ranging from paresthesias to tetany and seizures, or they can be asymptomatic.

The following describes the index case of Family A (Mannstadt et al., 2013): A 15 year-old boy presented with muscle cramps and tremulousness. Past laboratory studies revealed normal calcium at age 2. He experienced generalized seizures at age 5 which were treated with carbamazepine for 1 year and did not re-occur thereafter. Studies on presentation revealed mildly low serum calcium, high phosphorous, and inappropriately low PTH concentration (**Table 1**).

His mother, cousin, grandmother, and great-grandmother were also affected with hypoparathyroidism revealing an autosomal dominant inheritance pattern. Neither the patient nor any of his family members had a history of mucocutaneous candidiasis, hearing loss, renal abnormalities, or skin changes. He was clinically diagnosed with ADH and genetic sequencing ruled out mutations in the *PTH* and *GCM2* genes, but also in *CASR*.

TABLE 1 | Summary of the presenting laboratory values of the index case of Family A, a 15 year-old boy with ADH2, from Mannstadt et al. (2013).

	At presentation	Normal range
Calcium (mM)	2.10	2.25–2.65
Phosphorous (mM)	2.30	0.90–1.70
PTH (pM)	1.2	1.2–5.5

Exome sequencing revealed a novel mutation in *GNA11*, which was present only in affected family members (Mannstadt et al., 2013).

Few patients have been described in the literature with ADH2, limiting the number of observations (**Table 2**), but ADH2 might have a slightly milder phenotype with respect to hypocalcemia than patients with ADH1. Additionally, elevations in urinary calcium of individuals with ADH2 appear to be less pronounced than in those with activating CASR mutations in ADH1, raising the question whether the CASR in the renal tubules couples to G proteins other than G11.

The genetic basis of ADH2 was first elucidated by two groups. One study focused on two large unrelated families counting a total of 15 living patients with autosomal dominant isolated hypoparathyroidism, in whom mutations in the genes encoding the CASR, PTH, and GCM2 were ruled out (Mannstadt et al., 2013). Affected individuals in both families were hypocalcemic with inappropriately low PTH and elevated phosphorous. The urinary calcium:creatinine ratio was found to be normal in all of 8 patients measured across both families. Genetic linkage analysis revealed a linked interval of ∼10 Mb on chromosome 19p13.3 with a maximal LOD score of 3.0. Among the approximately 50 genes that reside at this locus, *GNA11* was a strong candidate gene. Sanger sequencing discovered a heterozygous missense mutation in *GNA11* exon 2 (c.178C->T) changing the conserved arginine 60 to a cysteine (p.Arg60Cys). Whole-exome sequencing of two members of this family revealed that this R60C was the only variant in the linked interval that was present in both subjects. In the second family, exome sequencing of two affected members uncovered a different mutation in *GNA11*, a heterozygous missense mutation in exon 5. This mutation (c.632C->G) leads to the substitution of the conserved serine 211 to a tryptophan (p.Ser211Trp). Analysis of exome data from both families confirmed the absence of other variants affecting the same gene in both families. Restriction site analysis of PCR products revealed the presence of the respective mutation in available affected members of each family, and the absence in unaffected members (Mannstadt et al., 2013).

Investigators of the other study discovered inactivating mutations in *GNA11* associated with the mirror-image disease FHH. They then hypothesized that activating mutations of *GNA11* could cause ADH (Nesbit et al., 2013). Eight unrelated patients with hypocalcemia and low or inappropriately normal PTH levels in the absence of *CASR* mutations were studied by Sanger sequencing. In two ADH patients, mutations in the *GNA11* gene were uncovered. These patients had normal magnesium levels, and normal urinary calcium to creatinine clearance. The mutations were heterozygous missense mutations in highly conserved residues, namely a G->A change at c.542 which predicts a protein change of Arg181Gln, and a C->G change at c.1023 which causes the Phe341Leu change (Nesbit et al., 2013).

Subsequently, additional mutations in *GNA11* in other families with ADH were published. In one large family, whole-exome-sequencing revealed a heterozygous mutation c.179G->T; p.R60L (Li et al., 2014). The affected individuals in this family were reported to have short stature relative to their unaffected family members. They also lacked hypercalciuria and had normal serum magnesium levels, which contrasts with patients with ADH1 (Li et al., 2014). A second large family from Iran with ADH was studied by whole-exome sequencing revealing a heterozygous mutation exon 7 of *GNA11* c1018G->A; p.Val340Met (Piret et al., 2016). The Val340Met mutation was also found in a family from Finland, in which all seven of the family members studied had short stature and three had intracranial calcifications (Tenhola et al., 2016).

In vitro studies of these *GNA11* mutants using HEK293 cells expressing the CASR have confirmed the hypothesis that they are gain-of-function mutations. A leftward shift in the intracellular calcium response curve to extracellular calcium was found, indicating increased intracellular response to lesser amounts of extracellular calcium in the setting of each mutant (Nesbit et al., 2013; Li et al., 2014; Piret et al., 2016). Likewise, cells expressing the R60L mutant demonstrated increased activation of the SRE promoter and increased phosphorylation of ERK and p38, downstream targets of G11/q signaling (Li et al., 2014). *In vitro* studies using HEK293 cells have shown that the calcilytic NPS2143, an allosteric modulator of the CASR, can rectify impaired calcium signaling in ADH2 despite the fact that the Gα11 protein is downstream of the CASR (Babinsky et al., 2016).

Molecular modeling has suggested different mechanisms through which these mutations confer a gain of function in Gα11 (Flock et al., 2015). The R60 residue (G.H1.9) is located in the GTPase domain and has polar interactions with Asp71 (H.HA.3), which stabilize the interaction of the GTPase and helical domain, thus stabilizing the GDP-bound inactive form of Gα11. Replacement of the R60 residue is predicted to loosen the tight clamshell leading to faster GDP-GTP exchange (Mannstadt et al., 2013). Substitution of Ser211 (G.H2.2) with the bulky tryptophan is predicted to disturb the interactions with the βγ subunits (Mannstadt et al., 2013). The Arg181 (H.HF.6) residue is located in the helical domain and forms hydrogen bonds with residue R183 that is important for GTPase activity and can confer oncogenic potential to G11 when mutated (Nesbit et al., 2013). The Val340Met (G.H5.7) and Phe341Leu (G.H5.8) mutations are located in the α5 helix of Gα11 therefore altering the contacts between H5 and H1, which are important for Gα subunit activation (Nesbit et al., 2013; Li et al., 2014).

To date, six mutations in GNA11 have been reported to cause ADH2 (Mannstadt et al., 2013; Nesbit et al., 2013; Li et al., 2014; Piret et al., 2016; Tenhola et al., 2016). The discovery of activating germline mutations in the ubiquitously expressed G11 protein was surprising, especially since apart from hypocalcemia, and small stature reported in two families, no other obvious

TABLE 2 | Summary of the biochemical characteristics of the families/patients described with ADH type 2.

	Family A (Mannstadt et al., 2013)	**Family B (Mannstadt et al., 2013)**	**Patient 3 (Nesbit et al., 2013)**	**Patient 4 (Nesbit et al., 2013)**	**Li et al., 2014**	**Piret et al., 2016**	**Tenhola et al., 2016**
GNA11 Mutation	Arg60Cys	Ser211Trp	Arg181Gln	Phe341Leu	Arg60Leu	Val340Met	Val340Met
No. of Individuals	6	8	1	1	3–4	1–4	7
Calcium (mmol/l) (nl range)	2.10 ± 0.07 (2.15–2.70)	1.91 ± 0.12 (2.10–2.55)	2.06 (2.1–2.5)	1.75 (2.1–2.5)	1.86[c] (2.20–2.60)	1.88[d] (2.20–2.60)	Adult-1.90[f] (2.15–2.51) Child-2.03[h] (2.05–2.70)
Phosphorus (mmol/l) (nl range)	1.92 ± 0.76 (0.70–1.4)	1.79 ± 0.37 (0.80–1.50)	1.09 (0.7–1.40)	1.54 (0.7–1.40)	2.16[c] (0.8–1.8)	1.42–1.81[e] (0.8–1.45)	Adult-1.21[g] (0.71–1.53) Child-2.03[h] (1.10–1.80)
PTH (nl range)	1.7 ± 0.93 (1.0–7.0 pmol/l)	1.33 ± 0.75 (1.6–6.9 pmol/l)	50 (10–65 ng/l)	1.3 (1.3–7.6 pmol/l)	1.3 (1.2–5.8 pmol/l)	7.78[d] (12–65 pg/ml)	Adult-12[g] (12–47 ng/l) Child 15[h] (12–47 ng/l)
Magnesium (mmol/l) (nl range)	0.77[a] (0.7–1.0)		0.77 (0.70–1.05)	0.76 (0.70–1.05)	0.83[c] (0.7–1.0)		Adult-0.81[i] (0.71–0.94) Child-0.82[h] (0.70–1.0)
Urinary calcium (nl range)	Normal[b] Ca:Cre Ratio	Normal[b] Ca:Cre Ratio	Ca:Cre Clearance = 0.002 >0.02	Ca:Cre Clearance = 0.012 >0.02	FE_{Ca} = 0.91 (1.0–2.0)	24 h UCa = 4.3–10.2[e] (2.5–7.5 mmol/24 h)	Adult 24 h UCa = 4.6[i] (1.3–6.5 mmol/24 h) Child Ca:Cre Ratio = 0.15[h] (<0.6)

[a] *Mean of 10 patients from both Family A and Family B.*
[b] *Measured in a total of 8 patients across both families.*
[c] *Mean taken from each individual's mean of 1–10 values.*
[d] *Average of 3–4 individuals, one of whom the median of a range is averaged.*
[e] *Range of one individual.*
[f] *One adult.*
[g] *Average of 3 adults.*
[h] *Average of 4 children.*
[i] *Average of 2 adults.*

phenotypes were found. Further, studies of these families and identification of new families, possibly with novel mutations, will increase our understanding of the action of Gα11 and refine the phenotypic characteristics of patients with ADH2.

CONCLUSIONS AND UNANSWERED QUESTIONS

In conclusion, much has been learnt from mutations in the CASR and its signaling molecule G11. Activating mutations of the CASR lead to ADH1 and activating mutations in Gα11 cause ADH2. Hypercalciuria is a particularly difficult problem in ADH1 due to two independent mechanisms: low PTH and activation of the CASR in the renal TAL. The use of small molecule negative CASR modulators (calcilytics) for treatment of ADH1 has the potential to correct defects both in the parathyroid glands and in the renal tubule. *In vitro* studies and experiments in mouse models of ADH1 have demonstrated the ability of calcilytics to stimulate PTH, increase serum calcium, and decrease urinary calcium. A preliminary report of a phase 2 clinical trial is consistent with the efficacy of calcilytics in ADH1 patients. Clearly, small molecule CASR inhibitors would be a very attractive treatment option and in line with the concept of "precision medicine" for patients with ADH1.

Many important questions about ADH1 and ADH2 remain and further research is needed. Do patients with germline activating mutations in Gα11 have other, perhaps more subtle phenotypes caused by the activation of Gα11 in other tissues? Do different mutations lead to different phenotypes? Do calcilytics alleviate the biochemical abnormalities *in vivo* in patients with Gα11 mutations? Is there a role for the recently described G11/q inhibitors YM254890 and FR900359 (Schrage et al., 2015; Xiong et al., 2016) as a treatment for ADH2? Could novel PTH agonists be useful in the treatment of ADH (Bi et al., 2015)? Investigating these questions could ultimately help define new drug targets and improve medical management of patients with ADH types 1 and 2.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Calcium-Sensing Receptor Gene: Regulation of Expression

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The human calcium-sensing receptor gene (*CASR*) has 8 exons, and localizes to chromosome 3q. Exons 1A and 1B encode alternative 5′-untranslated regions (UTRs) that splice to exon 2 encoding the AUG initiation codon. Exons 2–7 encode the CaSR protein of 1078 amino acids. Promoter P1 has TATA and CCAAT boxes upstream of exon 1A, and promoter P2 has Sp1/3 motifs at the start site of exon 1B. Exon 1A transcripts from the P1 promoter are reduced in parathyroid tumors and colon carcinomas. Studies of colon carcinomas and neuroblastomas have emphasized the importance of epigenetic changes—promoter methylation of the GC-rich P2 promoter, histone acetylation—as well as involvement of microRNAs in bringing about *CASR* gene silencing and reduced CaSR expression. Functional cis-elements in the *CASR* promoters responsive to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], proinflammatory cytokines, and the transcription factor glial cells missing-2 (GCM2) have been characterized. Reduced levels of CaSR and reduced responsiveness to active vitamin D in parathyroid neoplasia and colon carcinoma may blunt the "tumor suppressor" activity of the CaSR. The hypocalcemia of critically ill patients with burn injury or sepsis is associated with *CASR* gene upregulation by TNF-alpha and IL-1beta via kappaB elements, and by IL-6 via Stat1/3 and Sp1/3 elements in the *CASR* gene promoters, respectively. The *CASR* is transactivated by GCM2—the expression of which is essential for parathyroid gland development. Hyperactive forms of GCM2 may contribute to later parathyroid hyperactivity or tumorigenesis. The expression of the CaSR—the calciostat—is regulated physiologically and pathophysiologically at the gene level.

Keywords: gene, alternative transcripts, transcription, vitamin D, proinflammatory cytokines, glial cells missing-2, DNA methylation, microRNA

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THE *CASR* GENE

The single-copy *CASR* gene that maps to human 3q13.3-21 encodes a Class C G protein-coupled receptor family member (Pollak et al., 1993; Janicic et al., 1995). The T-cell antigen *CD86* gene lies upstream and the cysteine protease inhibitor *CSTA* gene downstream of the *CASR* gene and all are transcribed in the same 5′ to 3′ direction.

The *CASR* gene has eight exons and spans ∼100-kb (Yun et al., 2007) (**Figure 1**). Exons 2 to 7 encode the CaSR protein of 1078 aa (GenBank #U20759). Two different polyadenylation signal sequences within exon 7 may be used, to generate either a short (177-nucleotide) or a long (1304-nucleotide) 3′-untranslated region (UTR) (Aida et al., 1995; Garrett et al., 1995). Exon 2 encodes 242 nucleotides of the 5′-UTR upstream of the ATG translation initiation codon. Exons 1A and

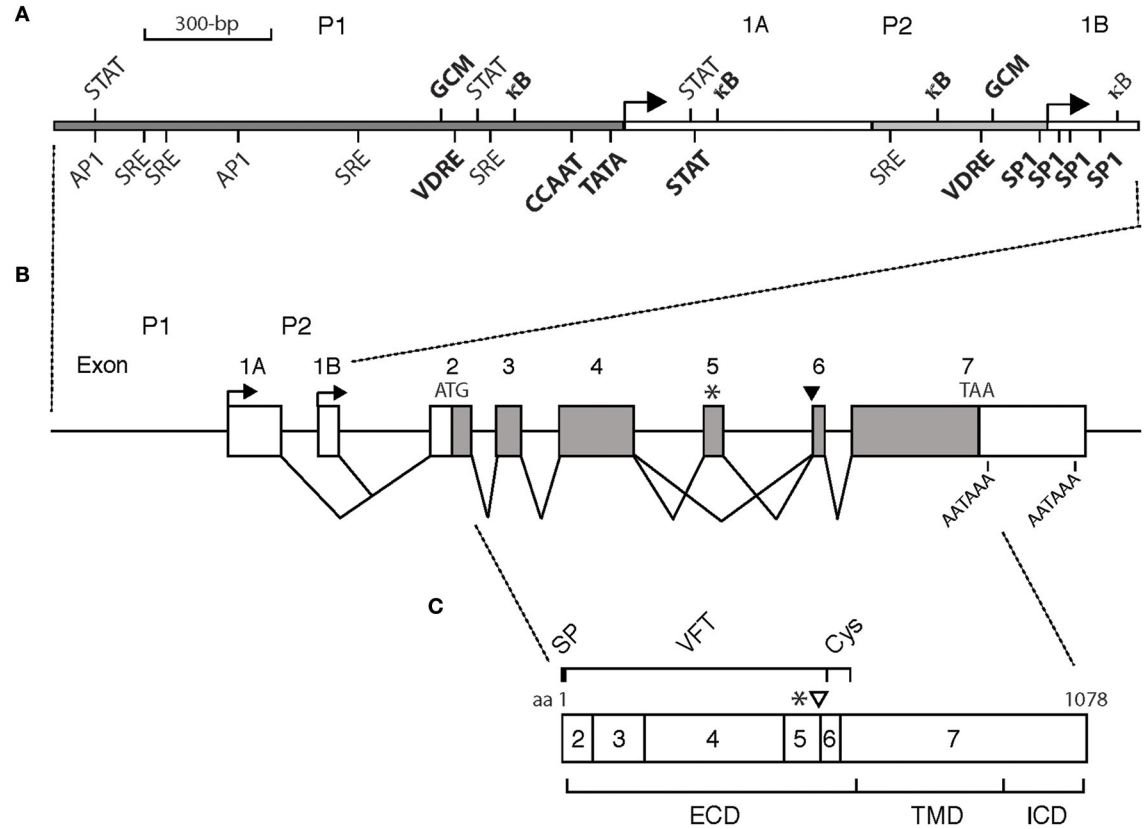

FIGURE 1 | Schematic of (A) the *CASR* gene promoters, (B) the *CASR* gene exon/intron organization, and (C) CaSR protein. (A) Promoter P1 and P2, gray bars. Exons 1A and 1B, white bars. Transcription start sites, arrows. CCAAT and TATA boxes, and SP-1 sites driving transcription of exon 1A and 1B, respectively, are bolded. Cis-acting elements are shown. VDRE, vitamin D response element; κB, kappa-B element responsive to nuclear factor kappa-light-chain-enhancer of activated B cells; STAT, signal transducer and activator of transcription; GCM, glial cells missing; AP1, activator protein 1; SRE, serum response element. Bolded: those shown to be functionally active. Not bolded: those predicted but either not functionally active or not yet evaluated. Not all predicted cis-acting elements are shown. **(B)** Exon/intron organization of the *CASR* gene. Exons are drawn to scale introns are not. White bars: mRNA untranslated (exons; 1A, 1B, part of 2, part of 7). Gray bars: mRNA protein coding (exons; part of 2, 3-6, part of exon 7). ATG: initiation codon. TAA: stop codon. AATAAA: polyadenylation signals. Alternative splicing of exons 1A and 1B to exon 2 is shown. Asterisk, *: alternative transcript lacking exon 5. Black arrowhead: alternative transcript having additional 30 bases at the beginning of exon 6. **(C)** CaSR protein: 1078 amino acid (aa) protein encoded by exons 2-7. Asterisk, *: minus 77 aa encoded by exon 5. Open arrowhead: additional 10 aa encoded by extra 30 bases of alternative RNA transcript. SP, signal peptide; VFT, venus flytrap domain; Cys, cysteine rich domain; ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain.

1B encode alternative 5′-UTRs that splice to a common segment encoded by exon 2 (Garrett et al., 1995; Chikatsu et al., 2000).

This organization of exons, conserved around primary protein domains, is first seen in aquatic vertebrates (Naito et al., 1998). With diversification from the teleost fishes to tetrapods, mammals, and primates, the evolutionary changes have been greatest in the 5′ and 3′ domains. While overall exonic structure is preserved a striking increase in intron size has occurred from teleost fish to higher species (Loretz, 2008). Linkage disequilibrium analysis across the human gene shows a central haploblock extending from exon 2 to 7 that is distinct from separate haploblocks for genetic variants in the 5′ and 3′ flanking regions (Yun et al., 2007).

Thus, the mouse, rat and human genes are organized in a similar manner. Both rodent genes comprise at least 7 exons and the translational start site is in exon 2. Alignment of expressed sequence tags (ESTs) and cloned rodent cDNAs reveal that, just like the human *CASR* gene, mouse and rat *CASR* genes have at least 2 distinct 5′UTRs (Exons 1A and 1B), suggesting the presence of at least 2 promoters. The mouse and rat *CASR* genes share >85% nucleotide identity (exons and introns) and 40% with the human *CASR*. The VDREs and κB elements (see below) that we have characterized in our studies of the human *CASR* gene promoters are conserved in the rodent *Casr* promoters.

Alternative Transcripts

The *CASR* gene is highly expressed in the parathyroid gland and renal tubule (Brown et al., 1993). However, the gene is widely expressed at lower levels in other tissues, for example in liver (Canaff et al., 2001), bone (Goltzman and Hendy, 2015) and in lung, breast, placenta, vasculature and gut (Brennan et al., 2013). Several alternative transcripts have been identified, raising the question of their function and regulation of their expression.

Coexistence of transcripts encoding the same 1078 amino acid protein in human parathyroid but different 5′ UTRs suggested alternative splicing (Garrett et al., 1995). Moreover, the transcripts may have either a short or long 3′ UTR. One low abundance transcript encodes an additional 10 amino acid stretch inserted after amino acid 536 in the exodomain (GenBank #U20760) but does not alter CaSR function (Garrett et al., 1995). The predominant transcript is 5.4-kb in length, while less abundant transcripts of 10, 4.8, and 4.2-kb are also found (Garrett et al., 1995). In human kidney, the 5.4-kb transcript is similarly predominant with the less abundant 10-kb transcript also present (Aida et al., 1995).

The human *CASR* gene has two promoters driving transcription of alternative 5′ UTR exons (1A and 1B) (Chikatsu et al., 2000). By northern blot analysis of human parathyroid adenomas and normal glands, the 5.4 and 10-kb transcripts (see above) appeared to be exclusive to exon 1A use, while the 4.2-kb transcripts are derived from either 1A or 1B (Chikatsu et al., 2000). Real-time PCR (qPCR) analysis of human parathyroid cells revealed that exon 1B-transcripts were much more highly expressed than exon 1A-containing transcripts (Mizobuchi et al., 2009). Transcripts of the 5.4 and 4.2-kb size derive from use of the two alternative polyadenylation sites in the 3′ UTR tract (Chikatsu et al., 2000).

An exon 3-deleted CASR transcript has been reported in thyroid TT cells (Freichel et al., 1996), in placental cytotrophoblast (Bradbury et al., 1998), and in parathyroid, thyroid, and kidney (D'Souza-Li et al., 2001). Fusion of exon 2 to exon 4 results in a truncated protein that is poorly expressed and not trafficked to the cell surface.

In human keratinocytes, an alternatively spliced transcript lacking exon 5 encodes a variant CaSR with a 77-amino acid in-frame deletion in the exodomain (Oda et al., 1998). This variant exerts a dominant negative effect on the full-length protein making it less responsive to calcium. In addition, the relative amounts of full-length vs. alternatively spliced transcript decrease during keratinocyte differentiation (Oda et al., 1998).

Consideration of alternatively spliced forms are of importance in phenotype evaluation of *Casr* mice knocked out by deletion of exon 5 (Ho et al., 1995). In the growth plate (Rodriguez et al., 2005), skin (Oda et al., 2000) and kidney (Oda et al., 2000) of the knockout mice, the *Casr* message lacking exon 5 is upregulated and compensates for the absence of the full-length counterpart in bone and cartilage (Rodriguez et al., 2005). Studies of *Casr* knockouts in which both full-length and exon 5 transcripts are deleted have suggested that the exon 5-deletion model might be hypomorphic with respect to CaSR actions in the skeleton (Chang et al., 2008).

Transcriptional Control of the *CASR* Gene

Regulated *CASR* gene expression is important in growth and development (Riccardi et al., 2013), and in normal adult physiology (Brown, 2013) and in disease pathogenesis (Hannan and Thakker, 2013). Some of the factors and mechanisms involved in transactivation of the *CASR* gene have been identified (Hendy et al., 2013).

Human *CASR* transcription is driven by either promoter P1, with a TATA box at nucleotide −26 and a CCAAT box at −110 relative to the start site, or P2 with an Sp1/3 site at the transcriptional start site (**Figure 1**). The rat and mouse *Casr* genes have a similar organization. Both promoters drive significant levels of basal activity, with promoter P2 being 2.5-fold more active than P1 in most cell types examined as assessed by transfected promoter-reporter analysis (Canaff and Hendy, 2002). Also nuclear run-on assays that directly measure transcripts suggest greater exon 1B relative to exon 1A transcripts in human thyroid C-cells and renal proximal tubular cells. The presence of multiple promoters provides the potential for tissue-specific and/or developmental/temporal-specific regulated expression from one promoter vs. responsiveness to hormonal or nutritional stimuli from the other. Evidence for this has yet to be fully realized for the *CASR* gene. In fact, from studies done so far the opposite seems to be the case in that both promoters respond to active vitamin D, cytokines, and the parathyroid cell-specific regulator, GCM2 (see below). However, pathophysiological differences in usage of the *CASR* gene promoters have been suggested.

Chikatsu et al. (2000) showed the expression of exon 1A transcripts driven by the upstream promoter (P1), was decreased in adenomas, whereas expression of exon 1B transcripts driven by the (normally stronger) promoter P2 was unchanged. These results suggested that P1 activity was reduced in parathyroid adenomas and is qualitatively in agreement with several studies showing a decrease in CaSR mRNA and protein expression in parathyroid adenomas relative to normal glands (Kifor et al., 1996; Farnebo et al., 1997; Gogusev et al., 1997; Cetani et al., 2000; Corbetta et al., 2000). However, other mechanisms yet to be identified are likely to contribute to the greater relative decreases in mRNA and protein expression that are not accounted for quantitatively by the ∼50% decrease of the apparently weaker P1 promoter. In a study of colorectal tumors, Kállay et al. (2003) found exon 1A transcript expression to be greater in the adjacent "normal" mucosa of colon cancer patients than in the tumor itself, and a highly significant decrease of exon 1A expression was found during progression from well-differentiated to poorly differentiated cancers whereas exon 1B transcript expression was not significantly altered. More recent studies have found hypermethylation of promoter P2 that drives the exon 1B transcripts (Fetahu et al., 2014a) and therefore reduced exon 1B transcript levels would be predicted. Further studies will be needed to resolve this apparent discrepancy.

Vitamin D

Active vitamin D increases CaSR expression and this has been documented in several studies in rodents. Parathyroid CaSR mRNA was reduced by 40% in vitamin D-depleted relative to replete rats, and $1,25(OH)_2D_3$ administration to vitamin D-replete rats enhanced parathyroid and kidney CaSR mRNA levels further (Brown et al., 1996). In another study administration of $1,25(OH)_2D_3$ to rats upregulated renal CaSR mRNA levels in a dose-and time-dependent manner (Yao et al., 2005). Kidney CaSR expression (microarray analysis) was downregulated in VDR null mice relative to their wildtype littermates (Li et al.,

2003) and likewise (ribonuclease protection assay) for *Cyp27−/−* mice lacking the 25-hydroxyvitamin D-1α-hydroxylase enzyme (Canaff et al., 2009a). Injection of $1,25(OH)_2D_3$ in either normal mice or the *Cyp27b1−/−* mice caused upregulation of CaSR expression in parathyroid/thyroid and kidney (Li et al., 2003; Canaff et al., 2009a). Nuclear run-on assays that measure RNA transcripts showed that upregulation in thyroid and kidney cells occurred via gene transcription (Canaff and Hendy, 2002). Functional vitamin D response elements (VDREs) are present in both promoters, P1 and P2, of the human *CASR* gene (Canaff and Hendy, 2002) and are conserved in the rodent (Hendy et al., 2013). The VDREs of the *CASR* are typical in that they consist of two 6-bp half-sites separated by 3-bp that are bound by the vitamin D receptor (VDR)-retinoic acid X receptor (RXR) dimer. However, the VDREs are atypical in that the orientation of the half-sites is inverted to that which is normally found. VDREs of this type are found in the 25-hydroxyvitamin D-24-hydroxylase (*CYP24A1*) gene.

In the parathyroid gland, the $1,25(OH)_2D$-upregulated CaSR makes the gland more responsive to extracellular Ca^{2+} and PTH secretion is reduced. The negative action of extracellular Ca^{2+} on PTH synthesis and secretion and parathyroid cell proliferation is reinforced by the active vitamin D metabolite. Impaired extracellular Ca^{2+}-sensing that drives increased parathyroid cell proliferation may contribute to parathyroid neoplasia. Somatic *CASR* mutations are rare, but parathyroid glands of patients with primary or severe uremic secondary hyperparathyroidism often have reduced CaSR expression (Kifor et al., 1996; Cetani et al., 2000; Corbetta et al., 2000). Thus, reduction in components of the vitamin D system, active vitamin D ligand either circulating levels or produced by parathyroid intracrine action, and/or target VDR levels, that normally decrease PTH synthesis and secretion, could play an additional role by negating the normal inhibitory effects of extracellular Ca^{2+} on PTH via decreased CaSR expression.

The activated kidney CaSR that can act independently of PTH to directly determine the circulating Ca^{2+} concentration (Loupy et al., 2012) inhibits the paracellular uptake of cations in the cortical thick ascending limb of the distal nephron and promotes hypercalciuria. Autosomal dominant hypocalcemia type 1 (ADH1) patients are heterozygous for a gain-of-function mutant *CASR*. Treatment of ADH1 patients with active vitamin D upregulates the oversensitive renal CaSR stimulating Ca^{2+} excretion provoking nephrocalcinosis, nephrolithiasis, and potential renal damage (Pearce et al., 1996; Lienhardt et al., 2001). For such patients a better treatment option is vitamin D itself whereby the amount $1,25(OH)_2D$ formed is limited by product inhibition of the 25-hydroxyvitamin D-1alpha-hydroxylase enzyme in the proximal tubule (Obermannova et al., 2016). In some genetic forms of hypercalciuria, altered regulation of CaSR expression by vitamin D metabolites may be a critical factor contributing to stone formation. In a genetic stone-forming rat model of hypercalciuric nephrolithiasis, nephron VDR levels are elevated, CaSR levels are increased and calcium reabsorption reduced (Yao et al., 2005; Bai and Favus, 2006).

Alterations of CaSR expression have been implicated not only in hyperparathyroidism but also other neoplasms. The CaSR is expressed in human colon epithelium and regulates cell proliferation and differentiation. Cells of the colon crypt acquire CaSR expression as they differentiate and migrate toward the apex of the crypt (Chakrabarty et al., 2005). CaSR expression is weak or absent in colon carcinomas and is inversely correlated with differentiation status. Extracellular Ca^{2+} and $1,25(OH)_2D$ upregulate *CASR* transcription and cyclin-dependent kinase inhibitor expression in the colon and Ca^{2+} and $1,25(OH)_2D$ may exert their chemopreventative actions with respect to colon cancer, in part, through the CaSR (Chakrabarty et al., 2005). Fetahu et al. (2014b) demonstrated the upregulation of CaSR expression by $1,25(OH)_2D_3$ in colon cancer cells. This would be consistent with the active vitamin D metabolite exerting its antiproliferative, prodifferentiating effects in part by inducing expression of the tumor suppressor, CaSR.

Vascular calcification occurs during aging and pathologically in atherosclerosis and chronic kidney disease. There is an inverse relationship between vascular calcification and expression of the CaSR (Alam et al., 2009) and maintenance of its expression can protect against calcification (Molostvov et al., 2015). Treatment of vascular smooth muscle cells with active vitamin D increases CaSR mRNA and protein levels (Mary et al., 2015). This would be consistent with $1,25(OH)_2D$ transactivation of the *CASR* gene via its VDREs (Canaff and Hendy, 2002). Treatment with cinacalcet that enhances sensitivity of the CaSR to Ca^{2+} promotes CaSR expression at the cell surface while overall CaSR expression is not altered (Hénaut et al., 2014). This would be consistent with ligand induced trafficking of the CaSR to the cell surface (Breitwieser, 2013) and Ca^{2+} within the normal range and higher having no marked effect on *CASR* gene expression (see below for further detail of potential regulation of *CASR* by extracellular Ca^{2+}).

Calcium

In addition to $1,25(OH)_2D$, a potential regulator of *CASR* gene expression is extracellular Ca^{2+} itself. Initial studies did not find an effect of extracellular Ca^{2+} on parathyroid gland or whole kidney CaSR mRNA in the rat *in vivo* (Rogers et al., 1995; Brown et al., 1996). A lack of effect on *CASR* expression by circulating Ca^{2+} is perhaps not unexpected in tissues such as parathyroid gland or kidney, where the CaSR plays a critical role in Ca^{2+} homeostasis as a calciostat to sense small changes in extracellular Ca^{2+} concentration. Even slight alterations in the extracellular Ca^{2+} set-point (the extracellular Ca^{2+} concentration at which PTH secretion from the parathyroid gland or calcium reabsorption across the kidney tubule is half-maximal) brought about by changes in CaSR synthesis could disturb overall calcium homeostasis.

CaSR expression did not change in rats having altered circulating calcium levels as a result of infusion of $CaCl_2$ or fed vitamin D-deficient diets supplemented with Ca^{2+} (Rogers et al., 1995; Brown et al., 1996). In these two studies the extracellular calcium levels ranged from 0.7 to 1.9 mM. However, potentially, lower or higher Ca^{+} concentrations could regulate CaSR expression and perhaps in a tissue-specific manner.

CASR gene transcription was modulated by extracellular Ca^{+} in studies of transfected *CASR* promoter-reporter constructs in human kidney proximal tubule cells and mouse distal convoluted tubule cells (Canaff, 2004). In both cell types, when

the concentration of Ca^{2+} in the media was varied from 1 to 5 mM, there was only a slight increase in P1 and P2 promoter activity. However, there was markedly reduced transcriptional activity of P1 (by 50%) and P2 (by 30%) in both proximal and distal convoluted tubule cell types when cultured in a very low (0.25 mM) Ca^{2+} concentration. Therefore, from normal to high Ca^{2+} there was little regulation but from normal to low calcium there was significant regulation. The results from these *in vitro* experiments would be consistent with the lack of obvious effect of circulating Ca^{2+} in the *in vivo* studies referred to above (Rogers et al., 1995; Brown et al., 1996). In other cells—differentiated human colon carcinoma cells—upregulation of the CaSR occurred when the Ca^{2+} concentration of the culture medium was increased from very low to normal (Chakrabarty et al., 2005). Stimulatory effects of Ca^{2+} and $1,25(OH)_2D$ were additive for promoter P2 but not for promoter P1 (Chakrabarty et al., 2005).

What would be the significance in the kidney of a reduced CaSR expression at low extracellular Ca^{2+} concentration? A decrease of CaSR expression in the proximal tubule would decrease the effect of calcium on the 25-hydroxyvitamin D-1alpha-hydroxylase activity i.e., the negative impact of Ca^{2+} in decreasing enzyme activity would be minimized. In the ascending limb and distal convoluted tubule, the combination of a reduced CaSR expression and a low extracellular Ca^{2+} would favor maximal Ca^{2+} reabsorption from the tubule lumen and allow PTH to stimulate Ca^{2+} reabsorption without being antagonized by the CaSR.

The precise pathways by which extracellular Ca^{2+} might transactivate the *CASR* gene are not known. If the extracellular Ca^{2+} effect is mediated via the CaSR then a transcription factor activated by mitogen activated protein kinase (MAPK) (e.g., Elk1) could be involved. In addition, the involvement of transcription factors in the reduced expression of the CaSR in neoplasia of parathyroid and colon, for example, are not known. In an *in vivo* murine model of primary hyperparathyroidism in which cyclin D1 overexpression is targeted to the parathyroid, expression of the CaSR in the gland is markedly reduced and correlates with the severity of the hyperparathyroidism (Kawata et al., 2005). Treatment of the mice with the allosteric CaSR activator cinacalcet to mimic high levels of extracellular Ca^{2+} suppressed serum PTH and Ca^{2+} and parathyroid cell proliferation but had no effect on the levels of parathyroid CaSR mRNA (Imanishi et al., 2011). This reinforces the view that normal or higher extracellular Ca^{2+} concentrations do not significantly modulate parathyroid CaSR expression levels.

Proinflammatory Cytokines

Evidence has accumulated that the increased levels of circulating cytokines such as IL-1β and IL-6 occurring in conditions of inflammation could provoke altered systemic Ca^{2+} homeostasis by modulating the setting of the calciostat—the expression of the CaSR. In critically ill patients such as those with sepsis and major burn injury hypocalcemia is common (Zaloga, 1992; Zivin et al., 2001; Steele et al., 2013) and in non-acutely ill patients undergoing surgery (Lepage et al., 1999). Serum IL-1β and IL-6 levels increase within hours of severe burns and infection (Kowal-Vern et al., 1994; Klein et al., 1995; Caldwell et al., 1997), and are inversely related to the falls in serum Ca^{2+} concentration (Lind et al., 2000) that may correlate with a poor prognosis (Guo et al., 1990; Nijsten et al., 1991; Schlüter et al., 1991; Ohzato et al., 1993; Yamada et al., 1996; Remick et al., 2002). Several factors such as decreased secretion of PTH and/or resistance to PTH action in kidney and bone may contribute to the hypocalcemia (Katz et al., 1992; Klein et al., 1997). In addition, the metabolism and action of vitamin D can be impaired. Calcitonin precursors are increased in the circulation of critically ill patients with sepsis (Lind et al., 2000; Müller et al., 2000; Becker et al., 2004, 2010). Certainly altered PTH secretion and action and vitamin D metabolism will be most important, whereas calcitonin plays a minor role in Ca^{2+} homeostatic control in human.

In patients with rheumatoid arthritis impaired secretion of PTH is inversely related to the inflammatory activity (Ekenstam et al., 1990). PTH secretion from bovine parathyroid cells was suppressed by IL-6 *in vitro* (Carlstedt et al., 1999) and the same effect was demonstrated for clinically relevant doses of IL-1β acting specifically via the IL-1 receptor in bovine parathyroid tissue slices *in vitro* with the added observation of upregulation of CaSR mRNA levels (Nielsen et al., 1997). Parathyroid CaSR mRNA levels were upregulated in an *in vivo* sheep model of burn injury in which increased circulating cytokine levels would occur (Murphey et al., 2000). Some proinflammatory cytokines can stimulate (rather than inhibit) the release of PTH. The IL-8B receptor (CXCR2) was identified in the bovine parathyroid gland (Angeletti et al., 1998). Cultured bovine parathyroid cells responded to IL-8 with increases in PTH mRNA levels and PTH secretion, although any role of altered CaSR expression in these effects is as yet unknown.

Dysregulated PTH release and altered circulating Ca^{2+} levels occurs in septic horses (Toribio et al., 2001; Hurcombe et al., 2009). Equine parathyroid cells in culture responded to elevations in extracellular Ca^{2+} (0.8–2 mM) with decreased PTH mRNA while CaSR mRNA expression was unaltered consistent with other studies (see above) showing lack of an effect of extracellular Ca^{2+} on CaSR expression within this concentration range. IL-1β acting via the IL-1 receptor decreased both PTH secretion and increased CaSR mRNA expression (Toribio et al., 2003). In rats *in vivo* serum PTH and $1,25(OH)_2D$ decreased significantly 12 h after intraperitoneal (ip) injection of IL-1β, followed by significantly decreased levels of serum Ca^{2+} at 15 h (Canaff and Hendy, 2005). In the same model, decreases in serum PTH and $1,25(OH)_2D_3$ and calcium occurred after ip injection of IL-6 (Canaff et al., 2008b).

The CaSR in part mediates the antiproliferative and prodifferentiation actions of Ca^{2+} in colonocytes and can be considered as a tumor suppressor in the colon. Fetahu et al. (2014b) showed that in two differentiated colonocyte cell lines CaSR mRNA and protein levels increased in response to the cytokines, TNFα, IL-1β, and IL-6. The robust increase of CaSR expression could represent a defense against inflammation similar to what had been shown in murine macrophages, in which lipopolysaccharide-induced TNFα release upregulated CaSR leading to negative feedback inhibition of synthesis of the cytokine (Kelly et al., 2011).

Proinflammatory cytokines, like IL-1β and TNF-α, work through NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). NF-κB exists in the cytoplasm of cells in an inactive form bound to an inhibitor, IκB. Upon receipt of a signal generated by activation of cytokine receptors, IκB is phosphorylated, and NF-κB is released from IκB and translocates to the nucleus to upregulate specific gene transcription via specific κB response elements. NF-κB is responsible for the expression of many immune and inflammatory response (and other) proteins (Ghosh et al., 1998; Baldwin, 2001).

In the rat *in vivo* IL-1β upregulates parathyroid, thyroid, and kidney CASR mRNA and protein levels and stimulates endogenous *CASR* gene transcription in human thyroid and kidney cell lines (Canaff and Hendy, 2005). In addition, we showed that IL-1β and TNF-α upregulate *CASR* gene transcription via NF-κB with functional κB response elements being present in both promoters of the *CASR* gene (Canaff and Hendy, 2005).

At the cell surface, IL-6 binds the IL-6 receptor (IL-6R) consisting of an IL-6 binding α chain (gp80) and the gp130 signal transducer that is shared among the IL-6-related cytokine subfamily members. IL-6 binding to its receptor activates Janus kinase (JAK) family members that phosphorylate and activate signal transducers and activators of transcription (STAT) family members. The STATS dimerize, translocate to the nucleus and bind specific gene STAT-response elements activating transcription (Horvath, 2000; Levy and Darnell, 2002). STAT-1 and STAT-3 are responsible for IL-6 signaling. The JAKS may also couple to the MAPK pathway to modify the activity of transcription factors including STATs and others (Heinrich et al., 2003).

Intraperitoneal injection of IL-6 to rats *in vivo* caused decreases in serum PTH, $1,25(OH)_2D$, and Ca^{2+} that were maintained over 24 h (Canaff et al., 2008b). Parathyroid, thyroid and kidney CaSR mRNA and protein levels were upregulated. IL-6 increased *CASR* gene transcription in human thyroid C-cell and kidney proximal tubule cells *in vitro*. *CASR* gene P1 and P2 promoter-driven transcripts were upregulated. Activation of the MAPK pathway contributed modestly to basal activity of both P1 and P2 promoters, but it was not involved in a major way in the IL-6 induction of either of them (Canaff et al., 2008b). For promoter P1 a STAT1/3 element downstream of the transcriptional start site accounted for the IL-6 induction. For promoter P2 that has no STAT elements, IL-6 rapidly promotes a complex containing both Sp1/3 and STAT1/3 on GC-rich elements that are clustered at the transcription start site.

The IL-6 administration to rats resulted in a more rapid decrease in serum PTH, $1,25(OH)_2D$ and Ca^{2+} levels than that occurring after IL-1β administration (Canaff and Hendy, 2005; Canaff et al., 2008b). The quicker response to IL-6 may relate to this cytokine's ability to induce rapid changes in mRNA translation and protein synthesis via the eukaryotic initiation factor-4F, as has been shown in sensory neurons, for example (Melemedjian et al., 2010). While IL-6 clearly regulates *CASR* gene transcription, uncovering any contribution of the cytokine at the post-transcriptional level with respect to the resetting of the calciostat will require further study.

Other Considerations

At sites of injury or infection the inflammatory response increases circulating proinflammatory cytokines leading to increased bone resorption. This occurs by enhanced osteocytic and osteoblastic production of receptor of activated NF-κB ligand (RANKL) resulting in increased osteoclastic bone resorption that releases Ca^{2+} from the bone (see Klein et al., 2016). One purpose of the increased extracellular Ca^{2+} concentration likely relates to its action as a chemokine on the one hand to recruit macrophages to sites of cell death and on the other to play a role in amplifying the inflammatory response via stimulating the assembly of a cytoplasmic multiprotein complex inflammasome that mediates proinflammatory cytokine maturation by activation of caspase-1 (see Hendy and Canaff, 2016 for further details).

Therefore, the CaSR in parathyroid gland and kidney executes its role in Ca^{2+} homeostasis but it is also expressed in monocytes and macrophages. This allows the CaSR to play critical roles in promoting and mediating the inflammatory response to tissue injury as well as minimizing or limiting these effects via its role in systemic Ca^{2+} homeostasis. Whether there is a place for the use of CaSR allosteric modulators in relevant clinical areas, for example, in reducing the degree of hypocalcemia in critically ill patients, remains to be explored. While extracellular Ca^{2+} is an activator of the NOD-like receptor family, pyrin domain-containing protein-3 (NLRP3) inflammasome, influenza virus infection also activates this particular inflammasome with resultant production of IL-1β (Allen et al., 2009; Owen and Gale, 2009; Thomas et al., 2009). This may be relevant to the observation that ADH1 patients with gain-of-function *CASR* mutations often become symptomatic (e.g., with seizures) during periods of intercurrent illness (Hendy et al., 2009). Enhanced expression of the activated CaSR by the increased circulating IL-1β cytokine would result in extracellular Ca^{2+} levels to drop further to symptomatic levels.

An additional pathological role for Ca^{2+}, CaSR, proinflammatory cytokines, and obesity has been suggested (Villarroel et al., 2014). It is proposed that Ca^{2+} activation of the CaSR in white adipose tissue preadipocytes increases proinflammatory cytokine production, proliferation and differentiation but decreased liped accumulation and adipose tissue dysfunction (Cifuentes et al., 2012). The obesity associated cytokines like TNF-α and IL-1β increase adipocyte CaSR expression (Cifuentes et al., 2010) by releasing NFκB to translocate to the nucleus and activate the *CASR* gene at κB response elements in its promoters (Canaff and Hendy, 2005). This represents a positive feed forward loop.

Glial Cells Missing-2

In Drosophila the transcription factor glial cells missing (*GCM*) acts as a developmental binary switch between glia and neurones. In mammals, there are two orthologs *GCM1*/*GCMA* and *GCM2*/*GCMB* critical for parathyroid and placental development, respectively (Kim et al., 1998; Kammerer et al., 1999; Kanemura et al., 1999). The five exons of the *GCM2* gene (OMIM# 603716) on chromosome 6p24.2 encode a protein of 506 amino acids. GCM2 is expressed in the PTH-secreting cells of the parathyroid glands and is critical for their development

in terrestrial vertebrates, and continues to be expressed in the adult (Günther et al., 2000; Maret et al., 2004; Okabe and Graham, 2004; Liu et al., 2007). From NH_2 to COOH termini of the GCM2 protein there is a DNA-binding domain, transactivation domain 1, an inhibitory domain, and transactivation domain 2.

Homozygous or heterozygous inactivating mutations occur in familial isolated hypoparathyroidism (FIH) inherited in an autosomal recessive or dominant manner, respectively (Hendy and Cole, 2015). Autosomal recessive mutations include missense, stop, frameshift, and gene deletion, (e.g., Ding et al., 2001; Baumber et al., 2005; Thomée et al., 2005; Bowl et al., 2010; Tomar et al., 2010; Doyle et al., 2012). Autosomal dominant mutations include missense and single-nucleotide deletion (e.g., Mannstadt et al., 2008; Canaff et al., 2009b; Mirczuk et al., 2010; Yi et al., 2012). *In vitro* functional studies of some of these mutants have demonstrated loss of GCM response element binding and/transcriptional activity in the case of recessive mutations, as well as the ability of dominant mutants to inhibit activity of wild-type GCM2 when the two are transfected together into cells (Mannstadt et al., 2008; Canaff et al., 2009b; Mirczuk et al., 2010).

Genetic defects affecting GCM2 are rare in FIH: a study of 20 unrelated FIH cases (10 familial and 10 sporadic) found several polymorphic variants, but did not find *GCM2* mutations that segregated with the disease and/or led to loss of function (Maret et al., 2008).

The CaSR is an early parathyroid differentiation marker (Liu et al., 2007). Absence or reduction of parathyroid GCM2, as in mice null for *Gcm2* (Günther et al., 2000) or in cultured human parathyroid cells treated with GCM2 siRNA (Mizobuchi et al., 2009), correlates with lack or decreased expression of the CaSR. GCM2 transactivates the *CASR* gene via GCM response elements in promoters P1 and P2 (Canaff et al., 2008a, 2009b). Thus, GCM2 and CaSR are mechanistically linked with respect to the development of the evolutionarily related parathyroid glands (in terrestrial vertebrates) and gills (in fish) (Okabe and Graham, 2004).

v-maf musculo-aponeurotic fibrosarcoma oncogene homolog B (MafB), a transcriptional activator, is present in developing and mature parathyroid glands (Kamitani-Kawamoto et al., 2011). MafB acts downstream of GCM2 and ensures that the developing parathyroid glands properly localize between the thyroid and the thymus (Kamitani-Kawamoto et al., 2011). GCM2 associates with MafB to synergistically activate *PTH* gene expression (Kawahara et al., 2010; Kamitani-Kawamoto et al., 2011).

Haploinsufficiency of the dual zinc-finger transcription factor, GATA3, results in the congenital hypoparathyroidism-deafness-renal dysplasia (HDR) syndrome (Ali et al., 2007). *Gata3* knockout mouse embryos lack *Gcm2* expression and have gross defects in the third and fourth pharyngeal pouches including absent parathyroid-thymus primordia (Grigorieva et al., 2010). GATA3 transactivates the *GCM2* gene by binding specifically to a double-GATA-motif within the *GCM2* promoter. In addition, GATA3 cooperates with GCM2 and MafB to activate *PTH* gene expression by interacting with the ubiquitous specificity protein-1 (SP1) transcription factor (Han et al., 2015). Thus, GATA3, GCM2 and MafB are part of a critical transcriptional cascade in the parathyroid morphogenesis and CaSR and PTH expression pathway (Grigorieva and Thakker, 2011; Han et al., 2015).

The persistance of GCM2 expression in the adult parathyroid raises the question of whether its overactivity or reduced expression could play a role in the development of parathyroid hyperfunction or tumorigenesis. In support of the first notion, increased expression of GCM2 has been noted in some adenomas of primary parathyroid patients (Kebebew et al., 2004). Furthermore, the allele frequency of a common Y282D polymorphism is significantly higher in Italian cohorts of primary hyperparathyroid patients than in normal individuals and the 282D variant exhibits higher activity on a GCM element promoter than Y282 (D'Agruma et al., 2014). In support of the second supposition, reduced levels of GCM2 mRNA have been reported in adenomatous tissue from some patients with primary hyperparathyroidism (Correa et al., 2002). This may contribute to the reduced CaSR expression commonly found in these tumors that might itself contribute to dysregulated control of parathyroid cell proliferation.

CASR Expression and Kidney Stones

Evidence has been provided that CaSR polymorphisms such as R990G can increase the susceptibility to hypercalciuria and urolithiasis (Liu et al., 2015). But single nucleotide polymorphisms (SNPs) within the regulatory regions of the *CASR* gene have also been associated with kidney stone risk, for example, in primary hyperparathyroidism (Vezzoli et al., 2011). A comparison of idiopathic calcium stone formers and healthy controls revealed one SNP (rs6776158 [A>G]) located in the *CASR* P1 promoter that was associated with nephrolithiasis (Vezzoli et al., 2013). Sr^{2+} handling has been proposed to be a mirror of Ca^{2+} metabolism and reduced Sr^{2+} excretion after an oral load was observed in GG homozygous stone formers. Activity of the G allele *CASR* P1 promoter-luciferase reporter was lower (than the A allele promoter) in cell transfection studies and CaSR mRNA levels were lower in kidney medulla samples in GG homozygous individuals than A allele carriers (Vezzoli et al., 2013). Therefore, the minor allele at rs6776158 may predispose to calcium stones by decreasing transcriptional activity of the *CASR* P1 promoter and CaSR expression in kidney tubules. Claudin-14 is a member of a superfamily of proteins that regulate paracellular transport of ions and small solutes at epithelial tight junctions. A mechanistic link between *CASR* gene activity was suggested by the finding that claudin-14 mRNA levels were lower in the *CASR* rs6776158 GG homozygous subjects Therefore, in these cases the predisposition to calcium nephrolithiasis is by a mechanism independent of hypercalciuria (Vezzoli et al., 2013). This hypothesis received further support by a comparison of another *CASR* gene regulatory SNP and R990G with respect to stone risk in primary hyperparathyroidism (Vezzoli et al., 2015).

In a genome-wide association study (GWAS) of Icelandic kidney stone cases a suggestive association of (rs7627468 [A]) in a *CASR* intron1 regulatory region was found in a large population set (Oddsson et al., 2015). A strong association was found for a polymorphism (rs199565725 [delAC]) in intron 1 of the *CLDN14* gene encoding claudin-14 that might mediate a decrease in *CLDN14* gene function. These data reinforced that of the same

group in an earlier study (Thorleifsson et al., 2009). The authors observed uncorrelated genome-wide significant association of variants at the *CASR* locus that also influence biochemical traits, serum total and ionized calcium, for example, but do not associate with the kidney stone disease. This implies that the risk is not mediated solely through the serum level of calcium or other biochemical trait (Oddsson et al., 2015), and provides further indirect support for the *CASR* directed Claudin-14 mechanism (Gong et al., 2012; Gong and Hou, 2014; Toka et al., 2015; Riccardi and Valenti, 2016).

CASR and Epigenetic Modification

Altered CaSR expression occurs in benign and malignant tumors suggesting it as either a tumor suppressor or oncogene. The epigenetic inactivation of the *CASR* may underlie the reduced CaSR expression in some cancers. The *CASR* P2 promoter that is GC-rich, is methylated to a greater extent in colorectal tumors relative to adjacent mucosa and this correlates with the reduced CaSR levels in the tumors (Hizaki et al., 2011; Fetahu et al., 2014a). Use of histone deacetylase inhibitors in colon cancer cell lines most of which poorly express CaSR has implicated the involvement of H3K9 deacetylation in the silencing of CASR in colorectal cancer (Hizaki et al., 2011; Fetahu et al., 2014a). Similar findings with respect to methylation of the *CASR* P2 promoter were reported for unfavorable neuroblastomas in which CaSR is barely detectable (Casalà et al., 2013). In addition, monosomy of chromosome 3 where *CASR* resides was found in the vast majority of primary neuroblastic tumors of all types (Casalà et al., 2013). Hence the *CASR* is silenced by both genetic and epigenetic means in these tumors. However, no evidence has been found of loss of *CASR* alleles (Farnebo et al., 1997) or *CASR* gene methylation being responsible for the reduced CaSR levels in primary and secondary hyperparathyroidism (Hofman-Bang et al., 2012; Sulaiman et al., 2013; Varshney et al., 2013). Moreover, the molecular mechanisms underlying the enhanced expression of CaSR in breast and prostate tumors that implicates an oncogenic role for CaSR in these tissues have yet to be deciphered (Tennakoon et al., 2016).

CaSR And MicroRNAs

MicroRNAs (miRNAs) are short non-coding RNAs that function in RNA silencing and post-transcriptional regulation of gene expression. A further cause of silencing of the CaSR in colorectal tumors has been proposed to be increased expression of miR-135b and miR-146b that are considered to be oncogenic (Fetahu et al., 2016). In colon cancer cell lines other miRNAs, miR-21, miR-145, and miR-135a, are inversely correlated with CaSR expression (Singh and Chakrabarty, 2013; Singh et al., 2013). Altered expression of miRNAs may well have an important role in development of parathyroid tumors and miRNAs have been implicated in parathyroid function. Dicer-dependent maturation of parathyroid miRNAs is responsible for the dysregulated control of PTH secretion in a uremic mouse model of secondary hyperparathyroidism (Shilo et al., 2015). However, specific miRNAs that directly affect parathyroid CaSR expression have yet to be identified.

PERSPECTIVES AND CONCLUSIONS

The CaSR is expressed in the central nervous system and the roles it may play in the brain are being explored (Ruat and Traiffort, 2013). It will be important in future to understand the precise mechanisms underlying the postnatal upregulation of the brain CaSR during development and whether altered regulation of the CaSR plays a role in some cases of epilepsy. Within neurons the CaSR may play a role in susceptibility to Alzheimer's Disease (AD) and its progression (Chiarini et al., 2016). *CASR* expression may be altered in AD as it is regulated by the same transcription factors (e.g., SP1/3, AP1, STAT1/3, NF-κB, TFIID) already known to modulate other AD-regulated genes (Chiarini et al., 2016). However, this has yet to be directly evaluated.

The blood-brain barrier defends extracellular Ca^{2+} in the brain from changes in serum Ca^{2+} and at rest brain extracellular Ca^{2+} is maintained at 1.1 mM. During neuronal activity extracellular Ca^{2+} can fall sharply as Ca^{2+} moves to intracellular compartments. Following action potentials, Ca^{2+} can fall as low as 0.3 mM in the synaptic cleft (Jones and Smith, 2016). Any potential changes on *CASR* gene expression and their consequences have yet to be examined.

Further insights to those described in the present review, can be anticipated with respect to identification of additional transcriptional factors and their cis-elements in the *CASR* gene promoters, epigenetic changes involving direct methylation of the *CASR* DNA as well as histone modifications and chromatin remodeling. The direct effect of miRNAs on degradation of CaSR mRNA or inhibition of its translation will become clearer.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Cross Talk between the Calcium-Sensing Receptor and the Vitamin D System in Prevention of Cancer

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There is epidemiological evidence for the cancer preventive effect of dietary calcium (Ca^{2+}) and vitamin D. This effect is strongest in colorectal cancer (CRC). The active vitamin D metabolite, 1,25-dihydroxyvitamin D_3 ($1,25D_3$), bound to its receptor, the vitamin D receptor (VDR) regulates the expression of hundreds of different genes in a cell- and tissue-specific manner. While Ca^{2+} acts through multiple mechanisms and pathways, some of its effects are mediated by the calcium-sensing receptor (CaSR). The joint action of Ca^{2+} and $1,25D_3$ is due to the fact that both regulate some of the main processes involved in the development of various cancers, such as proliferation, differentiation, apoptosis, migration, and inflammation. Moreover, $1,25D_3$, bound to VDR can induce translation of the CaSR, while the amount and activity of the CaSR affects $1,25D_3$ signaling. However, the complexity of the cross-talk between the CaSR and the vitamin D system goes beyond regulating similar pathways and affecting each other's expression. Our aim was to review some of the mechanisms that drive the cross-talk between the vitamin D system and the CaSR with a special focus on the interaction in CRC cells. We evaluated the molecular evidence that supports the epidemiological observation that both vitamin D and calcium are needed for protection against malignant transformation of the colon and that their effect is modulated by the presence of a functional CaSR.

Keywords: CaSR, VDR, VDRE, colorectal cancer, calcium, vitamin D

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INTRODUCTION

Epidemiological and preclinical studies suggested that dietary calcium and vitamin D are able to prevent several forms of cancer, with strongest effect observed in prevention of colorectal cancer (CRC; Zhang and Giovannucci, 2011). Low calcium intake and vitamin D insufficiency were considered independent risk factors for cancer, until Garland et al. showed that their colon cancer preventing effect is interdependent (Garland et al., 1985). Both calcium and vitamin D affect several hallmarks of cancer: enhance differentiation, adhesion, activate apoptosis, inhibit proliferation and inflammation, and decrease metastatic potential. Therefore, understanding the interactions between them would enhance the possibility of exploiting their cancer preventing potential.

Epidemiological Evidence

Low calcium intake was linked to the pathogenesis of several chronic diseases and is a recognized risk factor for total cancer incidence (Park et al., 2009; Peterlik et al., 2013). In a population-based, double-blind, placebo-controlled randomized trial among community-dwelling women dietary calcium (1400–1500 mg) and vitamin D (1100 IU) reduced all-cancer risk (Lappe et al., 2007). Calcium and vitamin D supplementation reduced melanoma risk in women with a history of non-melanoma skin cancer (Tang et al., 2011). High serum calcium levels at baseline were associated with lower breast cancer mortality in a Swedish cohort, while serum 25 hydroxyvitamin D_3 ($25D_3$) levels and breast cancer mortality showed a u-shaped correlation (Luo et al., 2013; Huss et al., 2014). A recent study showed that high calcium-sensing receptor (CaSR) expression in primary prostate tumors was associated with lethal progression of the disease if the tumors expressed low vitamin D receptor (VDR) levels, but not if the tumors had high VDR levels (Ahearn et al., 2016).

Expression level and activity of the CaSR were linked to risk, incidence, recurrence, or lethality of various cancers, such as prostate, breast, colorectal, ovarian cancer, or neuroblastoma (Tennakoon et al., 2016), cancers where vitamin D insufficiency might also be involved in etiology (**Table 1**).

In CRC there is ample evidence supporting the cancer preventive effects of calcium and vitamin D (World Cancer Research Fund/American Institute for Cancer Research, 2007; Tarraga Lopez et al., 2014). In adenomatous polyposis patients high doses of dietary calcium and vitamin D significantly reduced the rate of polyp formation after 6 months (Holt et al., 2006). Pooling data from 10 cohort studies showed that only high intake of both vitamin D and calcium reduced risk of CRC (Cho et al., 2004). Calcium supplementation in a placebo-controlled randomized multi-center clinical trial reduced risk of adenoma recurrence (RR = 0.71) only in subjects with $25D_3$ levels above the median (29.1 ng/mL). Moreover, serum $25D_3$ levels correlated inversely with adenoma risk only in subjects receiving calcium supplementation (Grau et al., 2003). Usually changes in serum Ca^{2+} levels are minimal; however dietary calcium causes high fluctuations in fecal Ca^{2+} levels that affect tumorigenesis. Ca^{2+} in the intestine forms insoluble salts with potentially carcinogenic bile acids, such as deoxycholic and lithocholic acid. Lithocholic acid is able to bind VDR, induce expression of the vitamin D degrading enzyme (CYP24A1) and 1,25 dihydroxyvitamin D_3 ($1,25D_3$) degradation (Hobaus et al., 2013a).

While interventional studies are sometimes inconclusive, many animal studies convincingly demonstrated the cancer-preventing effect of vitamin D_3 and calcium, suggesting that calcium and vitamin D regulate the dynamic balance between proliferation, differentiation, adhesion, motility, and apoptosis in the colon (Holt et al., 2006; Hummel et al., 2013) and both are needed for optimal effectiveness. In a dietary colon cancer model, where the so-called new western diet (NWD) fed for 2 years led to development of spontaneous colorectal tumors, supplementation with dietary calcium and vitamin D reduced significantly both colon tumor incidence and multiplicity (Newmark et al., 2009). Moreover, the high Ca^{2+} and vitamin D intake prevented colonic tumor formation even in the mice bearing the $Apc1638N^{+/-}$ mutation and fed the NWD (Yang et al., 2008). How far the effect of calcium was mediated by the CaSR is not yet clear.

Mechanism of Action

The mechanism of action of vitamin D is clear: vitamin D (synthesized in the skin or ingested through food) is transformed in the liver to 25 hydroxyvitamin D_3 and in the kidney (or other tissues), to its most active form 1,25 dihydroxyvitamin D_3. The enzymes involved in this process are the vitamin D_3 25 hydroxylases (e.g., CYP2R1 and CYP27A1) and the 25 hydroxyvitamin D_3 1 alpha hydroxylase (CYP27B1). $1,25D_3$ binds to its nuclear receptor, the transcription factor VDR and regulates target gene expression. Both $1,25D_3$ and its precursor $25D_3$ are catabolized by the 1,25 dihydroxyvitamin D_3 24 hydroxylase (CYP24A1) (Christakos et al., 2016). VDR, CYP27B1, and CYP24A1 are almost ubiquitously expressed, suggesting that $1,25D_3$ can be synthesized and degraded in most of the tissues. Effectiveness of $1,25D_3$ depends on the local expression level and activity of all these molecules.

The mechanism of action of extracellular calcium is by far much more complex. There are numerous different molecules (different types of Ca^{2+} channels, Ca^{2+} pumps, exchangers, etc.) and pathways that are involved (Capiod, 2016). The

TABLE 1 | Effect of calcium, vitamin D and involvement of the CaSR in development of cancer.

Cancer type	Effect of calcium	Effect of vitamin D	CaSR involvement
Parathyroid adenoma/Carcinoma	↓ Proliferation	↓ Proliferation	Tumor suppressor (Miller et al., 2012)
Colorectal cancer	↓ Incidence	↓ *Incidence**	Tumor suppressor (Aggarwal et al., 2015b)
	↓ Risk	↓ *Risk*	
Neuroblastoma			Tumor suppressor (Casala et al., 2013)
Breast cancer	↓ *Cancer risk*	↓ *Incidence*	↑ Metastasis to bone (Vanhouten and Wysolmerski, 2013)
		↓ *Risk*	
		↑ *Survival*	
Prostate cancer	↑ *Cancer incidence*	↑ *Protection against aggressive cancer*	↑ Metastasis to bone (Liao et al., 2006; Chakravarti et al., 2009)
Kidney cancer			↑ Metastasis to bone (Joeckel et al., 2014)

**Italics: limited evidence.*

extracellular calcium-sensing receptor (CaSR) is one of the candidates that mediate the cancer preventive effects of dietary calcium (Tennakoon et al., 2016). Whitfield suggested that the CaSR serves as the molecular switch that turns on differentiation and turns off proliferation in colonic epithelial cells as these migrate along the colonic crypt (Whitfield, 2009).

The main physiological role of this G protein-coupled receptor is regulating calcium homeostasis (Brown et al., 1993) by maintaining the balance between Ca^{2+} absorption in the intestine, Ca^{2+} excretion by the kidneys, and the release of Ca^{2+} from bone. The maintenance of calcium homeostasis is orchestrated by the intricate cross talk among Ca^{2+}, the CaSR, parathyroid hormone (PTH), and the active vitamin D hormone $1,25D_3$ (Brown and MacLeod, 2001). The CaSR regulates PTH secretion, dependent on extracellular Ca^{2+} concentrations. When Ca^{2+} levels are high in the serum, the receptor is activated, leading to a decrease in PTH synthesis and secretion. When serum Ca^{2+}concentration drops, CaSR is inactive, and PTH is secreted into the serum, leading to increased urinary Ca^{2+} resorption, stimulation of $1,25D_3$ production in the kidney, which enhances Ca^{2+} uptake from the intestine and calcium release from the bone. As an effect of this, serum Ca^{2+} concentration is increased again, activating the receptor and the cycle continues. This function is described in more detail elsewhere in this Research Topic (Roszko et al., 2016; Conigrave, in review).

The CaSR is expressed not only in calciotropic organs, such as the parathyroid, kidney, bone, and small intestine, but also in several organs not directly involved in maintaining calcium homeostasis. In these tissues, the CaSR is involved in a multitude of cellular processes including secretion, chemotaxis, cell–cell adhesion, and control of proliferation, differentiation, and apoptosis (Brown and MacLeod, 2001; Brennan et al., 2013).

The CaSR and the vitamin D system cooperate not only in calciotropic organs, but also in the skin (Tu and Bikle, 2013), in the colon (Canaff and Hendy, 2002; Chakrabarty et al., 2005; Aggarwal et al., 2016), and probably in other tissues as well. The cross-talk between CaSR and vitamin D in the skin is presented in detail by Bikle and colleagues in this Research Topic (Bikle et al., 2016). Therefore, this review concentrates mainly on the interdependence of CaSR and vitamin D signaling in the colon and focuses on the CaSR-mediated crosstalk between Ca^{2+} and vitamin D.

EFFECT OF VITAMIN D ON CaSR EXPRESSION

The CaSR and the vitamin D system become deregulated during tumorigenesis through different mechanisms. CaSR expression is lost in colorectal tumors (Sheinin et al., 2000; Chakrabarty et al., 2005; Fetahu et al., 2014a), mainly due to epigenetic silencing i.e., DNA hypermethylation, histone deacetylation, increased expression of the mircroRNAs miR-135b, miR-146b (Hizaki et al., 2011; Fetahu et al., 2014a, 2016). In unfavorable neuroblastomas CaSR is silenced by epigenetic and genetic mechanisms (Casala et al., 2013), while in parathyroid tumors CaSR loss is independent of DNA methylation (Varshney et al., 2013).

Shortly after the discovery of the CaSR, it was shown that on one hand, in vitamin D-depleted rats the CaSR expression was significantly reduced; on the other hand, intraperitoneal administration of $1,25D_3$ upregulated parathyroid, thyroid, and kidney CaSR expression (Canaff and Hendy, 2002). We have shown that dietary vitamin D was able to upregulate CaSR expression also in the colon of mice (Aggarwal et al., 2016). *In vitro* $1,25D_3$ increased CaSR expression in a thyroid C cell line, in the proximal tubule human kidney cells (HKC) (Canaff and Hendy, 2002), and in colon cancer cells (Chakrabarty et al., 2005; Fetahu et al., 2014b). An essential prerequisite for the direct modulation of transcription by $1,25D_3$ is the location of at least one liganded VDR protein close to the transcriptional start site (TSS) of the primary target gene. It was Canaff and her colleagues who have demonstrated that the *CaSR* gene has two functional promoters (P1 and P2), and both contain a vitamin D response element (VDRE) upstream of the TSSs (Canaff and Hendy, 2002).

Both VDREs are often methylated in colon cancer (Fetahu et al., 2014b), and the level of silencing of the CaSR varies depending on the level of DNA methylation and of histone acetylation at distinct residues. The epigenetic landscape of the CaSR promoter affects also its transcriptional and translational upregulation by $1,25D_3$ (Fetahu et al., 2014b). In two colon cancer cell lines expressing undetectable levels of CaSR 1.4 mM Ca^{2+} or 1 μM $1,25D_3$ were able to reduce CaSR promoter methylation and thus contribute to the upregulation of CaSR expression (Singh et al., 2015). Whether high dietary vitamin D and calcium would reduce or prevent methylation of the CaSR promoter also *in vivo* needs to be tested, as 1 μM concentrations of $1,25D_3$ in the tumor microenvironment would be difficult to obtain.

EFFECT OF THE CaSR ON EXPRESSION OF THE VITAMIN D SYSTEM

Although the kidney is the main source of serum $1,25D_3$ levels, the extra-renally synthesized $1,25D_3$, which acts locally in an autocrine and paracrine manner, is an indispensable source for the cancer-preventive action of vitamin D. However, during tumor development the expression of the different molecules of the vitamin D system in the affected tissue becomes deregulated. In undifferentiated colorectal adenocarcinomas not only CaSR expression, but also expression of VDR and CYP27B1 is lower than in differentiated tumors (Bareis et al., 2002; Bises et al., 2004; Giardina et al., 2015). Whether these phenomena are linked or not, needs to be determined. Nevertheless, loss of CaSR expression in an epidermis-specific CaSR knock-out mouse model led to significantly lower vdr and cyp27b1 expression in the skin compared with the wild type controls (Tu et al., 2012), suggesting that intact CaSR expression and function is needed for proper expression of the vitamin D system.

One of the causes of VDR loss in colorectal tumors is the increased expression of the transcription factor SNAIL1, one of the main regulators of the epithelial-to-mesenchymal transition (Palmer et al., 2004). Finding ways to prevent SNAIL1 upregulation would prevent VDR loss and preserve sensitivity to the anti-proliferative effects of $1,25D_3$. We were able to

show that transfection of the HT29 colon cancer cell line with the functional CaSR prevented epithelial-to-mesenchymal transition and upregulation of SNAIL1. Similar effects were seen by activating the receptor with the allosteric CaSR activator NPS-R568 (Aggarwal et al., 2015a).

In colorectal tumors the expression of the vitamin D degrading enzyme, CYP24A1 is significantly higher when compared with the adjacent normal tissue (Horvath et al., 2010). This higher expression was due, at least in part, to more copies of the *CYP24A1* gene. In these tumors, CYP24A1 expression correlated with proliferation rate of the tumors (Hobaus et al., 2013b). CYP24A1 overexpression conferred a more aggressive growth to colon cancer tumor xenografts (Hobaus et al., 2016). In human patients CYP24A1 has been suggested to be a biomarker for progression and prognosis of CRC (Sun et al., 2016). Upregulation of CYP24A1 is common in different solid tumors, such as lung, prostate, breast, cervical, ovary tumors (Anderson et al., 2006; Luo et al., 2013; Ahn et al., 2016). In these tumors, CYP24A1 reduces the half-life of $1,25D_3$, shortens the duration of the vitamin D signal, and thus reduces the anti-tumorigenic effects of the active $1,25D_3$. We have shown previously that low dietary calcium intake (0.04%) increased CYP24A1 expression in the colon of mice, while high calcium (0.9%) prevented CYP24A1 increase (Kállay et al., 2005). Whether the CaSR mediated this effect was not demonstrated, but our observation that in the colon of CaSR and PTH double knock-out mice cyp24a1 expression was higher compared with wild-type mice suggests a contribution of the CaSR.

EFFECT OF CaSR EXPRESSION AND FUNCTIONALITY ON VITAMIN D ACTION

The complexity of the cross-talk between the CaSR and the vitamin D system goes beyond affecting expression mutually. In CRC cells the anti-tumorigenic effects of $1,25D_3$ are influenced by the amount of CaSR expressed in a cell and by the activity of this receptor (Singh et al., 2013; Aggarwal et al., 2016). Liu et al. (2010) have shown that 1 μM $1,25D_3$ reduced cell number and inhibited invasion only in cells expressing the CaSR, but not in cells where the CaSR expression was down-regulated by transfection with CaSR-specific shRNA. In Caco-2 cells, transfection of a mutated CaSR harboring the inactivating mutation R185Q, abolished the anti-proliferative effect of $1,25D_3$ while the overexpression of the wild-type CaSR significantly intensified the anti-proliferative $1,25D_3$ effect. Treating the cells with the allosteric CaSR activator NPS R-568 increased the effect of 1 nM $1,25D_3$ on cell number even in the cells expressing the mutant CaSR (Aggarwal et al., 2016).

$1,25D_3$ has strong apoptosis-promoting effects, similar to Ca^{2+}. We have shown that 1 nM $1,25D_3$ effectively induced caspase 3/7 activity in colon cancer cells in the presence of 1.8 mM Ca^{2+}. This effect was significantly stronger when the cells overexpressed the wild type CaSR. NPS R-568 treatment almost doubled the effect of Ca^{2+} in the cells overexpressing the CaSR and this effect was further enhanced by $1,25D_3$. The inactivating CaSR mutant (R185Q) significantly reduced the apoptotic effect of $1,25D_3$ (**Figure 1**) (Aggarwal et al., 2016), although the mechanism is not clear. Inactivating mutations of the CaSR can cause disorders of calcium metabolism including familial hypocalciuric hypercalcemia type 1 (FHH1) and neonatal severe hyperparathyroidism (NSHPT). The impact of CaSR mutations on the colon physiology is not known. To date, no CaSR mutations have been associated with CRC risk or mortality, although there are suggestions that certain polymorphisms (including A986S, R990G, Q1011E) could be linked to CRC risk (Dong et al., 2008; Jacobs et al., 2010).

One apoptosis-inducing mechanism of $1,25D_3$ is the upregulation of the expression of the inducers of cell cycle arrest and apoptosis: the cyclin-dependent kinase inhibitors Cdkn1a and Cdkn1b (Chu et al., 2008; Karimian et al., 2016). In a colon cancer cell line the effect of $1,25D_3$ on Cdkn1a was abolished by knocking down the CaSR by CaSR-shRNA transfection. Moreover, the expression of survivin, a key anti-apoptotic protein, and the activity of the survivin promoter was inhibited

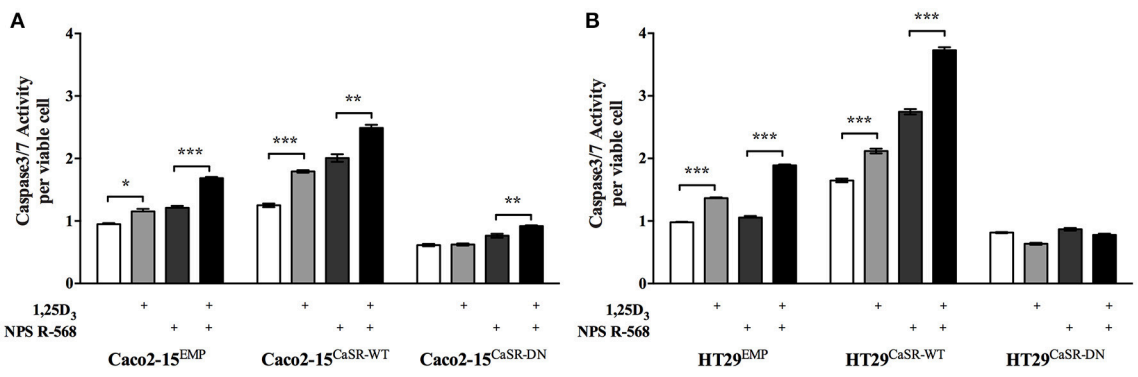

FIGURE 1 | Impact of the inactivating CaSR mutation on the apoptotic effect of $1,25D_3$ and NPS R-568 in the colon cancer cell lines Caco2-15 (A) and HT29 (B), stably transfected with wild type (CaSR-WT) or the dominant negative R185Q mutant (CaSR-DN). Cells transfected with and empty vector (EMP) were used as controls. One week post confluence, the cells were treated with $1,25D_3$ (1 nM), NPS R-568 (1 μM) or a combination of $1,25D_3$ and NPS R-568 for 48 h. Apoptosis was assessed by measuring caspase3/7 activity. Bars represent mean ± *SEM*. Statistical significance was calculated using two-way ANOVA followed by Tukey's correction. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Modified from Aggarwal et al. (2016).

by $1,25D_3$ only in cells expressing the wild-type CaSR and not in cells transfected with CaSR-shRNA (Liu et al., 2010).

Overexpression of the CaSR prevented the mesenchymal transition and the acquisition of stem cell-like properties in the HT29 colon cancer cell line (Aggarwal et al., 2015a) reducing the metastatic properties of the cells. Another mechanism by which CaSR might regulate adhesion and migration is the regulation of the activity of integrins, adhesion receptors that mediate cell migration (Tharmalingam and Hampson, 2016). Interestingly, $1,25D_3$ also affects integrin signaling, as it has been shown that $1,25D_3$ inhibited ionizing radiation-mediated upregulation of several integrins in keratinocytes (Muller et al., 2006).

Colon cancer cells transfected with CaSR express lower levels of thymidylate synthase (TS) (Liu et al., 2010), an enzyme involved in *de novo* DNA synthesis which is the target of the main colon cancer drug 5-fluoro uracil (5-FU) (Chu et al., 2003). $1,25D_3$ further inhibits TS expression and promoter activity and thus strengthens the cytotoxic 5-FU effect, however only in the cells expressing endogenous CaSR but not if the CaSR was knocked down (Liu et al., 2010). These data warrant for further studies to explore the possibility of using $1,25D_3$ and calcimimetics in supporting combinatorial anticancer therapy.

INTERPLAY BETWEEN CaSR AND $1,25D_3$ SIGNALING IN REGULATING THE Wnt PATHWAY

In a recent clinical trial, supplementation of the Western-style diet with $1,25D_3$ (0.5 μg/day) with or without 2 g calcium showed that in human colorectal mucosa $1,25D_3$ upregulated expression of multiple genes, such as those involved in inflammation and immunity, while Ca^{2+} modulated this effect (Protiva et al., 2016). The joint action of Ca^{2+} and $1,25D_3$ is due to the fact that both regulate some of the main processes involved in the development of various cancers. Among the best characterized pathways involved in many of these processes is the Wnt pathway.

Wnt proteins are secreted glycoproteins that signal by interacting with the receptors called Frizzled and Lipoprotein-related peptide 5/6 to release ß-catenin from its destruction complex. Colon cancer is regarded as a disease of defective Wnt-signaling (Gregorieff and Clevers, 2005; Clevers, 2006; Cancer Genome Atlas Network, 2012). The adenomatous polyposis coli (APC), a member of the ß-catenin destruction complex, is one of most often mutated components of the canonical Wnt-signaling (Powell et al., 1992) and leads to accumulation of ß-catenin and constitutive activation of the Wnt pathway (Behrens, 2005). The activated Wnt pathway induces proliferation by upregulating expression of target genes involved in DNA replication, cell cycling, such as cell division cycle 6 (CDC6), mini chromosome maintenance (MCM) 3/5, cyclin D1, c-myc. Both calcium and vitamin D regulate the Wnt pathway (Palmer et al., 2001; Shah et al., 2006; Cross and Kallay, 2009), usually inhibiting the canonical and activating the non-canonical Wnt pathway (Macleod, 2013).

In the intestine the CaSR expression inversely correlated with activity of the canonical Wnt/ß-catenin pathway. Loss of CaSR increased ß-catenin phosphorylation on Ser-552 and Ser-675 residues, which led to increased nuclear localization of ß-catenin in colonocytes and promoted its transcriptional activity (Rey et al., 2012). In colorectal tumors CaSR expression inversely correlated with expression of several proliferation markers, such as CDC6, MCM2, MCM5, MCM6, and these markers were also reduced in the colon of mice lacking the CaSR (Aggarwal et al., 2015b). Increasing CaSR expression in colon cancer cell lines led to reduced nuclear translocation of ß-catenin and to higher E-cadherin protein levels (Aggarwal et al., 2015a). In the CBS CRC cell line 1 mM Ca^{2+} alone, or in combination with 100 nM $1,25D_3$ inhibited the Wnt/ß-catenin pathway by upregulating E-cadherin and inhibiting T-cell factor 4 (TCF-4) expression (Chakrabarty et al., 2005). E-cadherin is a type-1 transmembrane protein that binds ß-catenin thus sequestrating it to the membrane and preventing its nuclear translocation, while TCF-4 is the transcription factor that together with ß-catenin regulates the transcription of the target genes of the Wnt pathway (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

In myofibroblasts, activation of CaSR with various agonists, such as Ca^{2+}, poly-L-Arginine, spermine, or neomycin induced secretion of the Wnt antagonists Dickkopf 1 (Dkk-1) and Wnt5a. This could be prevented by transient transfection of the cells with CaSR-specific siRNA. In colon cancer cells CaSR activation increased expression of the low density lipoprotein receptor-related protein 6 (Lpr6). Dkk-1 bound to Lpr6 upregulated E3 ubiquitin ligase expression leading to degradation of ß-catenin and thus to inhibition of Wnt signaling (MacLeod et al., 2007; Macleod, 2013). Moreover, activation of the CaSR upregulated the Wnt5a receptor, the receptor tyrosine kinase-like orphan receptor 2 (Ror-2), leading to reduced expression of the tumor necrosis alpha receptor (Kelly et al., 2011).

$1,25D_3$ regulates Wnt signaling also by multiple mechanisms, some very similar to CaSR effects. In colon cancer cells $1,25D_3$ inhibited TCF-4 transcription, upregulated expression of E-cadherin and of other adhesion proteins, and induced nuclear export of ß-catenin. The direct binding of the liganded VDR to ß-catenin inhibited its interaction with TCF-4 (Palmer et al., 2001). $1,25D_3$ was also able to upregulate DKK-1 expression in colon cancer cell lines similar to the effect of CaSR in myofibroblasts (Pendas-Franco et al., 2008).

Thus, functional CaSR and vitamin D system can reduce the impact of an overactive Wnt pathway overcoming, at least in part, the loss of APC activity.

CONCLUSIONS

This review provides molecular evidence for the interaction between the vitamin D system and the CaSR (**Figure 2**). It supports the epidemiological observation that vitamin D and calcium are both needed to protect against malignant transformation, at least in the colon and that their effect depends, at least in part from the presence of a functional CaSR.

It is feasible that in NSHPT or FHH1 patients with inactivating CaSR mutations vitamin D is less effective in regulating proliferation, differentiation, and apoptosis. These

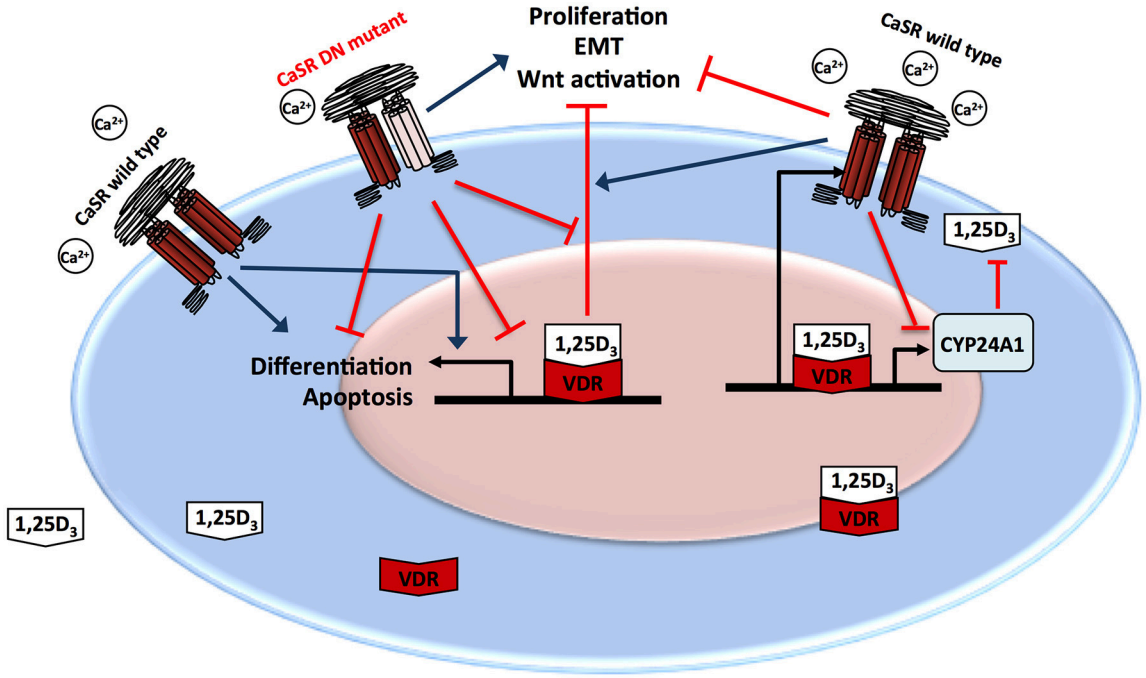

FIGURE 2 | Interactions between the vitamin D system and the CaSR. Ca/CaSR and the $1,25D_3$/VDR cross talk to protect colonic epithelial cells from malignant transformation. $1,25D_3$ is able to up regulate expression of both, CaSR and Cyp24a1. The wild type CaSR has a tumor suppressive role in the colon promoting (blue arrows) differentiation and apoptosis and suppressing (red arrows) proliferation and EMT and potentiates the tumor preventive effects of $1,25D_3$. The presence of a DN mutant CaSR abrogates the tumor preventive effects of both Ca and $1,25D_3$.

patients might therefore have a higher risk to develop cancer. The anti-proliferative effects of $1,25D_3$ should be studied also in the parathyroid of FHH1 patients. The marked parathyroid hyperplasia in FHH1 or NSHPT might be caused both by the less effective CaSR and by loss of the anti-proliferative function of $1,25D_3$ due to mutated CaSR.

AUTHOR CONTRIBUTIONS

EK and AA have written the manuscript. AA has made the figures.

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frontiers in Physiology

MINI REVIEW
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Calcium-Sensing Receptor: A Key Target for Extracellular Calcium Signaling in Neurons

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Though both clinicians and scientists have long recognized the influence of extracellular calcium on the function of muscle and nervous tissue, recent insights reveal that the mechanisms allowing changes in extracellular calcium to alter cellular excitability have been incompletely understood. For many years the effects of calcium on neuronal signaling were explained only in terms of calcium entry through voltage-gated calcium channels and biophysical charge screening. More recently however, it has been recognized that the calcium-sensing receptor is prevalent in the nervous system and regulates synaptic transmission and neuronal activity via multiple signaling pathways. Here we review the multiplicity of mechanisms by which changes in extracellular calcium alter neuronal signaling and propose that multiple mechanisms are required to describe the full range of experimental observations.

Keywords: calcium sensing receptor, nervous system, synaptic transmission, action potentials, ion channels, calcium, excitability

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CALCIUM AND EXCITABLE TISSUES

The importance of extracellular calcium in regulating the behavior of excitable tissues was first recognized by Sydney Ringer when he became aware that a very effective physiological saline he developed was contaminated with calcium (Ringer, 1883). Upon this discovery, Ringer quickly determined that calcium at a concentration of approximately 1 mM was essential to maintain the viability and function of isolated frog hearts and solutions derived from Ringer's work have been employed by physiologists studying the heart and many other organ systems ever since (Miller, 2004). It was some time before the importance of calcium on neuronal excitability was recognized, but despite more than 100 years of inquiry, the mechanisms by which calcium alters the excitability of neurons remain incompletely elucidated. In this mini-review we will examine some of the mechanisms by which extracellular calcium influences neuronal signaling by altering both intrinsic excitability and synaptic transmission.

EXTRACELLULAR CALCIUM IN THE BRAIN IS DYNAMIC

The distribution of calcium in the brain is characterized by steep transmembrane electrochemical gradients that are transiently attenuated as a result of large activity-dependent changes in both the intracellular and extracellular calcium concentration. The blood brain barrier defends extracellular calcium in the brain from changes in serum calcium (Jones and Keep, 1988) and at rest brain extracellular calcium is maintained at 1.1 mM (Hansen, 1985; Zhang et al., 1990; Nilsson et al., 1993, 1996). At the same time, neuronal intracellular calcium is orders of magnitude lower ranging

between 50 and 100 nM, but rising rapidly to 10–100 μM in microdomains near open voltage-gated calcium channels when action potentials invade presynaptic terminals (Zucker, 1996). In contrast, during neuronal activity extracellular calcium can fall sharply as calcium is displaced to the intracellular compartment. These transient drops in calcium are facilitated by the small extracellular volume of the brain (only 12–20% of total volume; Rusakov et al., 1998) and restricted diffusion (up to five-fold slower than in free solution; Kullmann et al., 1999). The small volume and limited accessibility of the synaptic cleft led to the prediction that pre- and postsynaptic calcium influx during neurotransmission will significantly reduce calcium in the cleft following an action potential, possibly to as low as 0.3 mM (Smith, 1992; Vassilev et al., 1997b; Egelman and Montague, 1998, 1999; Rusakov et al., 1998). At the surface of the brain, ion-selective electrodes have shown that extracellular calciumfalls to 0.8 mM for tens of seconds following focal stimulation at rates of 20 Hz (Nicholson et al., 1978) and decreases to 0.1 mM have been recorded as a result of focal brain trauma (Nilsson et al., 1996). Importantly, it has been shown that short trains of action potentials can reduce extracellular calcium and impact synaptic transmission (Rusakov and Fine, 2003). The observed fall in extracellular calcium in the cortex at times of high activity (Nicholson et al., 1978) along with the extreme sensitivity of synaptic mechanism to extracellular calcium (Dodge and Rahamimoff, 1967) prompts us to ask how neurons respond to this change.

EXTRACELLULAR CALCIUM AND THE INTRINSIC EXCITABILITY OF NEURONS

Even before calcium was identified as the trigger for exocytosis, reductions in extracellular calcium were known to increase the likelihood of action potential initiation (Frankenhaeuser and Hodgkin, 1957) by altering the properties of the neuronal ion channels. These effects on ion-channel activity can be divided into two major categories: those mediated by direct activity of calcium on ion-channel biophysics and those mediated indirectly by second messenger systems that are coupled to extracellular calcium concentrations by specific receptors. While many types of ion channels are involved in fine tuning neuronal excitability, voltage-gated sodium channels are central. Early work showed changes in excitability were mediated by shifts in the activation properties of the sodium conductance (Frankenhaeuser and Hodgkin, 1957) though subsequently extracellular calcium was found to alter the activity of other types of ion channels (Hablitz et al., 1986; Immke and McCleskey, 2001; Ma et al., 2012).

Direct Actions Of Calcium on Neuronal Excitability

The most widely recognized model for the impact of calcium on sodium channel activity is surface charge screening (aka, surface potential theory), whereby interactions between multivalent cations like calcium and negatively charged phospholipids in the neuronal membrane serve to alter the intramembrane electric field that regulates the activity of voltage-gated ion channels in a concentration dependent manner (Hille, 2001). The idea is that membrane bound negative charges influence the local potential (**Figure 1A**, solid blue curve) and thereby reduce the intramembranous electric field (broken blue line) established by the transmembrane electrochemical gradient (black broken line). Extracellular divalents are adsorbed to the membranous negative charges and attenuate their impact on the existing voltage field (red broken line). Hence voltage-dependent channels within the intramembranous electric field have altered activity. In the case of a high extracellular calcium concentration, the intramembranous field is increased and the probability of channels being activated is decreased resulting in a reduction in excitability.

However, surface charge screening does not account for all of the calcium-dependent gating phenomena exhibited by voltage-gated channels. At its simplest the surface potential theory predicts a uniform action on all types of voltage-dependent channels. While sodium channel activation is enhanced by reductions in extracellular calcium other types of voltage-gate ion-channels exhibit different dependence on extracellular calcium, ranging from sensitive to indifferent (Han et al., 2015). Beyond charge screening, other work has identified at least two other distinct biophysical mechanisms through which changes in extracellular calcium can alter the activity of voltage-gated sodium channels (Armstrong and Cota, 1991). Voltage-gated sodium channels have a number of extracellular moieties that also interact directly with calcium and so alter channel kinetics through changes in conformation or stability (Armstrong and Cota, 1990). Also, calcium ions are able to directly block sodium channels likely through interactions with specific amino acid residues lining the channel's pore (Santarelli et al., 2007).

Direct activation of non-selective cation channels following reductions in extracellular calcium, also depolarizes neurons and increases excitability (Hablitz et al., 1986; Xiong et al., 1997; Immke and McCleskey, 2001). Calcium homeostasis modulator 1 (CALHM1) is another non-selective cation channel that is both voltage- and calcium-dependent (Ma et al., 2012) positioning it to be a strong mediator of calcium-dependent excitability. In fact, neurons deficient in CALHM1 lost all calcium-dependent excitability (Ma et al., 2012) implying that in some neurons surface charge screening may not contribute to calcium-dependent excitability.

Receptor-Mediated Signaling of Extracellular Calcium

The excitability of neurons is also influenced indirectly by complex second messenger systems coupled to membrane receptors (**Figure 1B**). The calcium-sensing receptor (CaSR), a widely expressed G-protein coupled receptor, exhibits a punctate staining pattern in the cortex and cerebellum consistent with localization to nerve terminals (Ruat et al., 1995). Direct recordings from neocortical terminals, demonstrated that extracellular calcium regulated a membrane receptor and indirectly modulated a non-selective cation channel (Smith et al., 2004). Using a combination of pharmacological probes and a mutant mouse the terminal extracellular calcium receptor was identified as CaSR (Chen et al., 2010). In hippocampal

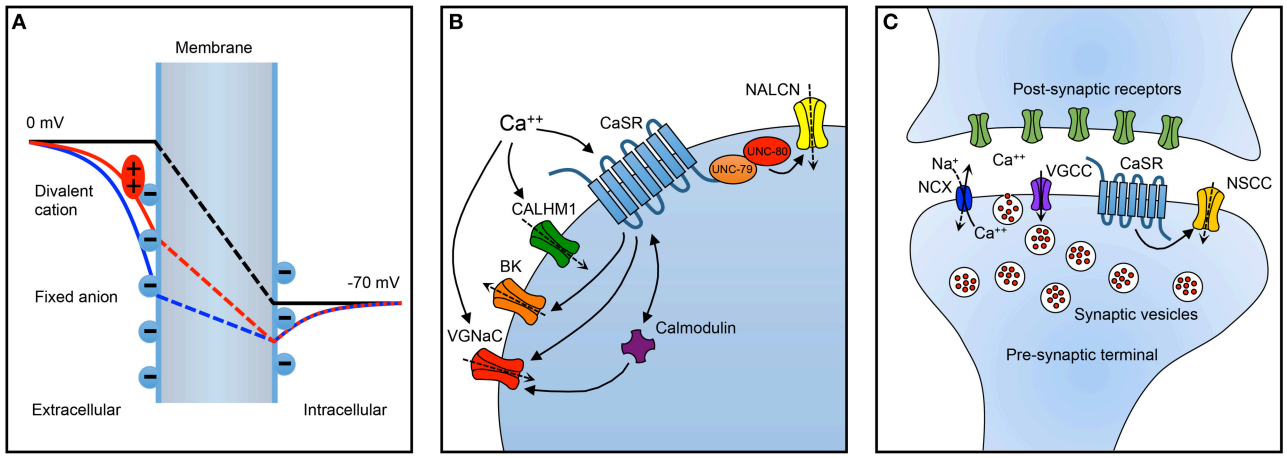

FIGURE 1 | Summary of important neuronal targets of extracellular calcium. (A) Impact on resting membrane electric field of surface charge screening. The transmembrane potential is illustrated in three scenarios: without fixed anions (black line), with fixed anions (blue line), and with fixed anions and divalent cations (red line). The electric field produced by the transmembrane electrochemical gradient alone (black line) is attenuated by membrane associated negative charges in the absence of divalent cations (blue line). When divalent cations interact with (screen/adsorp to) the fixed anions, the influence of the fixed charges on intramembranous electric field is reduced (red line). Consequently, the activity of ion channels within the membrane and sensitive to the electric field may be altered by changes in the concentration of divalent cations. **(B)** Summary of targets of extracellular calcium on neuronal excitability and **(C)** synaptic transmission. BK, calcium-activated potassium channel; CALHM1, calcium homeostasis modulator 1; CaSR, calcium-sensing receptor; NALCN, Na-leak channel non-selective; NCX, sodium/calcium exchanger; NSCC, non-specific cation channel; VGCC, voltage-gated calcium channel; VGNaC, voltage-gated sodium channel. Dashed arrows reflect direction of current under typical conditions. Inwards arrows are depolarizing/excitatory. During high activity levels, the NCX replenishes extracellular calcium in the synaptic cleft.

neurons reductions in extracellular calcium increased neuronal excitability via another indirect mechanism. A non-selective cation channel (NSCC) NALCN (Na-Leak Channel Non-selective), was activated by decreases in extracellular calcium and mediated the vast majority of calcium-dependent excitability (Lu et al., 2010). This signaling pathway required two intracellular proteins, UNC-79 and UNC-80, and an unidentified membranous receptor (Lu et al., 2010). The authors went on to hypothesize that CaSR may be the receptor that detected and transduced changes in extracellular calcium into changes in neuronal excitability. In this model, low extracellular calcium was transduced into activation of a depolarizing current mediated by NALCN and increased neuronal excitability, controversially minimizing the contribution of surface charge screening (Lu et al., 2010). Other consequences of extracellular calcium signaling are suggested by work showing that CaSR may inhibit some neuronal potassium channels (Vysotskaya et al., 2014). Interestingly, CaSR activation was also proposed to activate other types of neuronal potassium channels (Vassilev et al., 1997a). Similarly, an unusually non-selective channel in neuronal soma was reported to be activated by CaSR agonists (Ye et al., 1996). The impact of decreased extracellular calcium on CaSR modulation seems to favor channel activation but the overall effect will depend on the balance of channel activation and block.

Intracellular changes in calcium, as a result of changes in extracellular calcium, may modulate channel activity and neuronal excitability. Calmodulin, a calcium sensitive signaling protein that is modulated by calcium entry, regulated sodium channel activity (Kim et al., 2004). Specifically, calmodulin interacted with an intracellular domain of voltage-gated sodium channels and so modified their gating behaviors (Sarhan et al., 2012). Notably, calmodulin has also been shown to regulate the cell surface expression and signaling from the CaSR providing a potential mechanism for cross-talk between these distinct calcium signaling pathways in the modulation of neuronal excitability (Huang et al., 2010). Thus, there are multiple direct and indirect mechanisms by which extracellular calcium can impact the intrinsic excitability of neurons and while surface charge theory provides a common mechanism across all neurons it would be surprising if these other mechanisms did not operate in parallel and mediate variability in calcium dependent excitability between neuronal types.

EXTRACELLULAR CALCIUM AND SYNAPTIC FUNCTION

Calcium is a Key Determinant of Synaptic Efficacy

Calcium is an important signal on both the pre- and post-synaptic sides of the synapse where it triggers exocytosis (Douglas, 1968; Katz, 1969), plasticity (Lynch et al., 1983; Malenka et al., 1988; Bliss and Collingridge, 1993) and alters gene expression (Greenberg et al., 1992). The early and reproducible observation that synaptic efficacy is dependent on the fourth power of extracellular calcium highlights the importance of calcium in the exocytotic process and has been confirmed in a number of preparations (Dodge and Rahamimoff, 1967; Dudel, 1981; Augustine and Charlton, 1986; Zucker et al., 1991; Bollmann et al., 2000; Schneggenburger and Neher, 2000). Calcium activates the exocytotic machinery after entry

through N-, P/Q-, and R- type voltage-activated calcium channels (Wheeler et al., 1994; Jun et al., 1999; Wu et al., 1999; Rozov et al., 2001). Numerous forms of synaptic plasticity have been described with varied rates of onset and durations lasting from milliseconds to hours (Katz and Miledi, 1969; Lynch et al., 1983; Malenka et al., 1988; Bliss and Collingridge, 1993; Zucker, 1993; Fisher et al., 1997; DeMaria et al., 2001; Kreitzer and Regehr, 2001; Rozov et al., 2001), all of which are affected by cleft calcium emphasizing its important regulatory role on synaptic function.

The Impact of Falls in Cleft Calcium

The broad dynamic range of extracellular calcium along with the exceedingly steep dependence of synaptic release probability on extracellular calcium (Dodge and Rahamimoff, 1967) leads to the hypothesis that even modest falls in cleft calcium will render the synapse much less effective at conducting signals. Indeed, a widely observed fourth-order proportionality implies that a reduction of the cleft calcium by one third could reduce synaptic efficacy by up to 80%. Accordingly, maneuvers reducing cleft calcium reduce synaptic efficacy (Borst and Sakmann, 1999a). Nevertheless, sustained phasic synaptic transmission has been observed at rates of up to 800 Hz (Taschenberger and von Gersdorff, 2000), indicating that either falls in cleft calcium do not occur at all synapses or there are compensatory mechanisms to reduce the effect of the fall of extracellular calcium at the synaptic cleft. The mechanism by which reductions in extracellular calciumreduce release probability and potential compensatory mechanisms remain incompletely understood, but similar to the impact of extracellular calcium on synaptic transmission can be divided into direct biophysical mechanisms and indirect mechanisms mediated by second messenger systems.

Direct Compensatory Mechanisms

Dissociation of calcium from negative charged macromolecules, release from synaptic vesicles, and extracellular cation exchangers have been proposed to attenuate the fall in cleft calcium during episodes of high activity (Grohovaz et al., 1996; Borst and Sakmann, 1999a; Hartig et al., 2001), but the functional impact is uncertain. Similar to its impact on overall neuronal excitability, at the terminal reduced calcium is predicted to left-shift the voltage-dependence of sodium and calcium channels increasing the probability of release. Another putative, but incompletely understood compensatory mechanism observed at the calyx of Held and hippocampal nerve terminals is the broadening of presynaptic action potentials with repeated stimulation (Borst and Sakmann, 1999b; Geiger and Jonas, 2000). As calcium entry occurs during the repolarization phase of an action potential, spike broadening is a highly effective way of increasing calcium entry by prolonging depolarization (Sabatini and Regehr, 1997). Ion exchangers may also provide a mechanism to sustain synaptic transmission during periods of high activity. In parallel fiber-to-Purkinje neuron synapses, transient reversal of the sodium/calcium exchanger promotes calcium influx and enhanced glutamatergic transmission (Roome et al., 2013).

Indirect Compensatory Mechanisms

There is considerable evidence that the CaSR is intimately involved with regulating synaptic transmission **Figure 1C**. The CaSR is present in 80–90% of nerve terminals in the cerebral cortex (Smith et al., 2004; Chen et al., 2010) and its impact on synaptic transmission is complex indicating that it may be mediated by several mechanisms. In acutely isolated neocortical nerve terminals, decreases in extracellular calcium activated voltage-dependent NSCC currents indirectly via the CaSR (Smith et al., 2004; Phillips et al., 2008; Chen et al., 2010). Theoretically, NSCC activation at the nerve terminal following decreased CaSR activation (Smith et al., 2004) may depolarize the local membrane potential, inactivate voltage-dependent calcium channels, and thereby reduce the probability of evoked release. However, the voltage-dependence of the terminal NSCC means very few of the NSCCs would be activated at negative potentials making this unlikely to be a major effect. Another possibility is that NSCC activity following reduced CaSR activation could lead to action potential broadening which might prolong the duration of calcium entry and facilitate synaptic transmission. The absence of delay of activation of NSCC currents following rapid depolarizations (sub millisecond) and the ability of action potential waveforms to trigger these currents supported this hypothesis (Smith et al., 2004). Consistent with this idea, CaSR activation reduced excitatory transmission between pairs of neocortical neurons (Phillips et al., 2008). Furthermore, deletion of CaSR substantially increased excitatory synaptic transmission in neocortical neurons, and variance-mean analysis indicated this was due to an increase in release probability (Phillips et al., 2008). Thus, CaSR-NSCC signaling in nerve terminals would seem ideally placed to serve to increase release probability in situations where extracellular calcium was low thereby maintaining the fidelity of synaptic transmission during periods of high activity. However, although the NSCC currents were rapidly activated and likely to influence action potential shape the CaSR is a GPCR and unlikely to respond rapidly. Indeed in isolated terminals the pathway took a few seconds to respond to changes in extracellular calcium. These relatively slow kinetics indicate the CaSR-NSCC signaling pathway in terminals is more likely to detect and respond to sustained changes in calcium that persist for a few seconds and not those that develop over a few milliseconds (Smith et al., 2004; Chen et al., 2010). Endogenous modulators of CaSR in the periphery include magnesium, L-amino acids, polyamines, and γ-glutamyl peptides besides calcium (Leach et al., 2015). It remains unclear how much these agents modulate signaling in neurons but identification of central actions may reveal other physiological roles for CaSR in neurons as suggested for beta-amyloid (Conley et al., 2009).

Increasing attention has turned to spontaneous release of neurotransmitters with the recognition that action potential-evoked and spontaneous release mechanisms are distinct (Kavalali, 2015). Interestingly, CaSR activation by direct and allosteric agonists stimulate release of glutamate independent of intracellular calcium (Vyleta and Smith, 2011). In addition, deletion of CaSR substantially reduced spontaneous glutamate release. In other words, CaSR activation had opposite effects

on evoked and spontaneous release of the major excitatory neurotransmitter (Phillips et al., 2008; Vyleta and Smith, 2011). It is as yet unclear how CaSR could have opposite effects on exocytosis of these apparently distinct populations of vesicles that reside in the same nerve terminals. However, we recognize that these apparently opposite actions mechanistically mirror the actions that CaSR stimulation has on release of parathyroid hormone and calcitonin (Garrett et al., 1995). The importance of CaSR signaling at nerve terminals has also been emphasized by the finding that spontaneous release of GABA, the major inhibitory neurotransmitter, is also strongly enhanced by CaSR activation (Smith et al., 2012).

Given the apparent abundance of CaSR it seems surprising that a role for CaSR was not suggested sooner. However, CaSR signaling may have been difficult to detect because "physiological" experiments frequently employed supraphysiological levels of calcium and magnesium concentrations (Smith et al., 2004; Chen et al., 2010). This approach ensured CaSR was at near-saturation attenuated our ability to detect changes in CaSR signaling. Another confounder is that in studies of the effects of decreased extracellular calcium, magnesium concentrations were often increased with the presumption that magnesium would only obviate the effects of surface charge screening. Since CaSR and spontaneous release are stimulated by magnesium (Vyleta and Smith, 2011; Smith et al., 2012) this experimental approach minimized the contribution of CaSR. The importance of employing physiological concentrations of divalent ions was emphasized by comparing neuronal activity and synaptic transmission *in vivo* and in acute brain slices (Sanchez-Vives and McCormick, 2000; Lorteije et al., 2009).

CLINICAL RELEVANCE IN THE NERVOUS SYSTEM

Over the past decade a number of reports have underlined the potential of CaSR as a therapeutic target in diseases of the nervous system. Familial idiopathic epilepsy was linked to dominantly inherited CaSR mutations across three generations (Kapoor et al., 2008). The signaling pathways by which changes in CaSR activity might relate to epilepsy are not known, but the evidence implicating the CaSR in neuronal excitability and maintenance of high-frequency synaptic transmission suggests a plausible mechanism by which changes in CaSR activity could underpin a disorder of neuronal activity. In parallel, CaSR levels have been found to be increased in animal models following induction of seizures as well as traumatic brain injury (Mudo et al., 2009; Kim et al., 2011) hinting at a potential role for CaSR in the development of epilepsy following status epilepticus or traumatic brain injury. Intriguingly, CaSR antagonists were shown to reduce CaSR expression levels, brain tissue loss and neurological deficits, in animal models of traumatic brain injury and cerebral ischemia (Kim et al., 2013, 2014). Furthermore, links between beta amyloid and CaSR signaling may be important in the development of Alzheimer's disease and hypoxic brain injury (Bai et al., 2015; Dal Pra et al., 2015).

CONCLUSIONS

Extracellular calcium ions are recognized, like intracellular calcium ions, as important regulators of neuronal function in the central and peripheral nervous systems. The action of extracellular calcium is complex and its actions via CaSR and surface charge screening affect numerous ion channels impacting neuronal excitability and many forms of synaptic transmission. An important goal for the field is to determine the relative contributions of these signaling pathways to neuronal function to facilitate our understanding behind the role of CaSR signaling in pathogenesis of acute neurological diseases like stroke, traumatic brain injury, and epilepsy.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Regulation of Differentiation by Calcium-Sensing Receptor in Normal and Tumoral Developing Nervous System

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During normal development of the nervous system (NS), neural progenitor cells (NPCs) produce specialized populations of neurons and glial cells upon cell fate restriction and terminal differentiation. These sequential processes require the dynamic regulation of thousands of genes. The calcium-sensing receptor (CaSR) is temporally and spatially regulated in both neurons and glial cells during development of the NS. In particular, CaSR expression and function have been shown to play a significant role during differentiation of NPCs toward the oligodendrocyte lineage and also in maturation of cerebellar granule cell precursors (GCPs). Moreover, CaSR regulates axonal and dendritic growth in both central and peripheral nervous systems (PNSs), a process necessary for proper construction of mature neuronal networks. On the other hand, several lines of evidence support a role for CaSR in promotion of cell differentiation and inhibition of proliferation in neuroblastoma, a tumor arising from precursor cells of developing PNS. Thus, among the variety of NS functions in which the CaSR participates, this mini-review focuses on its role in differentiation of normal and tumoral cells. Current knowledge of the mechanisms responsible for CaSR regulation and function in these contexts is also discussed, together with the therapeutic opportunities provided by CaSR allosteric modulators.

Keywords: development, nervous system, neuroblastoma, calcium-sensing receptor, differentiation, differentiation and proliferation

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The calcium-sensing receptor (CaSR), a G protein-coupled receptor (GPCR) whose primary ligand is calcium, was initially identified in the parathyroid gland, where it regulates calcium homeostasis (Brown et al., 1993). Subsequent studies found that this GPCR is present in many other organs (Riccardi and Kemp, 2012) and in some cancers (Brennan et al., 2013), where it plays versatile roles. CaSR also participates in a wide range of cellular functions that are important for proper development of the nervous system (NS; Bandyopadhyay et al., 2010; Ruat and Traiffort, 2013), and any alterations in its expression and/or function may lead to disease, including tumors.

During development, cell fate restriction occurs in the NS where specialized neuronal and glial populations arise from neural progenitor cells (NPCs). This is achieved without changes of DNA sequence through the coordinated regulation of gene expression promoted

by cell-intrinsic programs, such as epigenetic mechanisms and transcription factors, as well as extracellular cues. Increasing evidence indicates that proper CaSR expression and function is important during differentiation of specific neural precursor cells upon commitment toward neuronal and glial fates. CaSR also plays significant roles during differentiation of neuroblastoma, a developmental tumor of the peripheral nervous system (PNS). Therefore, among many other functions of the NS in which it participates (Bandyopadhyay et al., 2010; Ruat and Traiffort, 2013), this mini-review focuses on the role of CaSR in differentiation, a cellular process that is crucial for both normal development and tumor biology.

ROLE OF CaSR IN DIFFERENTIATION OF NORMAL DEVELOPING NERVOUS SYSTEM

The rat *Casr* gene was cloned from a striatal cDNA library by homology screening (Ruat et al., 1995). Subsequent expression analyses identified its presence in almost all regions of the central nervous system (CNS) including hypothalamus, striatum, hippocampus, pituitary, cerebellum, brainstem, circumventricular organs, and spinal cord (Ruat et al., 1995; Chattopadhyay et al., 1997; Rogers et al., 1997).

The CNS is composed of neurons and three major populations of glial cells, astrocytes, oligodendrocytes, and microglia. Although, expression of CaSR has been reported in astrocytes (Chattopadhyay et al., 2000; Dal Pra et al., 2005) and microglia (Chattopadhyay et al., 1999a), the CaSR was first localized to nerve terminals of neurons and fiber tracts (Ruat et al., 1995). Nerve tracts consist of axons wrapped by myelin sheaths which are produced by oligodendrocytes in the CNS and by Schwann cells in the PNS. In order to be able to produce myelin, oligodendrocyes precursor cells (OPCs) progress through a series of differentiation steps, lose their capacity to proliferate and migrate, and finally generate mature oligodendrocytes (Barateiro and Fernandes, 2014).

Several lines of evidence support a role for CaSR in this differentiation process. First, a period of increased CaSR expression was identified during rat postnatal development in myelinated structures (Chattopadhyay et al., 1998; Ferry et al., 2000). By double *in situ* hybridization, *Casr* and myelin basic protein (*Mbp*) mRNAs were shown to co-localize in cerebellum, brainstem, corpus callosum, fimbria of the hippocampus, *stria medullaris,* and lateral olfactory tracts during myelogenesis. Second, Northern blot and reverse transcription polymerase chain reaction (RT-PCR) confirmed *Casr* mRNA expression in purified oligodendrocytes. Moreover, exposure to high Ca_o^{2+} and calcimimetic NPS R-568 resulted in phosphatidylinositol hydrolysis and intracellular Ca^{2+} (Ca_i^{2+}) mobilization (Chattopadhyay et al., 1998; Ferry et al., 2000). Altogether, these data indicate that a functional CaSR is present in olygodendrocytes and temporally regulated during OPCs differentiation.

The role of CaSR in the transition of neural precursor cells toward the oligodendrocyte lineage has also been established (Chattopadhyay et al., 2008). To this end, neural stem cells were isolated from fetal rat brains and induced to commit to neuronal, oligodendrocyte, or astrocytic lineages. *Casr* expression increased in OPCs, remained high during the premyelinating stage and declined in mature oligodendrocytes. Furthermore, *Mbp* mRNA levels increased in OPCs exposed to high Ca_o^{2+} or spermidine for 1–3 days. This phenotype was blocked by overexpression of a naturally-occurring dominant-negative CaSR variant p.Arg185Gln (Bai et al., 1997). Furthermore, *Mbp* mRNA levels were significantly reduced in the cerebellum of 2-week old *Casr*-null ($Casr^{-/-}$) mice as compared to age-matched $Casr^{+/+}$ mice. Altogether, these results indicate that prolonged CaSR activation promotes maturation of OPCs. However, a brief exposure to high Ca_o^{2+} induced OPCs proliferation, suggesting that acute and long-term activation of CaSR differentially affects cell proliferation and differentiation (Chattopadhyay et al., 2008).

Studies conducted in $Casr^{-/-}$ mice (Liu et al., 2013), a mouse model of human neonatal severe hyperparathyroidism (Ho et al., 1995), have provided direct *in vivo* evidence for CaSR roles during differentiation of CNS. In these mice, both brain weight and size were reported to be lower than that of wild-type littermates during the first 2 weeks of postnatal development. Small brain size was associated with a reduced number of cells and proliferation rates, but deletion of the parathyroid hormone (*Pth*) gene, which corrects hyperparathyroidism, hypercalcemia, and hypophosphatemia, normalized these alterations. Interestingly, decreased expression of neuronal (neuronal nuclear antigen, NeuN) and glial (glial fibrillary acidic protein and MBP) differentiation markers were detected in these brains, and levels of expression were not normalized upon deletion of the *Pth* gene, thus suggesting that CaSR is necessary for differentiation of neural progenitors toward neuronal and glial fates, but not for their proliferation.

More recently, the role of CaSR has also been evaluated in the developing cerebellum (Tharmalingam et al., 2016) during a period that includes initial proliferation of granule cell precursors (GCPs) in the external granule cell layer (EGL) followed by differentiation and cell cycle exit. At later stages, differentiated GCPs migrate within the EGL, a process called tangential migration, and then toward the internal granule cell layer (IGL) by radial migration. Immunoblots showed high rat Casr protein expression in the cerebellum from P7 to P18, a period during which maximal GCPs migration occurs. Moreover, CaSR allosteric activators NPS R-568 and R-467 increased GCPs migration *in vitro*, and these effects were blocked by calcilytic NPS 2143 (Bandyopadhyay et al., 2010). Also, calcimimetics promoted increased radial migration of GCPs from the EGL into the IGL. Specificity of this phenotype was corroborated by experiments conducted with NPS 2143. Interestingly, the number of cells positive for NeuN was higher in rats treated with CaSR allosteric activators and reduced in those receiving NPS 2143 when compared to controls. Moreover, rats exposed to the calcilytic also showed significantly increased numbers of Ki67-positive GCPs, a nuclear marker of cell proliferation. Together, these studies argue that CaSR expression and function are necessary for proper migration and differentiation of GCPs.

While several studies have analyzed the expression and function of CaSR in the CNS, much less attention has been devoted to the role of this receptor in the PNS. Several lines of evidence support that CaSR expression and function during normal formation of the PNS are critical for axonal and dendritic growth. Proper regulation of axon growth and branching are crucial for constructing functional, mature neuronal networks. These processes are regulated by extracellular cues, growth factors, and morphogens that signal through receptors, activate intracellular signaling cascades and regulate cytoskeletal dynamics (Kalil and Dent, 2014). Compelling data reported by Vizard et al. (2008) support that CaSR would be among receptors that integrate extracellular signals during axon growth and branching. These authors showed that a brief period of increased *Casr* mRNA expression occurs in mouse neurons of the superior cervical ganglion (SCG) from embryonic day 16 (E16) until E18, a time when murine sympathetic axon are branching at their targets. They functionally showed that neurons at this peak of *Casr* expression display enhanced axonal growth when exposed to high Ca_o^{2+} and calcimimetic NPS R-467, whereas this output is blunted by blocking CaSR function by either calcilytic NPS 89636, *Casr* deletion, or overexpression of a dominant-negative CaSR (Bai et al., 1997). Also, a significant reduction in the iris sympathetic innervation density was shown in $Casr^{-/-}$ mice. Furthermore, a similar role was demonstrated in hippocampal pyramidal neurons, thus providing evidence for a CaSR role in neuronal growth and branching in both PNS and CNS.

ROLE OF CaSR IN DIFFERENTIATION OF TUMORAL DEVELOPING NERVOUS SYSTEM

Childhood solid tumors have been recognized as a group of cancers significantly different from adult neoplasias (Scotting et al., 2005; Marshall et al., 2014). They arise from precursor cells during organogenesis and retain many of the morphological and biological features of their undifferentiated, highly proliferative, and sometimes migratory normal cells of origin.

Neuroblastomas originate from PNS precursor cells (Cheung and Dyer, 2013) which in turn derive from trunk neural crest cells. This is a transient population of embryonic cells (LeDouarin, 1982) that generate several derivatives including neurons and glia of the sympathetic NS (Bronner and LeDouarin, 2012). This process involves a period of cellular proliferation, followed by delamination, migration, specification, and terminal differentiation. To produce glial cells and neurons, a portion of trunk neural crest cells migrate along a ventral pathway (Henion and Weston, 1997). Several environmental cues contribute to their fate restriction and, upon terminal differentiation, they give rise to the sympathetic ganglia and medullary region of the adrenal gland (Anderson and Axel, 1986; Anderson et al., 1991).

The potential origin of neuroblastomas in neural crest precursor cells "blocked" at different stages of this process, as well as a combination of various molecular and genetic events, is thought to underlie the heterogeneity of this group of tumors that include both benign and malignant cases (Brodeur, 2003; Maris, 2010; van Noesel, 2012). The most relevant factors associated with these different clinical behaviors of neuroblastomas are age at diagnosis, clinical stage, *MYCN* amplification, alterations of ploidy, numerical and structural chromosomal abnormalities, and histological degree of differentiation (Ambros et al., 1996; Bown et al., 1999; Janoueix-Lerosey et al., 2008; Molenaar et al., 2012; Cheung and Dyer, 2013; Pugh et al., 2013).

The first genetic alteration to be described in neuroblastoma was the amplification of the oncogene *MYCN* (Schwab et al., 1983). It is present in only 22% of these tumors but it is the most significant genetic predictor of poor outcome (Brodeur et al., 1984). MYCN is part of the basic helix-loop-helix family of transcription factors that also includes MYC (c-MYC) and MYCL (Zimmerman et al., 1986; Gustafson and Weiss, 2010). MYCN is a critical promoter of cell proliferation while inhibiting differentiation and apoptosis in early post-migratory neural crest cells and also during CNS neurogenesis (Knoepfler et al., 2002). This is achieved by a complex network of interactions with other transcription factors and epigenetic mechanisms that cooperate to regulate a wide array of genes (Huang and Weiss, 2013). Intriguingly, MYC, which exhibits some structural and functional similarities with MYCN, is an important transcriptional regulator in the transition from proliferating to differentiating OPCs (Magri et al., 2014).

Neuroblastic tumors are composed of two main cellular components: neuroblasts, of neuronal origin, and glial, Schwannian-like cells. Classifications based on the degree of neuroblasts maturation and the extent of the glial component showed that differentiated tumors were associated with good clinical outcome (Hughes et al., 1974; Shimada et al., 1999, 2001). A variety of proteins participate in the differentiation processes of neuroblastomas (reviewed in Mohlin et al., 2011). Among them, the CaSR was found to be highly expressed in differentiated neuroblastic tumors and up-regulated upon differentiation induction (de Torres et al., 2009). CaSR was previously identified in adult CNS tumors (Chattopadhyay et al., 1999b, 2000), but it had not been reported in any developmental malignancy. In neuroblastoma, *CaSR* mRNA expression significantly correlated with several factors associated with good outcome such as age at diagnosis <1 year, low clinical stage and differentiated histology. Immunohistochemistry showed that undifferentiated neuroblasts were mostly CaSR-negative while even the earliest stages of neuroblast differentiation displayed CaSR immunostaining. When present, glial cells were also strongly positive for CaSR. Moreover, upon neuroblastoma differentiation induction, increased CaSR expression was seen both in clinical specimens obtained after treatment, and *in vitro*, at early phases of neuronal differentiation induced by retinoic acid.

In accordance with these data, *CASR* gene silencing by epigenetic mechanisms was found in undifferentiated, *MYCN*-amplified, aggressive neuroblastomas (Casalà et al., 2013). These mechanisms included promoter 2 hypermethylation and histone modifications. CpG islands are clusters of GC dinucleotides located in promoter regions, and also in intragenic regions, that are usually unmethylated (Deaton and Bird,

2011). Methylation of the fifth position of cytosines at CpG islands around promoter regions is a mechanism of gene silencing that inactivates tumor-suppressor genes (Herman et al., 1996; Timp and Feinberg, 2013) and also contributes to gene modulation during cell type specification and lineage commitment (Hirabayashi and Gotoh, 2010; Hu et al., 2014). A particular region of the CpG island encompassing *CASR* gene promoter 2 was found to be hypermethylated in 25% primary neuroblastomas, in association with reduced *CaSR* mRNA expression, *MYCN* amplification, undifferentiated histopathology and other factors of poor outcome. In neuroblastoma cell lines, treatment with demethylating agent 5′aza-2-deoxycitidine and/or histone deacetylase inhibitor trichostatin A decreased the percentage of methylated cytosines in this specific region of *CASR* gene promoter and concomitantly restored *CaSR* expression in *MYCN*-amplified cell lines. Association of *MYCN* amplification and epigenetic silencing of several genes in neuroblastoma had been previously reported (Alaminos et al., 2004), although the underlying mechanisms are still under investigation (Perini et al., 2005; Hervouet et al., 2009; Murphy et al., 2009, 2011; He et al., 2013). In addition, monosomy of chromosome 3, where the human *CASR* gene resides, was observed in >90% of primary neuroblastic tumors of all subgroups by interphase fluorescence *in situ* hybridization. Interestingly, other genes that exert tumor-suppressor functions in neuroblastoma, like *RASSF1A*, are also located on chromosome 3 and hypermethylated in neuroblastic tumors and phaeochromocytomas, which are also PNS tumors (Astuti et al., 2001). Furthermore, ectopic overexpression of full-length CaSR in two *MYCN*-amplified cell lines in which this gene was previously shown to be silenced by promoter hypermethylation significantly decreased their proliferative and tumorigenic capacities. Moreover, acute exposure to high Ca_o^{2+} concentrations prompted their apoptosis. In all, these data provided functional evidence of the biological relevance of CaSR epigenetic silencing in neuroblastoma biology.

In addition, when non-synonimous genetic variants located at the intracellular tail encoded by exon 7 of the *CASR* gene were analyzed in a cohort of neuroblastoma patients, a haplotype including a polymorphism considered to mildly reduce CaSR activity (Heath et al., 1996; Cole et al., 1999; Scillitani et al., 2004, 2007; Hu and Spiegel, 2007; Vezzoli et al., 2007; Yun et al., 2007) was associated with poor outcome (Masvidal et al., 2013).

Finally, a recent study has shown that cinacalcet, an allosteric activator of the CaSR approved for clinical use (Nemeth et al., 1998), inhibits neuroblastoma tumor growth *in vitro* and *in vivo* (Rodríguez-Hernández et al., 2016). Mechanisms involved include ER stress coupled to apoptosis dependent on phospholipase C activation in *MYCN*-amplified neuroblastoma cells and, irrespective of *MYCN* status, differentiation of surviving cells. Induction of differentiation was also observed upon prolonged exposure to cinacalcet *in vivo*. Genome-wide gene expression analysis by microarrays of xenografts showed up-regulation of numerous genes involved in neuroblastoma differentiation, like *NTRK3* (Nakagawara et al., 1993) and *GABRA3* (Roberts et al., 2004). Gene Ontology categories also unveiled up-regulation of genes involved in axon growth like doublecortin and ephrins (Kalil and Dent, 2014). Concomitantly, genes that critically support neuroblastoma proliferation were down-regulated, such as *MYCN*, inhibitor of differentiation 2 (*ID2*) and *MYB* (**Figure 1**). ID2 is a helix-loop-helix transcription factor controlled by MYC proteins that blocks differentiation and promotes cell proliferation (Lasorella et al., 2000), and MYB is a transcription factor that cooperates with MYCN in cell cycle regulation of *MYCN*-amplified neuroblastomas (Gualdrini et al., 2010). Quite unexpectedly, cinacalcet also promoted up-regulation of cancer-testis antigens, a family of proteins that are almost exclusively expressed in tumor cells and are thus considered ideal targets for immunotherapy (Fratta et al., 2011).

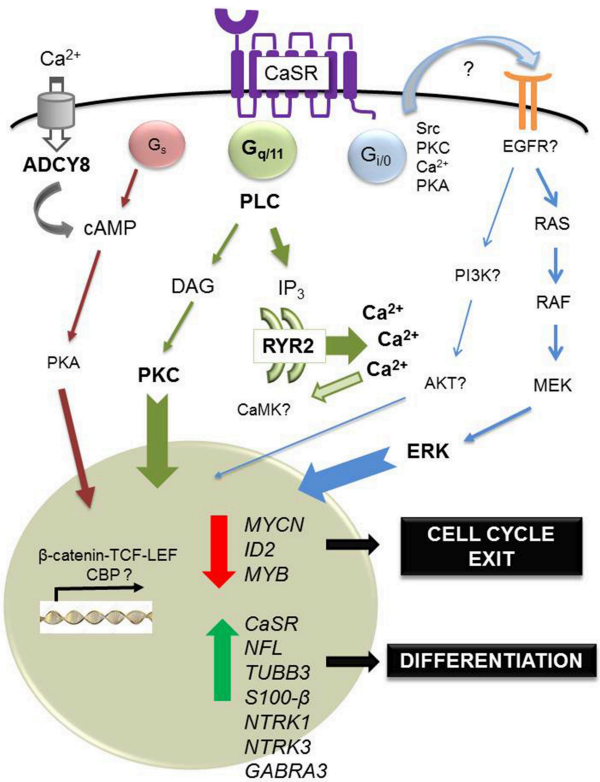

FIGURE 1 | Regulation of differentiation by calcium-sensing receptor in neuroblastoma. In neuroblastoma, the CaSR is expressed in benign, differentiated tumors. In this tumoral context, the main physiological ligand remains unknown. However, acute exposure to high extracellular concentrations of Ca^{2+} induces apoptosis of CaSR-positive, *MYCN*-amplified cells, dependent on sustained activation of ERK. Also, short *in vitro* exposure to cinacalcet, an allosteric activator of the CaSR approved for clinical use, induces endoplasmic reticulum (ER) stress coupled to apoptosis in CaSR-positive, *MYCN*-amplified neuroblastoma cells. This output is dependent on activation of phospholipase C (PLC). Massive Ca^{2+} exit from the ER up-regulates ryanodine receptor 2 (*RYR2*) and activation of adenylyl cyclase type 8 (ADCY8) via capacitative calcium entry, a mechanism triggered by depletion of intracellular Ca^{2+} stores. Furthermore, prolonged treatment with cinacalcet promotes up-regulation of genes associated with neuroblastoma differentiation (*NFL*, *TUBB3*, *S100-β*, *NTRK1*, *NTRK3*, *GABRA3*) and down-regulation of genes that are critical for proliferation of these tumors (*MYCN*, *ID2*, *MYB*). Concomitantly, sustained exposure to cinacalcet also induces up-regulation of CaSR and increased expression of cancer-testis antigens.

Although mechanisms involved are not yet understood, histone acetyltransferases such as p300 and CREB binding protein might be recruited, taking into account that these genes are mainly regulated by epigenetic mechanisms (Rao et al., 2011).

In summary, studies conducted in neuroblastoma indicate that CaSR promotes differentiation and inhibits proliferation in this malignancy. Also, they are in accordance with data obtained in developing NS supporting that CaSR plays a significant role in differentiation processes of specific NPCs upon commitment toward neuronal and glial lineages. More importantly, neuroblastoma models show that pharmacological modulation of CaSR activity can provide novel therapeutic opportunities.

CONCLUSIONS AND FUTURE DIRECTIONS

Over the past decades, the patterns of expression of CaSR in the NS have been described. However, our understanding of CaSR regulation and functions during normal and pathological development of CNS and PNS is incomplete. In the coming years, epigenetic mechanisms responsible for CaSR regulation during formation of NS will be elucidated. They will probably involve cytosines methylation and demethylation by DNA methyltransferases and 10–11 translocation enzymes (Hahn et al., 2013), histones modifications and non-coding RNAs. Also, the complex interplay of these mechanisms with transcription factors such as the MYC family will be characterized. This knowledge, together with a precise picture of signaling pathways downstream of CaSR during differentiation processes, will help to evaluate whether pharmacological modulation of this GPCR might be beneficial in the treatment of NS developmental diseases.

AUTHOR CONTRIBUTIONS

CdT is the Principal Investigator of the project. SM-L, CR-H, and MG are postdoctoral researchers working in the project, notably contributing to the development of both execution and conception of experiments.

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REVIEW
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Calcium-Sensing Receptors of Human Neural Cells Play Crucial Roles in Alzheimer's Disease

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In aged subjects, late-onset Alzheimer's disease (LOAD) starts in the lateral entorhinal *allocortex* where a failure of clearance mechanisms triggers an accumulation of neurotoxic amyloid-β_{42} oligomers ($A\beta_{42}$-os). In neurons and astrocytes, $A\beta_{42}$-os enhance the transcription of Aβ precursor protein (APP) and β-secretase/BACE1 genes. Thus, by acting together with γ-secretase, the surpluses of APP and BACE1 amplify the endogenous production of $A\beta_{42}$-os which pile up, damage mitochondria, and are oversecreted. At the plasmalemma, exogenous $A\beta_{42}$-os bind neurons' and astrocytes' calcium-sensing receptors (CaSRs) activating a set of intracellular signaling pathways which upkeep $A\beta_{42}$-os intracellular accumulation and oversecretion by hindering $A\beta_{42}$-os proteolysis. In addition, $A\beta_{42}$-os accumulating in the extracellular milieu spread and reach mounting numbers of adjacent and remoter teams of neurons and astrocytes which in turn are recruited, again via $A\beta_{42}$-os•CaSR-governed mechanisms, to produce and release additional $A\beta_{42}$-os amounts. This relentless self-sustaining mechanism drives AD progression toward upper cortical areas. Later on accumulating $A\beta_{42}$-os elicit the advent of hyperphosphorylated (p)-Tau oligomers which acting together with $A\beta_{42}$-os and other glial neurotoxins cooperatively destroy wider and wider cognition-related cortical areas. In parallel, $A\beta_{42}$-os•CaSR signals also elicit an excess production and secretion of nitric oxide and vascular endothelial growth factor-A from astrocytes, of $A\beta_{42}$-os and myelin basic protein from oligodendrocytes, and of proinflammatory cytokines, nitric oxide and (likely) $A\beta_{42}$-os from microglia. Activated astrocytes and microglia survive the toxic onslaught, whereas neurons and oligodendrocytes increasingly die. However, we have shown that highly selective allosteric CaSR antagonists (calcilytics), like NPS 2143 and NPS 89626, efficiently suppress all the neurotoxic effects $A\beta_{42}$-os•CaSR signaling drives in cultured cortical untransformed human neurons and astrocytes. In fact, calcilytics increase $A\beta_{42}$ proteolysis and discontinue the oversecretion of $A\beta_{42}$-os, nitric oxide, and vascular endothelial growth factor-A from both astrocytes and neurons. Seemingly, calcilytics would also benefit the other types of glial cells and cerebrovascular cells otherwise damaged by the effects of $A\beta_{42}$-os•CaSR signaling. Thus, given at amnestic minor cognitive impairment (aMCI) or initial symptomatic stages, calcilytics could prevent or terminate the propagation of LOAD neuropathology and preserve human neurons' viability and hence patients' cognitive abilities.

Keywords: calcium-sensing receptor, calcilytic, human, neurons, astrocytes, oligodendrocytes, microglia, Alzheimer's disease

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ALZHEIMER'S DISEASE (AD): AN INTRODUCTION

During the last decades, human lifespan has lengthened due to progress in medical knowledge and improvements in nutrition and hygiene. Unfortunately, this has been paralleled with an increased prevalence of age-related ailments, including neurodegenerative diseases, which have adversely impacted the quality of life. The *sporadic* or *late-onset Alzheimer's disease* (*LOAD*) is the most prevalent of these dementias striking ∼60 million people worldwide, half of which in the European Union and United States (Alzheimer's Association, 2012). AD has a lengthy (20–45 years) asymptomatic or preclinical phase, followed by an amnestic minor cognitive impairment phase (aMCI: 2–6 years) that in most subjects evolves into the terminal fully symptomatic phase (6–12 years; Selkoe, 2008a,b; Sperling et al., 2011). AD's less frequent (1–5% of all cases) *early onset* (around 60 years) *familial* (autosomal dominant) form (*EOFAD*) is caused by mutations in genes encoding the amyloid precursor protein (APP) or presenilin 1 (PSEN1) or presenilin 2 (PSEN2). These mutations trigger excess production and secretion of amyloid-β peptides (Aβs) and formation of toxic oligomers (Aβ-os) and polymers (fibrils). Most EOFAD cases result from PSEN1 mutations, those from APP and PSEN2 mutations being rarer (Selkoe, 2008a,b). The dramatic effects elicited by the Aβs excess due to such mutations have inspired the "*amyloid cascade hypothesis*" of AD which posits that Aβ-os precede the manifestation of toxic hyperphosphorylated (p)-Tau proteins and neurofibrillary tangles (NFTs) (Hardy and Selkoe, 2002; Selkoe, 2008a,b). Conversely, the "*Tau first hypothesis*" of AD posits that just the opposite happens (Attems et al., 2012; Braak and Del Tredici, 2013; Braak et al., 2013). An extended post-mortem survey revealed that AD cognitive decline is linked to both Aβs and p-Taues build-ups (Murray et al., 2015). However, Choi et al. (2014) provided evidence that, in a 3D human neural stem cells (NSCs) culture system, the accumulation of Aβ-os precedes any p-Tau/NFTs materialization thereby validating the "*amyloid cascade hypothesis*." Bilousova et al. (2016) confirmed that this Aβ-os ⇒ p-Tau sequence occurs also in advanced AD stages, strengthening the view that an anti-amyloid therapy must be started in advance of the tauopathy onset.

Albeit clinically both EOFAD and LOAD present with a similarly increasing memory failure, at variance with EOFAD's known mutations, LOAD's etiologic factors are manifold and controversial. The slow concurrence of several age-related metabolic and vascular defects presumably triggers LOAD by hindering the mechanisms which effect the brain's physiological clearance of Aβs (Domert et al., 2014). Two genetic factors only are known to aid LOAD's onset and progression, i.e., the heterozygous or homozygous presence of apolipoprotein E (APOE) ε4 allele(s) and TREM-2 mutations, especially the R47H one (Ising et al., 2015). AD's neuropathological hallmarks are accumulations of Aβs as senile plaques in the neuropil, intra-neuronal build-ups of p-Taues as insoluble NFTs, a chronic diffuse neuroinflammation, and the progressive death of neurons and oligodendrocytes. Such characteristics are detectable and more intense in wide cortical and subcortical regions starting at least 15 years ahead of EOFAD's clinical onset (Braak and Braak, 1991a; Armstrong, 2011; Benzinger et al., 2013). Conversely, LOAD starts from neuronal *foci* in the layer II of the lateral entorhinal cortex (LEC) of the middle temporal lobe in humans and AD-model transgenic (Tg) mice (Khan et al., 2014). Synaptically disconnected and deceasing neurons stuffed up with Aβs and NFTs appear first in the LEC *allocortex* and *subiculum*/CA1 areas (Braak and Braak, 1991a; Gómez-Isla et al., 1996; Khan et al., 2014) and later spread slowly to the parietal lobes and other cognition-related cortical areas of human AD brains (reviewed in Dal Prà et al., 2015a; **Figure 1A**). Remarkably, LEC is the portal through which the perforant pathway conveys multimodal data illustrating events of the outside world to the memory-recording hippocampus (Klemm, 2014). Next, the hippocampal *allocortex* and prefrontal *neocortex* mutually interact through the LEC to consolidate integrated memories (Klemm, 2014). These bidirectional exchanges are compromised by the ravages LEC suffers at the aMCI stage of LOAD. Hence, the first clinical harbingers of LOAD are worsening failures of the declarative memory.

The present lack of an anti-AD beneficial therapy is due to several concurrent causes: (i) LOAD's etiology is still hotly debated; (ii) EOFAD and LOAD are diseases typical of the human central nervous system (CNS) whose features can be only partially modeled in animals because of the huge differences in brain structures and cellular functions. The significant losses of hippocampal and neocortical neurons while the astrocytes survive are emblematic of human AD. Conversely, in most Tg rodents AD-models neurons are spared whereas astrocytes undergo an earlier cytotoxic injury and death. That's why any drug reportedly "successful" in AD-model animals has failed the test of clinical trials (reviewed in Han et al., 2015); and (iii) most previous clinical trials of candidate anti-AD drugs recruited patients already at the symptomatic stage of EOFAD or LOAD, *viz*. their cognitive cortical areas had already undergone irretrievable damage (Cummings et al., 2014). These failures have taught at a high cost that any anti-LOAD therapy must be started as early as possible, i.e., at the aMCI stage or just a little later for the time being (or earlier when it will be feasible).

Abbreviations: α7-nAChR(s), α7-nicotinic acetylcholine receptor(s); AD, Alzheimer's disease; (a)MCI, (amnestic) minor cognitive impairment; ANT(s), astrocyte-neuron team(s); APOE, apolipoprotein E; APP, Aβ precursor protein; Aβ(s), amyloid-β peptide(s); $Aβ_{42}$-os, amyloid-$β_{42}$ oligomers; β-S, BACE1/βS, β-secretase; CAA, cerebral amyloid angiopathy; CaSR, calcium-sensing receptor; CKD, chronic kidney disease; EOFAD, early onset familial (autosomal dominant) AD; fMRI, functional Magnetic Resonance Imaging; GABA, γ-amino butyric acid; γ-S, γ-secretase; GFAP, glial fibrillary acidic protein; GPCRs, G-protein-coupled receptors; LDL, low density lipoprotein; LEC, lateral entorhinal *allocortex;* LOAD, late-onset (sporadic) AD; LRP1, LDL receptor-related protein 1; LTP, long-term potentiation; MBP, myelin basic protein; NAHAs, normofunctioning adult human astrocytes (from cerebral cortex); n.d., not determined; NFTs, neurofibrillary tangles; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NSCs, neural stem cells; NVU(s), neurovascular unit(s); $p75^{NTR}$, p75 neurotrophin (receptor); PAR-4, prostate apoptosis response-4; pE, pyroglutamate; PET, positron emission tomography; PKA, protein kinase A; PSEN1/2, presenilin 1/2; (p)-Tau-os, hyperphosphorylated Tau protein oligomers; (p)-Tau(es), hyperphosphorylated Tau protein(s); PTH, parathyroid hormone; TF(s), transcription factor(s); Tg, transgenic; VEGF-A, vascular endothelial growth factor A; VFT, Venus Fly Trap; WT, wild-type; 7TM, seven transmembrane α-helices region.

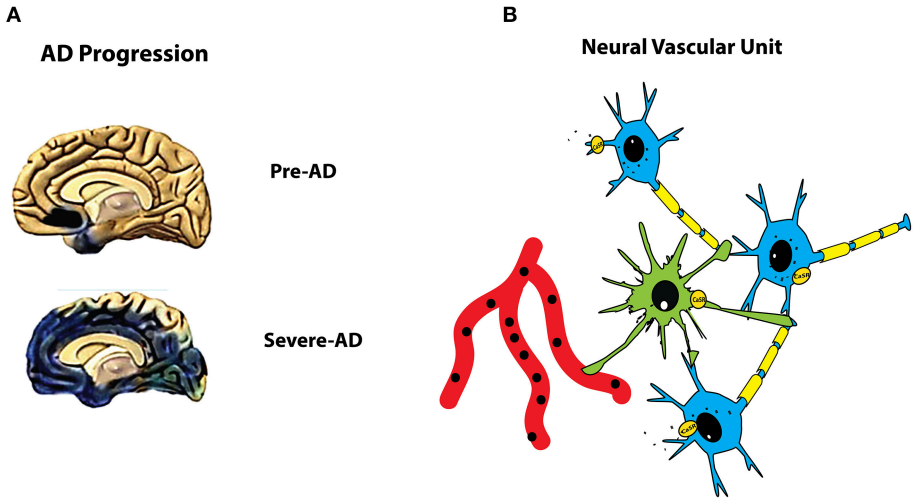

FIGURE 1 | (A) Late-onset AD (LOAD) neuropathology affects increasingly wider cerebral cortical areas. LOAD is a spreading disease which starts from the layer II neurons of the lateral entorhinal *allocortex* (LEC) of the temporal lobe and expands progressively to cognition-related upper neocortical areas. Involved brain tissues undergo deep changes due to concurrent neurotoxic, inflammatory, oxidative, and hypoxic-ischemic processes driven by accumulating $A\beta_{42}$-os and Aβ fibrils and later by p-Tau-os and causing the death of susceptible neurons. The diagram represents a view of the LOAD-affected areas (*in dark blue color*) from the medial-inferior hemispheric face at an early (*Pre-AD*), presymptomatic AD) and a late fully symptomatic stage of the illness. **(B)** The basic organization of the brain's neurovascular unit (NVU). NVUs are made up by cerebral astrocyte-neurons teams (ANTs) placed in close contact with capillary vessels. In this cartoon, a "master" astrocyte (*in green color*) emits numerous cytoplasmic processes (of which only a few are depicted here), the end-feet of which enshroud two neuronal synapses, touch the dendrite of a "client" neuron (*in blue color*), and cover a portion of the outer surface of a capillary vessel (*in red color*). The neuronal axons are endowed with myelin sheaths (*in yellow color*). Both neurons and the astrocyte express the CaSR (*yellow ovals*). By its placement, the astrocyte acts as a bridge between the capillary vessel and the neurons, provides the latter with nutrients brought up by the former, protects synapses, and partakes in the upkeep of the brain-blood barrier (BBB; not shown) functional integrity.

Hitherto, no specific marker of the preclinical stage of LOAD has been validated. However, changes in cerebrospinal fluid total Tau protein, p-Tau, and $A\beta_{42}$ levels, novel techniques of high-resolution functional Magnetic Resonance Imaging (fMRI), and genetic risk profiling show the potential of a future early diagnosis (Nordberg, 2015).

Aβs AND AD NEUROPATHOLOGY

In healthy human brains, neurons steadily produce physiologically low amounts (∼200 pM) of harmless monomeric $A\beta_{42}$s and release them during synaptic activity (Puzzo et al., 2008, 2011; Abramov et al., 2009; Garcia-Osta and Alberini, 2009). Aβs are synthesized via sequential enzymatic cleaving of the transmembrane Aβ precursor protein (APP) by BACE1/β-secretase (β-S) and γ-secretase (γ-S) (Takami and Funamoto, 2012). According to the "classical" view, only neurons express β-S in normal brains, whereas astrocytes do it only when hit by stressful insults (Kimura et al., 2005; Lee et al., 2014). However, proliferatively quiescent untreated normofunctioning adult human astrocytes (NAHAs) isolated from surgical leftovers of the temporal lobe cortex and cultured *in vitro* exhibit, at variance with rodent astrocytes, low basal levels of β-S and γ-S activity and hence produce and release trivial amounts of $A\beta_{42}$ and $A\beta_{40}$ (Dal Prà et al., 2011; Armato et al., 2013a).

Normally, the production of $A\beta_{40}$ prevails (90%) on that of $A\beta_{42}$ (10%), but in AD the $A\beta_{42}/A\beta_{40}$ ratio shifts in favor of $A\beta_{42}$ (Masters and Selkoe, 2012). $A\beta_{42}$'s two C-terminal hydrophobic amino acids, Ala and Ile, cause its greater proclivity to form aggregates and resist proteolysis with respect to $A\beta_{40}$ (Kim and Hect, 2005; Masters and Selkoe, 2012). At safe pM concentrations $A\beta_{42}$ monomers play important trophic functions by: (a) inducing an enhanced expression of proteins related to insulin-like growth factor (IGF) function or transcription factor (TF) regulation (IGFBP3/5, and Lim only domain protein 4, respectively); (b) favoring adult neurogenesis in the subgranular zone of dentate gyrus; (c) modulating synaptic plasticity, long-term potentiation (LTP), and memories recording in the hippocampus; (d) sealing blood vessels to preserve blood-brain barrier (BBB) integrity; and (e) fine-tuning Ca^{2+} homeostasis by binding α7-nicotinic acetylcholine receptors (α7-nAChRs) and enhancing *intra*cellular Ca^{2+} signals without triggering *inter*cellular Ca^{2+} waves in astrocytes. Thus, $A\beta_{42}$ monomers assist in the mutual modulation of neuron-astrocyte signals promoting long-term potentiation (LTP) and memory storing (Plant et al., 2003; Koudinov and Berezov, 2004; Puzzo et al., 2008, 2011; Garcia-Osta and Alberini, 2009; Morley et al., 2010; Cárdenas-Aguayo et al., 2014; Lee et al., 2014; Storck et al., 2016). $A\beta_{42}$s are kept at physiological pM levels via the activity of proteases like insulin-degrading enzyme, neprilysin, angiotensin-converting enzyme, endothelin-converting enzyme, and the ubiquitin-proteasome system (López Salon et al., 2003; Wang et al., 2006). Additional Aβ-disposing mechanisms are microglial phagocytosis, and dumping into the circulating blood through the α2-macroglobulin receptor/low density lipoprotein receptor-related protein 1 (LRP1) (Storck et al., 2016).

Such monomeric Aβ-clearing mechanisms become inadequate when mutations of *APP* or *PSEN1* or *PSEN2* genes cause an overproduction of Aβs as in EOFAD or when they significantly decline with age and fail in LOAD (Tarasoff-Conway et al., 2015). The resulting accumulation of $A\beta_{42}$s triggers the assembling of $A\beta_{42}$ monomers into an assortment of toxic $A\beta_{42}$ oligomers ($A\beta_{42}$-os) of growing sizes eventually forming Aβ fibrils (Braak and Braak, 1991b; Mawuenyega et al., 2010; Masters and Selkoe, 2012; Lesnè et al., 2013). In addition, the generation of long fatty acid–derived oligomers (LFA-os) via a prion-like mechanism (Kumar and Walter, 2011; Kumar A. et al., 2012), the increasing presence of the $A\beta_{43}$ isoform (Sandebring et al., 2013), and the Aβ-phosphorylating activity of membrane-bound or extracellular protein kinase A (Kumar and Walter, 2011; Kumar S. et al., 2012) accelerate the rate of Aβ-os assembly, reduce their proteolytic or microglia-mediated clearance, and step up their neurotoxicity. Another toxic species is pyroglutamate (pE)-$A\beta_{3-42}$, which amounts to ∼20% of the total Aβs in AD brains, but is missing among the Aβs extracted from aged yet cognitively normal brains (Gunn et al., 2010; Jawhar et al., 2011). In AD-developing human brains, pE-$A\beta_{3-42}$ engenders pure or mixed (with other Aβs) highly toxic oligomers, the amount of which tightly correlates with the actual rate of cognitive decline (Morawski et al., 2014). Additionally, the N-truncated $A\beta_{4-42}$ also abounds in AD brains and spawns stable $A\beta_{4-42}$-os which are as neurotoxic as $A\beta_{1-42}$-os and pE-$A\beta_{3-42}$-os *in vitro* and in the mouse Tg4-42 transgenic line (Bouter et al., 2013). Moreover, interactions with cell membranes increase the aggregation rate of $A\beta_{42}$-os and produce amyloid pores and Ca^{2+}-permeable channels resulting in an intracellular Ca^{2+} dyshomeostasis promoting the neurodegeneration (Mattson, 2007; Kawahara, 2010; Zhao et al., 2012; Berridge, 2014). However, being pathologically bound and activated by $A\beta_{42}$-os, the calcium-sensing receptor (CaSR) expressed by all types of neural cells is also involved in AD development via mechanisms implicating much more than Ca^{2+} influxes.

INTERACTIONS BETWEEN NEURONS AND ASTROCYTES IN LOAD

Neurons and astrocytes derive from embryonic radial glia acting as neural stem cells (NSCs) during development (Bonfanti and Peretto, 2007). Accumulating evidence has shown that human cortical astrocytes remarkably differ from their rodent counterparts. They are bulkier, own 10-fold more numerous primary processes, include the entirely new cortical polar and interlaminar subtypes, exhibit a different transcriptome as assessed by genome-wide unbiased comparisons, govern much broader synaptic domains, and perform more intense and complex metabolic tasks, e.g., faster Ca^{2+} waves propagation, than rodents' counterparts (Oberheim et al., 2006, 2009, 2012; Sherwood et al., 2006; Tsai et al., 2012; Zhang et al., 2016). Astrocytes' evolutionary changes have affected both human brain physiology and neuropathology, including AD and other neurodegenerative disorders. The increased learning capacity and activity-dependent plasticity of mouse brains engrafted with human astrocytes confirms this view (Han et al., 2013). Human brain evolutive changes prevent AD-model animals from fully emulating human LOAD. This hampers any successful translation of pharmacological results reaped from AD-model animals to human clinical settings (Cummings et al., 2014; Han et al., 2015). Astrocytes' roles in AD progression deserve a careful consideration. Such cells are more numerous (from 1.7- to 2.2-fold at least) than neurons, form gap junction-connected networks, partake in the assembly of tripartite synapses, and tightly nestle and chemically insulate neurons with which physiologically trade several indispensable compounds (Ullian et al., 2001). Each "*master*" astrocyte functionally integrates with up to a 30-odd "*client*" neurons forming *astrocyte-neuron teams* (*ANTs*; reviewed in Araque and Navarrete, 2010; Giaume et al., 2010; Halassa and Haydon, 2010). Neighboring ANTs are reciprocally connected via gap junctions astrocytes' processes bear. Other astrocytes processes get in touch by means of their end-feet with the walls of cerebral micro vessels forming physiologically integrated *neurovascular units* (*NVUs*) (**Figure 1B**; reviewed in Dal Prà et al., 2014b; Nelson et al., 2016). Physiologically, the synapses of ANTs "*client*" neurons are induced and stabilized by the shrouding end-feet of their "*master*" astrocytes. Moreover, the synapses pertaining to a single neuron can also be enveloped by the processes end-feet of astrocytes pertaining to neighboring ANTs. Importantly, the astrocytes of connected ANTs promote or reduce the release of neurotransmitters into the synapses they wrap thus modulating neural transmission by (a) sweeping up spilled over glutamate and K^+; (b) releasing "*gliotransmitters*" like glutamate, ATP, D-serine, γ-amino butyric acid (GABA), and taurine; and (c) letting out or taking up, respectively, Ca^{2+} ions during their Ca^{2+} waves (Antanitus, 1998; Bushong et al., 2002; Kettenmann and Ransom, 2013; Gundersen et al., 2015). The term *infotropism* defines the control of neurotransmitter release and hence of synaptic function by the astrocytes (Antanitus, 1998). Astrocytes' activation is coupled with *intra*cellular Ca^{2+} transients and *inter*cellular gap-junction-mediated Ca^{2+} waves and triggers both locally and remotely the secretion of gliotransmitters modulating astrocyte-astrocyte and astrocyte-neuron signaling (Lee et al., 2014). Moreover, astrocytes express receptors for other neurotransmitters—like purines, GABA, and N-methyl-D-aspartate (NMDA)—and control extracellular ion levels (e.g., K^+), pH, and water volume (reviewed in Kettenmann and Ransom, 2013). Because of these distinctive properties, human astrocytes likely play a role as neuronal partners in learning, memory, and cognition—all functions progressively lost in AD.

In AD, extracellularly accumulating Aβ-os and Aβ fibrils contact all cellular members of ANTs and NVUs. In Tg AD-model rodents, while acting as wardens, astrocytes sweep extracellular Aβs by engulfing them via several Aβ-binding receptors, like LRP1 and LRP2/Megalin, and next proteolyse them. Eventually, ingested Aβs are toxic for the astrocytes which before dying discharge them back into the extracellular milieu. This promotes the assembly of smaller senile plaques which are rich in glial fibrillary acidic protein (GFAP; Wyss-Coray et al., 2003; Nagele et al., 2004; Pihlaja et al., 2008). Thus, an

initially beneficial clearing of Aβs surpluses by the astrocytes eventually competes with and wrecks their role as supporters of neurons metabolism (Pihlaja et al., 2008; Araque and Navarrete, 2010; Giaume et al., 2010; Halassa and Haydon, 2010; Mulder et al., 2012). In 3 × Tg AD-model mice, as AD slowly yet inexorably progresses, the pattern of astrocytes' reactions changes. Astrocytes' processes rapidly wither and detach their shrouding end-feet from the tripartite synapses within the CA1 area and dentate gyrus. An early diffuse astrogliosis develops surrounding the senile plaques (Rodriguez-Vieitez et al., 2015).

Conversely, in human AD brains, astrocytes become hypertrophic, conserve their spatial domains, pierce with their processes Aβ senile plaques, lose part of their glutamate-metabolizing enzymes, over express GFAP, hyper polymerize actin, and make and release surplus amounts of cytokines and chemokines, such as S100β, TNF-α, IL-1β, IL-6, and IFN-γ-inducible protein-10 (IP-10). The sercreted chemokines induce circulating leukocytes to cross the BBB and sustain a chronic neuroinflammation (Perez et al., 2010). In aMCI patients, but not in healthy individuals, an astrogliosis can be detected (using the [11] CD-deprenyl marker and Positron Emission Tomography) which abates during the progression toward full-blown AD (Choo et al., 2014). This human AD-related astrogliosis co-occurs with oxidative stress, extracellular accumulation of glutamate and/or K^+, dyslipidemia, and/or folate deficit (Rojo et al., 2008; Li et al., 2011).

A belief has been prevailing for a long time, i.e., only a transneuronal diffusion of neurotoxic Aβ-os happens in AD. This view was experimentally modeled in retinoic acid–differentiated human SH-SY5Y neurons (Nath et al., 2012; Hallbeck et al., 2013). Conversely, astrocytes' own production and secretion of Aβs as well as their potential contribution to AD progression was generally neglected. According to such a "classical" view, astrocytes only played the role of onlookers or at most of concierges cleansing neuronal debris and/or Aβ fibrils. Reports of astrocytes stuffed with $Aβ_{42}$s in human brains with advanced LOAD strengthened this view (Nagele et al., 2004; Maragakis and Rothstein, 2006; Avila-Muñoz and Arias, 2014). Yet, because of their high numbers, even a token increase in astrocytes' Aβs secretion rate would remarkably raise brain's load of Aβs (Busciglio et al., 1993; Corbett and Buss, 2014). Nevertheless, recent studies have provided evidence that the intracerebral diffusion of $Aβ_{42}$-os results from chemical interactions of astrocytes with neurons, oligodendrocytes, and microglia (Skaper et al., 2009; Bero et al., 2011; Dal Prà et al., 2015a). Such reciprocal exchanges *activate* the astrocytes which then express surpluses of APP and of β-S which act with γ-S to trigger an overproduction of Aβs in several Tg AD-model mice (Rossner et al., 2005). The effects of exogenous Aβs on mouse, rat, and human astrocytes and neurons have been studied *in vivo* and *in vitro*. Physiological patterns of astrocytes' intercellular Ca^{2+} waves and synchronous hyperactivity are changed in Tg AD-model animals (Kuchibhotla et al., 2009). When exposed to Aβ-os, newborn rat hippocampal astrocytes exhibited an increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Hence, a Ca^{2+} dyshomeostasis occurs in the astrocytes activated by AD (reviewed in Abramov et al., 2003; Mattson and Chan, 2003; Bezprozvanny and Mattson, 2008; Berridge, 2014). Moreover, mouse astrocytes exposed to $Aβ_{25–35}$ produced and secreted ceramide-stuffed exosomes ("*apoxosomes*") and prostate apoptosis response-4 (PAR-4) protein which would trigger the apoptotic demise of nearby neurons releasing Aβs surpluses (Wang et al., 2012). In addition, primary cortical astrocytes from neonatal mouse pups treated with TNF-α + IFN-γ or $Aβ_{42}$ (either in soluble or fibrillar form) raised the cells' levels of APP and β-S and their secretion rate of endogenous $Aβ_{40}$ (yet, $Aβ_{42}$ secretion was not assessed). The authors surmised that neuroinflammation triggers a feed-forward mechanism pushing the production of endogenous Aβs in mouse astrocytes (Zhao et al., 2011).

As mentioned above, β–S and γ–S exhibit a discrete basal activity in untreated (control) NAHAs (Armato et al., 2013a). Once exposed to exogenous fibrillary or soluble $Aβ_{25–35}$—an $Aβ_{42}$ proxy having the physical and biological features of $Aβ_{42}$ (Kaminsky et al., 2010)—NAHAs start producing, accumulating, and secreting surplus $Aβ_{42}$/$Aβ_{42}$-os just as human cortical HCN-1A neurons do (Dal Prà et al., 2011; Armato et al., 2013a).

Under conditions of acute or chronic hypoxia, or during LOAD or when exposed to exogenous Aβs, APP levels and both β–S and γ–S activities raise significantly thereby increasing Aβs production and release (Perez et al., 2010; Dal Prà et al., 2011, 2014a,b; Takami and Funamoto, 2012). This might be due to $Aβ_{42}$-os entering the nuclei and binding Aβ-interacting domains (AβIDs) in the *APP* and *β–S* genes promoters sequences causing their transcriptional activation (Bailey et al., 2011; Maloney and Lahiri, 2011; Barucker et al., 2014). Lastly, Aβ-activated microglia release cytokines, like IL-1β or IFN-γ + TNF-α, that induce cultured adult human astrocytes to synthesize and secrete $Aβ_{40}$ and $Aβ_{42}$ (Blasko et al., 2000).

Aβs AS RECEPTORIAL LIGANDS

As mentioned, an exposure to fibrillar or soluble $Aβ_{25–35}$ elicits an excess production, accumulation, and secretion of $Aβ_{42}$ and $Aβ_{42}$-os in cultured NAHAs (Armato et al., 2013a; Dal Prà et al., 2015a). The primary molecular mechanism(s) underlying this exogenous Aβs ⇒ endogenous Aβs self-induction in NAHAs was (were) initially totally and still partly is (are) unclear. It appeared that exogenous Aβs interacted with "something" located at the outer surface of the cells' plasma membrane (Kam et al., 2014; Jarosz-Griffiths et al., 2016). At the same time, the question arose whether this Aβs self-inducing feed-forward mechanism worked in *human* neurons too, as it had been shown to do in Aβ-exposed rat cortical neurons and mouse hippocampal slices (Marsden et al., 2011). So would Aβs bind and activate the signaling of one or more receptors? So far, several receptors have been indicated to interact with Aβs (**Table 1**). Nevertheless, since Aβs are the unique ligands for none of them, these Aβ●receptor interactions have been debated. Yet, once bound to Aβs some of the receptors did undergo internalization and accumulated intracellularly (Kam et al., 2014; Jarosz-Griffiths et al., 2016). For example, highly specific soluble Aβ-os●CaSR complexes were shown to gather together and form patches at the plasma membrane of NAHAs prior to be internalized (Dal Prà et al., 2014a,b, 2015b).

TABLE 1 | Receptor interactions with various Aβ forms.

Aβ forms	Receptor	References
$Aβ_{42}$ monomers	Insulin-like growth factor-1 receptor (IGF-1R)	Giuffrida et al., 2012
$Aβ_{42}$ monomers	Low-density lipoprotein receptor-related protein 1 (LRP1)	Shibata, 2000; Kanekiyo et al., 2013, 2012
$Aβ_{42}$ monomers	Low-density lipoprotein receptor (LDLR)	Castellano et al., 2012
$Aβ_{42}$ monomers	Macrophage receptor with collagenous structure (MARCO)	Brandenburg et al., 2010
$Aβ_{42}$ and $Aβ_{40}$ monomers	Advanced glycation end products receptor (RAGE)	Du et al., 2012
$Aβ_{42}$ and $Aβ_{40}$ monomers	Apolipoprotein E (ApoE) receptor	Liu et al., 2013
$Aβ_{42}$ monomers, $Aβ_{42}$-os	α7 nicotinic acetylcholine receptor (α7nAChR)	Wang et al., 2000; Jurgensen and Ferreira, 2010
$Aβ_{40}$ monomers, $Aβ_{42}$-os	Cellular prion protein (PrP^{C})	Nygaard and Strittmatter, 2009; Pflanzner et al., 2012
$Aβ_{42}$	Formyl peptide receptor (FPR1) Formyl peptide receptor-like 1 (FPRL1)	Iribarren et al., 2005; Doens and Fernandez, 2014
Aβ globulomers	P/Q-type Ca^{2+} channels	Nimmrich et al., 2008
$Aβ_{42}$-os, $Aβ_{40}$-os	Frizzled (Fzd) receptor	Magdesian et al., 2008
$Aβ_{42}$-os	Insulin receptor	Zhao et al., 2008
$Aβ_{42}$-os	α-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid receptor (AMPAR)	Zhao et al., 2010
$Aβ_{42}$-os	Amylin 3 (AMY3) receptor	Fu et al., 2012
$Aβ_{42}$-os	NMDA-type glutamate receptor	Shankar et al., 2007
$Aβ_{42}$-os, Aβ fibrils	Calcium-sensing receptor (CaSR)	Ye et al., 1997; Conley et al., 2009; Dal Prà et al., 2014a,b, 2015b
$Aβ_{42}$-os, Aβ fibrils	p75 neurotrophin ($p75^{NTR}$) receptor	Perini et al., 2002; Chakravarthy et al., 2012
Aβ fibrils	SCARA1/2 (microglia) receptor	Wilkinson and El Khoury, 2012
Aβ fibrils	SCARB2/CD36 receptor	Stewart et al., 2010
Aβ fibrils	Toll-like receptor 2 (TLR2)	Doens and Fernandez, 2014
Aβ fibrils	Complement receptor type 3 (CR3)	Doens and Fernandez, 2014

Conversely, most of the fibrillar Aβ•CaSR complexes could not be internalized because of intrinsic mechanical hindrances and their persistent signaling likely altered crucial cellular functions with noxious and/or lethal consequences. This happened also in engineered SK-N-BE neuroblastoma cells over expressing the whole $p75^{NTR}$ receptor which bound fibrillar Aβs (Perini et al., 2002). Indeed, $p75^{NTR}$ is also over expressed in the hippocampi of full-blown LOAD patients (Chakravarthy et al., 2012).

A clue on the topic was offered by observations that a mixture of three cytokines (i.e., TNF-α, IL-1β, and IFN-γ) or soluble $Aβ_{40}$ or fibrillar $Aβ_{25-35}$ or $Aβ_{1-42}$ induced a MEK/ERK1/2-mediated surplus NO production in NAHAs that could be fully suppressed when the cells were co-treated with a CaSR antagonist (or *calcilytic*) like NPS 89686 or NPS 2143 (Nemeth, 2002; Chiarini et al., 2005; Dal Prà et al., 2005; Armato et al., 2013a). Such results prompted us to investigate CaSR's interactions with Aβs in human cortical astrocytes and neurons (Armato et al., 2012, 2013a).

THE CaSR IN THE SEVERAL NEURAL CELL TYPES

The readers looking for more details about the features of the CaSR are referred to other contributions in this special issue. Briefly, the CaSR is a member of family C of G-protein-coupled receptors (GPCRs). Its huge (∼612 amino acids) extracellular N-terminal domain, named Venus Flytrap (VFT), is linked via a cysteine-rich region to seven transmembrane α-helices (TM1–TM7) joined together by extracellular and intracellular loops altogether forming the 7TM region. Two domains of the CASR's intracellular C-terminal tail are necessary for its expression at the cell surface and its composite signaling functions which are mediated by G-proteins (Armato et al., 2012). The two huge VFT lobes of functional CaSR homodimers bind orthosteric (type I) *agonists* like Ca^{2+} (the physiological ligand), various other divalent or trivalent cations, polyamines, and aminoglycoside antibiotics (Silve et al., 2005; Armato et al., 2012; Zhang et al., 2015). The allosteric (type II) CaSR ligands, like aromatic L-α-amino acids and highly selective *agonists* (or *calcimimetics*) and *antagonists* (or *calcilytics*) bind various 7TM sites (Nemeth, 2002; see also below). The CaSR swiftly senses any change in the $[Ca^{2+}]_e$ (Nemeth, 2002). Orthosteric type I agonists switch CaSR's signaling on owing to a rearrangement of its 7TM region permitting the receptor's C-tails to interact with various G proteins. The manifold CaSR's signaling pathways involve (i) second messenger-producing enzymes (e.g., adenylyl cyclase); (ii) phospholipases A2, C, and D; (iii) protein kinases (e.g., AKT, PKCs, MAPKs,); (iv) Ca^{2+} influxes via TRPC6-encoded receptor-operated channels; and (v) transcription factors (TFs; reviewed in Zhang et al., 2015). Like other GPCRs, CaSRs display the "*ligand-biased signaling*" feature, i.e., a specific CaSR signaling pathway may be stably preferred over the others according to the ligand involved (Leach et al., 2015). Here we will briefly consider some pathophysiological effects of CaSR's signaling regarding the CNS.

The CaSR is expressed by all types of neural cells and by the endothelial cell and pericytes of the cerebral vessels

with a variable intensity, which for example is greater in the hippocampus (Chattopadhyay et al., 2000, 2008; Yano et al., 2004; Noh et al., 2015). In addition, cultured NAHAs also express functional CaSRs, less intensely when proliferating but more strongly when in mitotic quiescence. In any case, CaSR expression is unaffected by changes in the growth medium Ca^{2+} levels (Dal Prà et al., 2005).

CASR is a key player in genetic regulation of Ca^{2+} homeostatic system (Kapur et al., 2010). In addition, CaSR performs relevant roles outside the Ca^{2+} homeostatic system, as for example in the CNS (Riccardi and Kemp, 2012). Besides upholding local ionic homeostasis, brain cells' CaSRs modulate the proliferation, differentiation, and migration of neurons and oligodendrocytes during development; axonal and dendritic growth; axons myelination; neurons' and glial membrane excitability; olfactory and gustatory signal integration; presynaptic external Ca^{2+} signaling at *neocortex* nerve terminals; synaptic plasticity; and neurotransmission during perinatal and adult life. Importantly, an altered expression and/or dysfunction of the CaSR, as observed in CNS diseases like AD and ischemia/hypoxia/stroke, also deeply affects CaSR-dependent neurophysiological processes (**Figure 2**) (Chattopadhyay et al., 1999; Vizard et al., 2008; Bandyopadhyay et al., 2010; Chen et al., 2010; Armato et al., 2012, 2013a; Ruat and Traiffort, 2013; Kim et al., 2014; Dal Prà et al., 2014a,b, 2015a; Bai et al., 2015; Noh et al., 2015; Tharmalingam et al., 2016).

The first clue about a potential role for the CaSR in AD pathophysiology was the degeneration of hippocampal neurons ensuing Aβ-induced peaks of cytosolic (intracellular) Ca^{2+} concentration ($[Ca^{2+}]_i$) (Brorson et al., 1995). A second clue was the assumed ability of fibrillar $A\beta_{25-35}$ or $A\beta_{1-40}$ to open Ca^{2+}-permeable non-selective cation channels (NSCCs) in hippocampal neurons of wild type (WT) $CaSR^{+/+}$ rats but not of $CaSR^{-/-}$ rats (Ye et al., 1997). The authors posited that Aβs could bind the CaSR since like polyamines they are endowed with orderly spaced arrays of positive charges. However, the same authors had previously observed hefty changes in pipette cations concentrations in cell-attached recordings and their replacement with Ca^{2+} had not affected channel amplitude or reversal potential (Ye et al., 1996a,b). Taken together, these results would have suggested the channel was alike permeable to K^+ and Na^+ or, alternatively, impermeable to cations like a Cl^- channel. However, the authors did not test his hypothesis. In this regard, several other authors have reported NSCCs being activated by *decreases* of calcium or other CaSR agonists (Hablitz et al., 1986; Xiong et al., 1997; Immke and McCleskey, 2001; Smith et al., 2004; Lu et al., 2010; Ma et al., 2012). Overall such findings do not corroborate the suggestion that Aβs activate NSCCs in neurons. Rather, Aβs would lessen the likelihood of NSCC openings or possibly activate Cl^- channels like probably the data from Ye et al. (1997) had demonstrated.

Afterwards, Conley et al. (2009) investigated the association of *CASR* gene variations in AD susceptibility using a cohort of 692 AD cases and 435 controls. A polymorphic dinucleotide repeat marker within intron 4 associated with AD, while three non-synonymous SNPs within exon 7 of the *CASR* gene associated with AD only in non-APOE ε4 carriers. In addition, TF activation assays revealed that both apoE ε4 and ε3 (but not ε2) and exogenous $A\beta_{1-42}$ bound and activated CaSR's signaling. The authors concluded that the CASR plays a role in AD susceptibility in the absence of the APOE ε4 allele(s).

Subsequently, the formation of Aβs●CaSR complexes and their endocytosis was shown to occur in NAHAs by using the highly specific *in situ* proximity ligation assay (Dal Prà et al., 2014a,b, 2015b). As aforesaid, such Aβs●CaSR complexes elicited a surplus production and secretion of $A\beta_{42}$ and $A\beta_{42}$-os from cortical NAHAs and HCN-1A neurons (Armato et al., 2013a). These observations imply that all types of human neural and cerebrovascular cells are susceptible to the neurotoxic effect(s) elicited by Aβ●CaSR signaling.

CASR gene transcription is regulated by its promoters P1 and P2 which bind several TFs. Recently, the role of TFs in a number of genes associated with AD has been studied in detail. Interestingly, the *CASR* gene promoters bind several TFs which are involved also in the expression of AD-related genes. Thus, there exists a deeper than previously thought connection of CaSR expression regulation with AD pathophysiology (see **Table 2** and references in it). Although CaSR mRNA and protein levels have not yet been investigated in human AD brains, it is likely that *CASR's* expression be altered in AD because of its co-regulation by some of the TFs implicated in the disease.

Besides the CaSR, $A\beta_{42}$-os simultaneously link to many other surface receptors (**Table 1**) activating their signaling systems and changing ion balances prior to be endocytosed by all types of CNS cells. In so doing, $A\beta_{42}$-os spark a dense clutter of cellular responses including mitochondrial over release of toxic ROS, Ca^{2+} surges via NMDARs' activation driving further mitochondrial releases of ROS, and production of toxic p-Tau oligomers (p-Tau-os) (Mao and Reddy, 2011; Müller et al., 2011; Swerdlow, 2011; Kam et al., 2014; Jarosz-Griffiths et al., 2016). The outcomes are the disconnection of neuronal networks—a cause of cognitive deterioration—and the damage and death of susceptible neurons eventually leading to full blown AD (Crimins et al., 2013; Kayed and Lasagna-Reeves, 2013; Medeiros et al., 2013).

However, the earliest asymptomatic stages of AD are still hard to detect because the build-up of highly toxic, synapse destroying $A\beta_{42}$-os inside and outside neurons and astrocytes is imperceptible until senile plaques and NFTs remain undetectable (West et al., 2004; Selkoe, 2008a,b; Ferreira and Klein, 2011; Klein, 2013; Medeiros et al., 2013; Dal Prà et al., 2015a). Thus, in the course of several years the neurotoxic $A\beta_{42}$-os spread stealthily from LEC's layer II to higher cognitive cortical areas (Khan et al., 2014) and the emergence of AD's typical hallmarks (**Figure 1A**). As it will be discussed below, these events are related to $A\beta_{42}$-os●CaSR interactions whose signaling mechanisms are likely to underlie the developing amyloidosis in AD brains and hence have crucial therapeutic implications.

Some authors surmise a prion-like mechanism fostering the $A\beta_{42}$-os (and p-Tau-os) diffusion in AD brains (Nussbaum et al., 2013; Morales et al., 2015). $A\beta_{42}$-os extracted from AD brains could be passed on from retinoic acid–differentiated human SH-SY5Y donor neurons to similarly differentiated SH-SY5Y recipient neurons (Nath et al., 2012; Hallbeck et al., 2013). Most

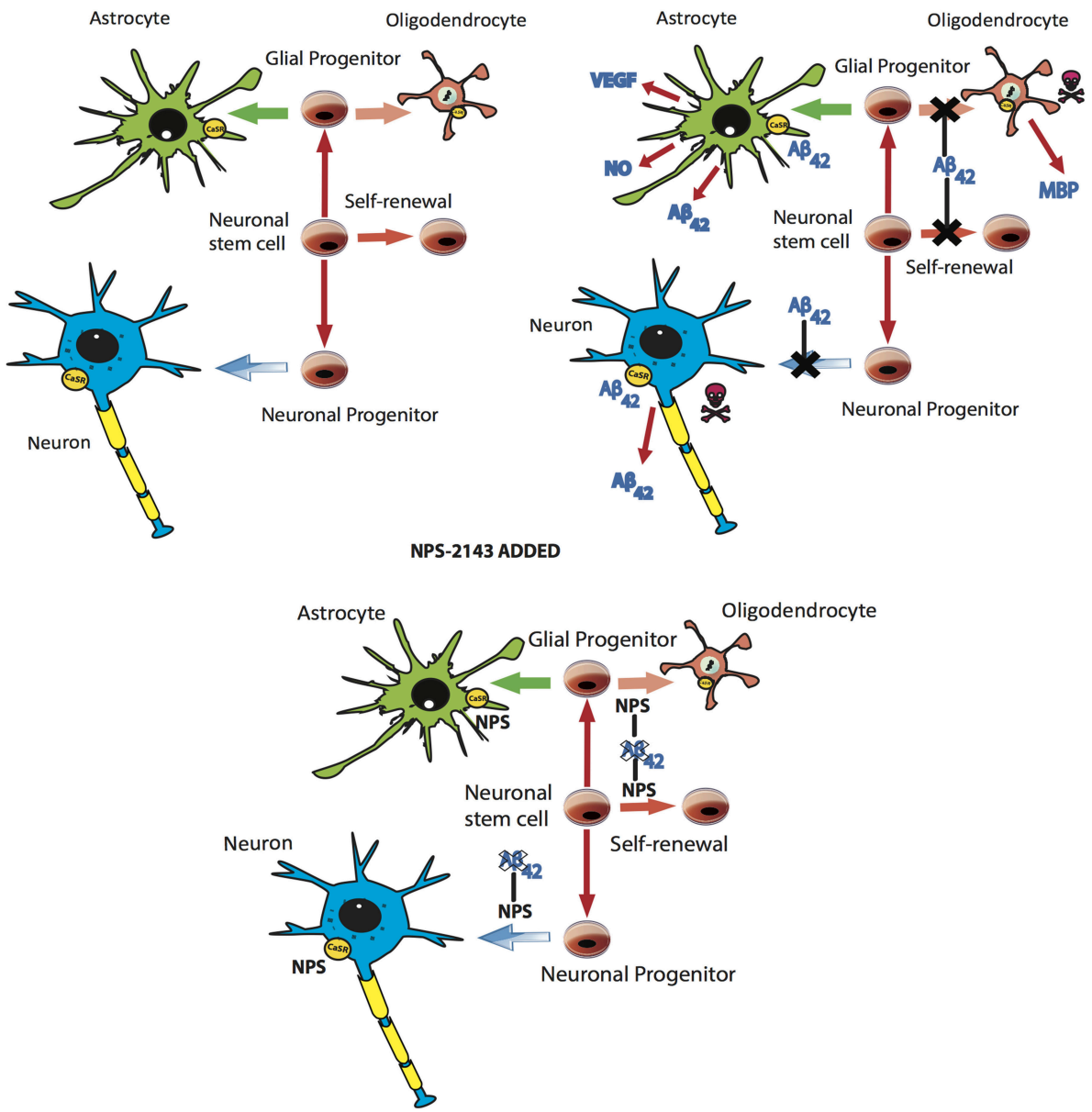

FIGURE 2 | The main neural cell types origins, and the effects of $A\beta_{42}$-os accumulation without or with an added calcilytic. Top left: During development and in the adult, neurogenesis starts from NSCs that self-renew while giving birth to neurons and glial progenitors. From the latter (also named NG2 cells) stem both astrocytes and oligodendrocytes. All these cell types express the CaSR (see the text for details). **Top right:** When $A\beta_{42}$-os start accumulating in the brain tissues they soon block the NSCs self-renewal and differentiation of both neurons and oligodendrocytes from their respective precursors. The interactions of $A\beta_{42}$-os with the CaSRs (*yellow ovals*) elicits a surplus production/release of $A\beta_{42}$-os from neurons and astrocytes, of NO and VEGF-A from astrocytes, and of MBP and $A\beta_{42}$-os (not shown) from oligodendrocytes. All these toxic compounds together with later appearing p-Tau-os (not shown), microglial proinflammatory cytokines, and hypoxia/ischemia due to damaged micro vessels eventually cause an increasing death of neurons and oligodendrocytes (*flanking skull and crossbones*). **Bottom center:** The addition of calcilytic NPS 2143 (short termed here as *NPS*) thwarts all of the toxic effects elicited by $A\beta_{42}$-os•CaSR signaling like surplus secretion and diffusion of additional $A\beta_{42}$-os, NO, and VEGF-A, hindered differentiation of NSCs, and most of all, the death of neurons and oligodendrocytes, vascular damage, the later p-Tau-os appearance, and likely microglial activation (the latter two not shown). The findings on neurons and astrocytes indicate the feasibility of calcilytics as anti-LOAD therapeutics capable of halting $A\beta_{42}$-os self-promoting and self-maintaining mechanisms (Dal Prà et al., 2015a).

TABLE 2 | Comparison between *CaSR* and AD-related genes transcriptional regulators.

Gene	Transcription factor	References
CaSR	**SP1/3***, **AP1**, **STAT1/3, NFκB, TFIID**, Vitamin D, GCM-2	Santpere et al., 2006; Hendy et al., 2013
APP	**SP1**, **AP1**, **STAT1/3, NFκB**, USF, CTCF, HSF1, SP1-like, UBP, **HIF-1α, CREB**, GATA1	Theuns and Van Broeckhoven, 2000; Santpere et al., 2006; Chen et al., 2013
BACE1	**SP1, STAT1/3, NFκB,** HIF-1α, PPAR γ	Santpere et al., 2006; Wen et al., 2008; Chen et al., 2013
PSEN1	**SP1**, Ets, **CREB**	Theuns and Van Broeckhoven, 2000; Santpere et al., 2006; Chen et al., 2013
APOE	**SP1**, **TFIID**, **AP-2**, URE3BP, PPARγ	Theuns and Van Broeckhoven, 2000; Santpere et al., 2006; Chen et al., 2013;
MAPT	**SP1, AP-2**, Nrf1, MTF1, MBF1, MepI, GCF	Santpere et al., 2006; Caillet-Boudin et al., 2015

*Shared transcription factors are in bold characters.

important, an $A\beta_{42}$-os propagation within the brains of Tg APP-model mice or WT rats or marmoset (*Callithrix jacchus*) monkeys also obtained via injections of AD brain extracts or punctures made with steel wires coated with the same extracts (Maclean et al., 2000; Meyer-Luehmann et al., 2006; Eisele et al., 2009; Langer et al., 2011; Hamaguchi et al., 2012; Rosen et al., 2012). In such animal models, the diffusion of the injected $A\beta_{42}$-os followed the same route as developing AD pursues in humans, i.e., LEC layer II ⇒ perforant pathway ⇒ hippocampal dentate gyrus and CA3 area ⇒ upper cortical regions (Morrison and Hof, 2007; Khan et al., 2014). Besides, a cerebrovascular amyloidosis was induced after a delay of various months by intraperitoneal injections of 1000-fold higher doses of $A\beta_{42}$-os-charged mouse brain extracts (Eisele et al., 2010). The mechanisms by which misfolded $A\beta_{42}$-os (and p-Tau-os) propagate within the brain are undetermined (Moreno-Gonzalez and Soto, 2011). So far, the prion-like intrabrain spreading potential of $A\beta_{42}$-os appears to be feeble as compared to the infectious capabilities proper of true prions and to require a direct contact with the neural cells (Aguzzi and Rajendran, 2009; Irwin et al., 2013). In addition, an $A\beta_{42}$-os amplifying mechanism has simultaneously to operate in order to assist the prion-like diffusion of the brain amyloidosis (Brettschneider et al., 2015). Hypothetically, this amplifying mechanism might result from or be aided by the $A\beta_{42}$-os own self-induction and self-spreading properties due to their interaction with the CaSRs of neurons, astrocytes, and other brain cell types (Dal Prà et al., 2015a).

Aβ●CaSR SIGNALING PROMOTES INTRA- AND EXTRACELLULAR TOXIC $A\beta_{42}$/$A\beta_{42}$-OS OVERLOADS

By using as preclinical models *in vitro* cortical nontumorigenic NAHAs and postnatal HCN-1A neurons brought to a complete proliferative quiescence, exogenous $A\beta_{25-35}$-os and $A\beta_{1-42}$-os were shown to bind the plasma membrane-inserted CaSRs with a high specificity. These bonds activated CaSR's intracellular signaling pathways which in turn elicited a whole set of pathophysiological effects in both cell types, including the death of the HCN-1A neurons (summarized in **Table 3**; (Dal Prà et al., 2011, 2014a,b, 2015a,b; Armato et al., 2013a; Ruat and Traiffort, 2013). As mentioned, an Aβ-os●CaSR-activated MEK/ERK-dependent pathway mediated NO overproduction in NAHAs; the same signaling stabilized the HIF-1α●HIF-1β TF which then entered the astrocytes' nuclei to trigger a VEGF-A surplus production and secretion (Dal Prà et al., 2005, 2014b).

By contrast, the mechanisms of the increased synthesis, accumulation, and release of $A\beta_{42}$-os elicited through Aβ-os●CaSR signaling in both NAHAs and HCN-1A neurons are not as yet fully understood and currently under investigation. In regard to this topic, an upregulation of the CaSR and an intensified Aβ-os●CaSR signaling induced the death of neurons in rodent models of cerebral ischemia/hypoxia/stroke (Kim et al., 2014). This is a second instance besides LOAD in which Aβ-os●CaSR signaling kills neurons.

NEUROTOXIC Aβ●CaSR INTERACTIONS IN OTHER GLIAL AND CEREBROVASCULAR CELLS

The extremely complex mammalian CNS harbors several distinct cell types. Generally, LOAD discussions focus most on neurons, less on microglia, but leave the other cell types in the shade. However, several authors have tried to broaden this restricted viewing by putting astrocytes into the fray (Busciglio et al., 1993; Blasko et al., 2000; Chiarini et al., 2005; Li et al., 2011; Zhao et al., 2011; Armato et al., 2013a; Dal Prà et al., 2015a). Indeed, in our work we have been using both NAHAs and HCN-1A neurons as separate models to clarify the responses evoked by an exposure to exogenous Aβ-os. Hence, we do not deem astrocytes being more important players than neurons in LOAD promotion. Instead, we have been endorsing a holistic view, i.e., all CNS cell types are likewise important players both in CNS physiology and LOAD pathophysiology. Consequently, we review below relevant knowledge concerning the CaSR with respect to LOAD in the remaining glial cell types and cerebrovascular cells.

Oligodendrocytes

Oligodendrocytes precursors (NG-2 glial cells) generate also protoplasmic astrocytes (**Figure 2**) and maybe neurons, and receive synaptic inputs. These precursors become functionally impaired and/or damaged with aging (reviewed in Cai and Xiao, 2016). When NSCs' differentiation is aimed at the oligodendrocyte lineage, the expression and activity of NSCs' CaSRs are up-regulated, which favors their expansion and differentiation (Chattopadhyay et al., 2008). Human oligodendrocytes keep amplifying their numbers up to 5 years of age, when they amount to ∼5–10% of the total glia. Thereafter, their turnover remains negligible. Typically, oligodendrocytes' myelin production and myelin sheaths' upkeep quickly adapt to ongoing needs, e.g., learning activities—a feature promoting

TABLE 3 | Harmful effects Aβ●CaSR signaling elicits in human neurons and astrocytes.

Cell type	Stimulus	Pathological effect	Effect of adding calcilytic to Aβs	Effect of calcimimetic alone
Neurons, astrocytes	$A\beta_{42}$-os Aβ fibrils	Overproduction and diffuse intracellular accumulation of endogenous $A\beta_{42}$ monomers and $A\beta_{42}$-os due to an increased β-S and γ-S activity and (likely) to decreases in Aβ proteolysis	Total suppression of intracellular accumulation of $A\beta_{42}$ monomers and $A\beta_{42}$-os due to increased Aβ-os proteolysis (no effect on increased β-S and γ-S activities)	No apparent intracellular accumulation of Aβs
Neurons, astrocytes	$A\beta_{42}$-os Aβ fibrils	Concurrent $A\beta_{40}$-os intracellular accumulation	Modest decrease of $A\beta_{40}$-os intracellular accumulation	n. d.*
Neurons, astrocytes	$A\beta_{42}$-os Aβ fibrils	Surplus secretion of $A\beta_{42}$-os, but not of $A\beta_{40}$-os, along the Golgi/*trans*-Golgi pathway and axons ⇒ extracellular $A\beta_{42}/A\beta_{40}$ ratios values shift to the cytotoxic range	Total suppression of surplus release of $A\beta_{42}$-os along the Golgi/*trans*-Golgi pathway and axons, but increased release of $A\beta_{40}$-os⇒ extracellular $A\beta_{42}/A\beta_{40}$ ratios values remain in the normal range (NPS 2143 by itself exerts no effect on basal $A\beta_{42}$-os secretion)	Significant surplus secretion of $A\beta_{42}$-os
Neurons	$A\beta_{42}$-os Aβ fibrils	Slow yet progressive death by apoptosis of the human cortical neurons (*in vivo* this is the cause of cognitive decline; Nelson et al., 2012).	Neurons remain alive and kicking	n. d.
Astrocytes	$A\beta_{42}$-os Aβ fibrils	NAHAs survive and keep making and releasing neuron-harming compounds (see below)	No apparent effect on survival	n. d.
Astrocytes	Aβ fibrils	Increased activity of the glycogen synthase kinase (GSK)-3β, one of the main Tau kinases (Armato et al., 2013b).	Total suppression of the surge of GSK-3β activity (Armato et al., 2013b).	n. d.
Astrocytes	$A\beta_{42}$-os Aβ fibrils	Stabilization and nuclear translocation of the HIF-1α●HIF-1β transcription factor ⇒ expression of VEGF-A, APP, and BACE1 genes ⇒ heightened synthesis/secretion of VEGF-A and $A\beta_{42}/A\beta_{42}$-os	HIF-1α destabilization ⇒ deep yet transient decrease of nuclear HIF-1α●HIF-1β transfer ⇒ no surplus production/release of VEGF-A, APP, and $A\beta_{42}/A\beta_{42}$-os	n. d.
Astrocytes	$A\beta_{42}$-os, Aβ fibrils	Significant yet transient surge of total CASR protein	Downregulation of total CaSR protein: modest and transient with NPS 2143 alone but fast, intense and persistent with Aβs + NPS 2143	No change in total CaSR protein
Astrocytes	$A\beta_{42}$-os Aβ fibrils	Induction and MEK/ERK-dependent activation of GTP cyclohydrolase-1 (GCH1) ⇒ production of BH4 (tetrahydrobiopterin) ⇒ dimerization and activation of the concomitantly induced NO synthase (NOS)-2 ⇒ excess release of NO	Inactivation of GCH1 ⇒ lack of BH4 ⇒ no dimerization and activation of the concomitantly induced NO synthase (NOS)-2 ⇒ no overproduction of NO	n. d.

**n.d., not determined.*

neural plasticity. During AD development, at neurotoxic levels (i.e., in the μM range) Aβ-os switch off the Wnt signaling of the precursors thereby hindering their differentiation into oligodendrocytes (**Figure 2**; Barateiro et al., 2016). In advanced AD, soluble and fibrillar Aβs and/or p-Taues/NFTs together with ongoing oxidative stress and neuroinflammation cause oligodendrocytes' to dysfunction and die via apoptosis (reviewed in Cai and Xiao, 2016). Consequently, myelin sheaths break down first in the hippocampus and fornix, and later in the other involved areas (Roth et al., 2005). The breakdown of myelin sheaths releases myelin basic protein (MBP), which by itself triggers a neurotoxic discharge of NO from cortical NAHAs. This MBP effect is synergistically amplified by a mixture of three proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ) or by soluble $A\beta_{40}$ (Chiarini et al., 2005). So far, the specific neurotoxic effects of Aβ-os●CaSR signaling have not been investigated in human oligodendrocytes, although doing it would be worthwhile. Interestingly, mature oligodendrocytes are able to express APP and to produce and secrete both $A\beta_{42}$ and $A\beta_{40}$ (Skaper et al., 2009). Therefore, besides neurons and astrocytes, oligodendrocytes are the third potentially relevant source of endogenous $A\beta_{42}$-os in LOAD—a source hitherto disregarded perhaps because of its progressive cytotoxic damage and destruction. Thus, a crucial role of Aβ-os●CaSR signaling in the neurotoxic responses and demise of oligodendrocytes in AD remains to be proven.

Microglia

At variance with neural cells, microglia arise from circulating myeloid monocytes which migrated into the CNS during gestation to act there as macrophage equivalents. In their physiologically "quiescent" phenotype, microglia promote

brain development and relevantly upkeep neural environment homeostasis, immunological surveillance, modulation of neuronal proliferation and differentiation, pruning of synapses, and clipping of apoptosing neurons (Saijo and Glass, 2011; Harry, 2013). Microglia become "activated" in response to neural tissue injury and then start sweeping up debris from degenerated neurons and infectious agents (when present), hence favoring tissue repair (Yang et al., 2010; Lee et al., 2011; Derecki et al., 2013; McGeer and McGeer, 2015). Moreover, in AD microglia are persistently activated, keep engulfing extracellularly accumulating fibrillar and soluble Aβs, and surround and infiltrate dense core senile plaques where they promote Aβs fibrillation (reviewed in Rosen et al., 2012). Additionally, both Aβ fibrils and $A\beta_{42}$-os trigger the microglia to secrete proinflammatory cytokines, (e.g., IL-1β, TNF-α, IFN-γ), chemokines (Lindberg et al., 2005; Färber and Kettenmann, 2006; Kawanokuchi et al., 2006; Saijo and Glass, 2011; Heneka et al., 2013; Prokop et al., 2013), ROS, NO, and even N-terminally truncated Aβ-os (Nagele et al., 2004; Mawuenyega et al., 2010; Oberstein et al., 2015). The interaction of ROS and NO generates hyper toxic peroxynitrites ($ONOO^-$). Therefore, both microglia and astrocytes contribute to kindle and keep going the chronic neuroinflammation proper of LOAD brains. Furthermore, the microglial cytokines bind and activate their specific receptors on the surface of the astrocytes which are thus stimulated to produce and release additional amounts of Aβ-os, NO, and VEGF-A besides the amounts of the same agents generated by the astrocytes own Aβ-os●CaSR signaling (Dal Prà et al., 2005, 2015a; Chiarini et al., 2010). In turn, these microglia-elicited astrocytes' secretions sustain and/or intensify microglia activation, starting vicious cycles of astrocytes ⇔ microglia reciprocal interactions. They also stimulate the adjoining ANTs to produce additional $A\beta_{42}$-os that keep spreading and via Aβ-os●CaSR signaling elicit the release of (a) further amounts of $A\beta_{42}$-os from neurons and astrocytes; (b) NO and VEGF-A from astrocytes; (c) MBP and $A\beta_{42}$-os from oligodendrocytes; and (d) proinflammatory cytokines, chemokines, ROS, NO, and N-terminally truncated Aβs from microglia (Blasko et al., 2000; Lindberg et al., 2005; Kawanokuchi et al., 2006; Mandrekar-Colucci and Landreth, 2010; Zhao et al., 2011; Prokop et al., 2013; Oberstein et al., 2015). Thus, self-maintaining and spreading vicious cycles of reciprocal interactions between microglia and ANTs' members keep exerting significant toxic effects on all neural cell types (Nath et al., 2012) strongly promoting as a result LOAD progression (Rojo et al., 2008; Nordberg, 2014). However, likely for cytotoxic reasons microglia's inflammogenic role decreases with the progressing of AD (Mizuno, 2012), and it may even become less relevant than astrocytes' role due to the greater numbers, stronger resistance to toxic agents, and longer-lasting functional activation of the astrocytes (reviewed in Rosen et al., 2012).

Moreover, the specific outcomes of the interactions between exogenous $A\beta_{42}$-os and microglial CaSRs of WT and AD-model rodents are still undefined notwithstanding their potential relevance to LOAD therapy (McGeer and McGeer, 2015). Notably, rat microglia express a functional CaSR capable of modulating a Ca^{2+}-activated K^+ channel (Chattopadhyay et al., 1999; Yano et al., 2004). In this regard, we recall that the BV-2 immortalized murine microglial cell line was reported to constitutively produce and release Aβs. Moreover, and remarkably, adding exogenous $A\beta_{25-35}$ or lipopolysaccharide increased the production and secretion of Aβs from BV-2 microglial cells (Bitting et al., 1996). The authors did not assess the CaSR's role in this process. Nevertheless, the findings of Bitting et al. (1996) and Oberstein et al. (2015) indicate microglia as a likely fourth *source* of Aβ-os in LOAD brains. As far as we know, no study about the CaSR in *human* microglia has been reported. Therefore, the role of Aβ-os●CaSR signaling in microglia deserves further investigations.

Cerebral Vessels

Astrocytic processes' end-feet envelop the cerebral micro vessels forming functional NVUs which govern the delivery of nutrients and oxygen required for the activities of ANTs' neurons (**Figure 1B**). In LOAD, accumulating Aβ42-os, Aβ fibrils, and NO harm the cells of the cerebral vessels eventually causing the onset of a cerebral amyloid angiopathy (CAA) which helps advance LOAD (Nelson et al., 2016). CAA's degenerative changes include perivascular ring-like Aβs casts staving off astrocytic processes' end-feet, increased vessel walls stiffness, weakened responses to astrocyte-released vasodilator agents, impeded neoangiogenesis, and changed BBB permeability, altogether causing local hypoxic/ischemic lesions (Kimbrough et al., 2015; Love and Miners, 2015). The latter favor the production/release of Aβ42-os surpluses, likely via Aβ42-os●CaSR signaling from adjoining ANTs' components thus hastening cognitive decline (reviewed in Helman and Murphy, 2016). The endothelial cells of human aorta and other vessels express the CaSR (Ziegelstein et al., 2006). By inference, the endothelia and other cellular components of the human brain vessels' walls should also express the CaSR. Then again, to our knowledge, no study has specifically addressed the potential toxic effects of Aβ-os●CaSR signaling on the cerebrovascular walls pericytes, smooth muscle cells, and endothelial cells in LOAD. Finally, yet importantly, LOAD-damaged blood vessels can block neurogenesis from NSCs in the subventricular zone and hippocampus (**Figure 2**) thereby thwarting the processing and storage of new memories (Licht and Keshet, 2015).

In summary, the interactive dysfunctional responses of all brain-resident cell types evoked via Aβ-os●CaSR signaling are likely to play significant roles in the promotion of LOAD (Brorson et al., 1995; Dal Prà et al., 2015a).

Aβ-os●CaSR INTERACTIONS ADVANCE LOAD PROGRESSION

During AD development, ANTs' vital functions can turn into grievously troublesome ones. When the "client" neurons and their "master" astrocytes of small *foci* in the LEC's layer II overproduce $A\beta_{42}$ monomers, they start releasing $A\beta_{42}$-os into the synaptic spaces surrounded by the astrocytes' shrouding end-feet and into the extracellular milieu. When $A\beta_{42}$-os spread to the CA1 area, the formation of new memories begins to fail

(Bushong et al., 2002). We already mentioned that ANTs' neurons and astrocytes are endowed with a variety of $A\beta_{42}$-os-binding receptors, including the CaSR (**Table 1**). Next, released $A\beta_{42}$-os scatter from the ANTs of origin to contiguous ANTs, in which they bind and activate the local neurons' and astrocytes' CaSRs (Dal Prà et al., 2015a). As a result, both cell types start producing and releasing additional $A\beta_{42}$-os, which will keep spreading and targeting the CaSRs of neurons and astrocytes of remoter ANTs (**Figure 3**). Remarkably, $A\beta_{42}$-os diffuse not only by contiguity, but also by apparent "jumps" because the blighted hippocampal neurons project their long axons carrying $A\beta_{42}$-os surpluses to the *neocortex* of far-off cerebral lobes (**Figure 1A**). Thus, reiterating feed-forward cycles of this kind which sustain and amplify themselves via $A\beta_{42}$-os•CaSRs interactions end up recruiting the neurons and astrocytes of ever-increasing numbers of ANTs. The latter will make and release still more $A\beta_{42}$-os which will spread to even farther off ANTs. Thus, from the LEC LOAD neuropathology would reach through this basic molecular mechanism upper cerebral cortical areas (Dal Prà et al., 2015a). $A\beta_{42}$-os•CaSRs and $A\beta_{42}$-os•PRP^Cs interactions would next favor the gradual appearance of p-Tau-os which at some later point will acquire via still undefined mechanism(s) the ability to self-induce themselves and spread independently of $A\beta_{42}$-os. Afterwards, both toxic drivers would hasten AD progression toward its gloomy conclusion (Dal Prà et al., 2015a).

PHARMACOLOGICAL CaSR MODULATORS AND LOAD

Various synthetic phenyl alkylamines derivatives endowed with two-to-four aromatic rings and NH^{3+} groups selectively act either as CaSR's type II allosteric *agonists* (or *calcimimetics*; e.g., NPS R-568, Cinacalcet, and AMG 416) or *antagonists* (or *calcilytics*; e.g., NPS 89636, NPS 2143). Such agents shift to the right or to the left, respectively, the CaSR's response curve to changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) (Nemeth, 2002; Saidak et al., 2009; Widler, 2011). These CaSR modulators bind distinct sites in the 7TM region—both calcimimetics and calcilytics between TM6 and TM7, but calcilytics alone between TM3 and TM5 (Petrel et al., 2004). The full therapeutic potential of CaSR modulators has yet to be gauged in human ailments (Saidak et al., 2009; Widler, 2011; Ward et al., 2012; Nemeth, 2015). These agents too can promote the "*ligand-biased signaling*" according to the specific cell type considered—a feature that might favor target-specific therapeutic approaches (Davey et al., 2012; Leach et al., 2015)

Calcimimetics

NPS R-568 and Cinacalcet are presently the best paradigms of allosteric CaSR agonists as they hinder PTH secretion (Nemeth, 2004, 2013). In clinical settings, Cinacalcet has been and still is used to manage primary hyperparathyroidism and secondary hyperparathyroidism due to chronic kidney disease (CKD). It has been used particularly in patients in chronic dialysis, although in some of these cases it failed to be effective (Nemeth and Goodman, 2015; Brunaud et al., 2016). Cinacalcet also averts or reverses parathyroid hyperplasia in rats and functionally rescues CaSR's loss-of function mutations (Nemeth, 2004, 2013; Miller et al., 2012; Nemeth and Shoback, 2013; Palmer et al., 2013; Nemeth and Goodman, 2015; Mayr et al., 2016). However, since CaSR expression is ubiquitous, one should not overlook that calcimimetics (and calcilytics) may exert PTH-independent effects in tissues, brain included, other than the parathyroid glands (Massy et al., 2014). As an example, a Cinacalcet-triggered protracted CaSR signaling curtailed the mitotic activity and interfered with the remodeling and barrier function of oesophageal epithelial cells via catenin-cadherin complexes disruption, actin cytoskeletal changes, and CaSR reallocation to the nuclei (Abdulnour-Nakhoul et al., 2015). Various pieces of evidence discussed in previous sections have denoted the CaSR's involvement in AD onset and progression. Remarkably, calcimimetic NPS R-568 mimics at least one pathological effect of Aβs•CaSR signaling: it significantly increases the amount of $A\beta_{42}$-os secreted by cortical NAHAs (Armato et al., 2013a; Dal Prà et al., 2015a; **Table 2**). The potential clinical implications of this NPS R-568 effect deserve further assessment.

Calcilytics

Compounds like NPS 2143, NPS 89636, Calhex, etc., desensitize the CaSR to $[Ca^{2+}]_e$ changes and characteristically increase PTH secretion (Nemeth, 2004, 2013). Various calcilytics were initially tested as therapeutics for postmenopausal osteoporosis. But, they lacked effectiveness because they elicited a PTH oversecretion which stimulated in parallel both osteogenesis and osteolysis. This stopped any further clinical testing concerning a potential anti-osteoporosis activity of calcilytics (Nemeth, 2004, 2013; Nemeth and Shoback, 2013; Nemeth and Goodman, 2015). Novel indications for calcilytics are (i) idiopathic hypercalciuria; and (ii) autosomal dominant hypocalcaemia due to CaSR's gain-of-function mutations; as for the latter condition calcilytic NPS P-795 is being tested as a therapeutic in clinical trials (White et al., 2009; Letz et al., 2010; Park et al., 2013; Nemeth, 2015; Nemeth and Goodman, 2015). In addition, calcilytics may mitigate the airways hyper responsiveness and inflammation proper of asthma (Yarova et al., 2015). Calcilytics also inhibit the cellular hyper proliferation typical of pulmonary artery idiopathic hypertension (Yamamura et al., 2012, 2015)

A further potential indication of calcilytics is LOAD (Armato et al., 2013a). In fact, we showed that in cultured *human* untransformed cortical NAHAs and HCN-1A neurons calcilytics NPS 2143 and NPS 89696 counteracted *all* the noxious consequences—death of the neurons included—brought about by Aβs•CaSR signaling (**Table 3**; Armato et al., 2013a; Dal Prà et al., 2014a,b, 2015a). These preclinical findings indicate that, by hindering the Aβs•CaSR signaling at the level of neurons, of all glial cell types, and of cerebrovascular cells, calcilytics would effectively suppress (or at least significantly mitigate) the intracerebral propagation of the amyloidosis and its concurrent neurotoxic effects. By keeping the neurons alive and functioning, calcilytics would safeguard the patients' cognitive faculties. Most remarkably, calcilytics would be the so far unique anti-LOAD therapeutics simultaneously targeting a number of

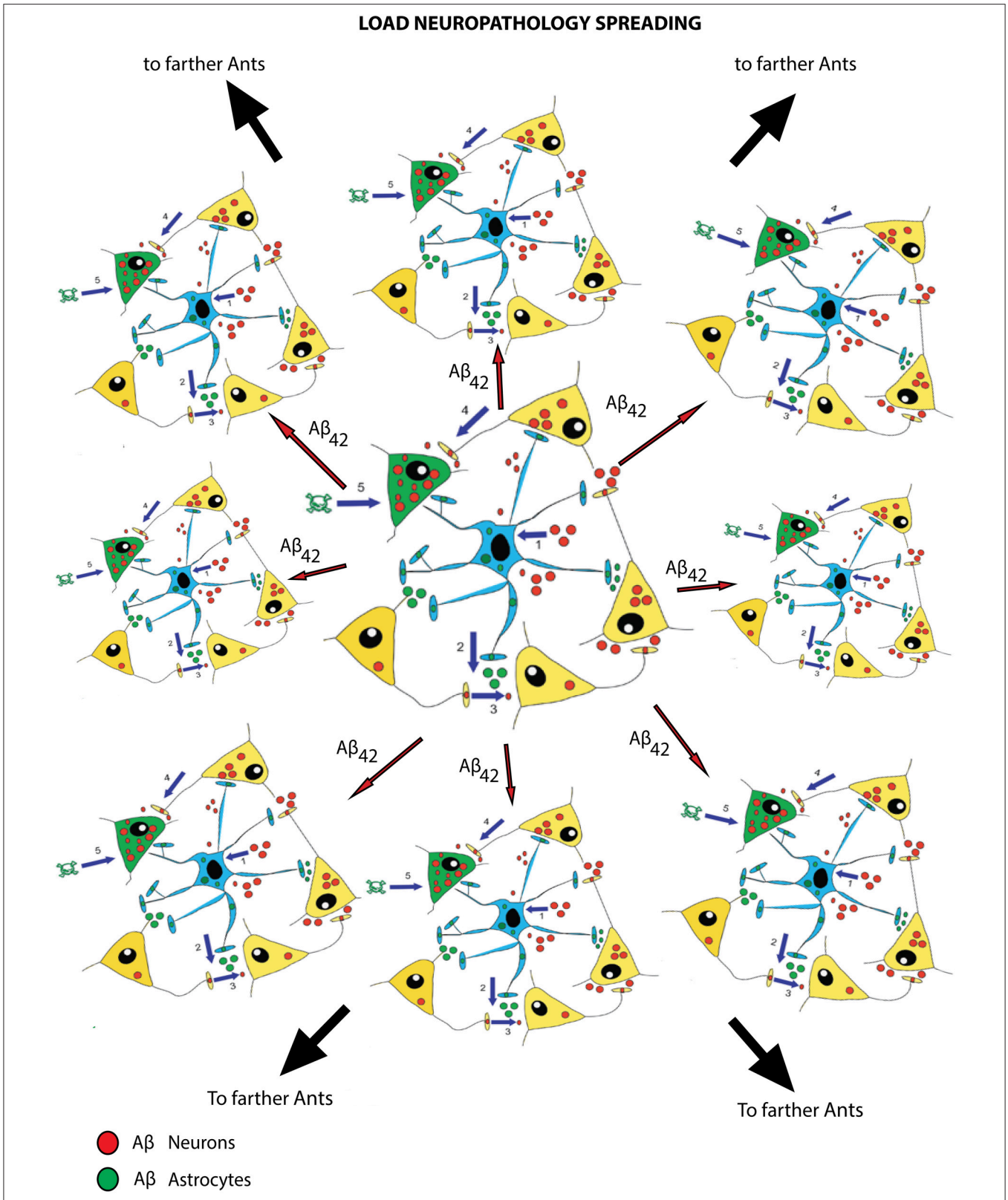

FIGURE 3 | Propagation of LOAD neuropathology to neighboring astrocyte-neurons teams (ANTs). The cartoon shows that an excess of exogenous $A\beta_{42}$-os (here short-termed as $A\beta_{42}$) supposedly reaches first the team of neurons and astrocytes (ANT) at the center and binds their CaSRs (not detailed) triggering signals that end up increasing the secretion of newly produced endogenous $A\beta_{42}$-os (*red and green circles*) from all of the ANT's cellular members *(# 1–5)*.

(Continued)

FIGURE 3 | Continued
Blue arrows indicate the diffusion of $A\beta_{42}$-os from neurons to astrocytes (red solid circles) and from astrocytes to neurons (green solid circles). Numbers 1–5 also suggest possible sequences of events both intra- and inter-ANTs. While the involved cells undergo cytotoxic changes, including the early death of some neurons (*in green color with skull and crossbones aside*), the newly released $A\beta_{42}$-os spread and reach both neighboring and remoter ANTs *(short and long red arrows)*, starting via $A\beta_{42}$-os•CaSR signaling new cycles of surplus production and secretion of endogenous $A\beta_{42}$-os. The latter will disperse and engage nearby and still farther away ANTs (not shown) again triggering the same kind of $A\beta_{42}$-os•CaSR signaling-triggered pathological responses, including additional $A\beta_{42}$-os oversecretion and neuronal deaths. Thus, $A\beta_{42}$-os spread can affect local ANTs (as embodied here by the *short and long red arrows*) or remoter ANTs via projecting axons carrying the $A\beta_{42}$-os (as exemplified here by the *big black arrows*).

LOAD-promoting processes which Aβs•CaSR signaling triggers in all types of CNS cells (Armato et al., 2012, 2013a; Dal Prà et al., 2014a,b, 2015a). Calcilytics reduce neuronal death also in animal models of ischemia/hypoxia/stroke, i.e., in conditions that increase Aβ-os production in the affected brain area(s) (Kim et al., 2014; Bai et al., 2015). These findings too strongly substantiate our hypothesis about the crucial role of the CaSR in AD.

Here, some pharmacological notations are in order. Being lipid-soluble, calcilytics cross the BBB, can be administered by any route (oral, etc.), and in the presence of exogenous soluble Aβ-os or fibrillar Aβs (which also release Aβ-os) selectively antagonize CaSR's signaling and intensely down regulate the CaSRs of human cortical astrocytes, and likely neurons and other CNS cells (Armato et al., 2013a; Dal Prà et al., 2015a). Calcilytic NPS 2143 is well tolerated by rodents (Nemeth, 2002; Kim et al., 2014). Recent NPS 2143 derivatives, which stimulate PTH secretion less intensely, were well withstood by human subjects during phase I and phase II clinical trials aimed at assessing the drugs' anti-osteoporosis activity (in such instances no consideration was given to brain-related effects; Nemeth and Shoback, 2013; John et al., 2014). Of late, the NMDA receptor inhibitors Memantine and Nitromemantine and the Fyn kinase inhibitor Saracatinib (AZD0530) were suggested as therapeutics to offset the neurotoxic actions brought about by extracellularly gathering Aβ-os (Talantova et al., 2013; Kaufman et al., 2015). It should be realized that the calcilytics' target, i.e., the CaSR, holds an *upstream* place with respect to NMDARs and Fyn. Therefore, calcilytics' ability to hinder any extracellular $A\beta_{42}$-os build-up would as well prevent any downstream $A\beta_{42}$-os harmful effects involving NMDARs and Fyn. Moreover, by keeping the extracellular $A\beta_{42}/A\beta_{40}$ ratio values within the physiological range, calcilytics would thwart any cytotoxic effects and hindrance of NSCs differentiation (**Figure 2**) and of functions necessary for neurogenesis to occur in the dentate gyrus subgranular zone. Calcilytics would also safeguard the structural and functional integrity of cognition-critical upper cerebral cortical areas (Choi et al., 2013; Lee et al., 2013; Barateiro et al., 2016). In short, calcilytics would preserve the patients' ability to store and retrieve memories and to cope with daily needs, thus improving her/his life's quality and prospects.

Calcilytics' failure as therapeutics for osteoporosis due to the double-edged effects of PTH was a stroke of ill-luck (Nemeth, 2004, 2013; Nemeth and Shoback, 2013; Nemeth and Goodman, 2015). In addition, calcilytics' potential side effects—e.g., mild hyperparathyroidism in humans, hypertension in rats—shied people away from considering their use in clinical settings. However, calcilytics' rather mild side effects must be carefully weighed against the harsh fact that *symptomatic LOAD inexorably kills the patient cognitively several years before her/his actual physical demise*. Hence, just as anticancer chemotherapeutics are used notwithstanding their potential side effects, once clinical trials have proven calcilytics therapeutic effectiveness, their side effects will be a trivial toll against preventing/stopping LOAD progression.

CONCLUSIONS AND FUTURE PERSPECTIVES

Astrocytes' and neurons' pathophysiology in LOAD brains are quite intricate and specific for each animal species, brain area, aging phase, and stage of the illness. Therefore, a deeper understanding of AD-related metabolic events occurring in human cortical untransformed astrocytes and neurons has helped and will help identify ground breaking therapeutic approaches to LOAD. Differently from neurons, human astrocytes survive for lengthy terms the exposure to toxic amounts (in the μM range) of soluble or fibrillar Aβs while undergoing complex and only partially understood functional changes collectively defined as *activation*. The latter include, amongst others, alterations of (a) the Aβ•α7-nAChR signaling affecting the intra- and intercellular Ca^{2+} signaling and gliotransmitters secretion, and (b) the Aβ•CaSR signaling triggering a surplus production and secretion of neurotoxic $A\beta_{42}$-os, VEGF-A, and NO. However, since the CaSR is endowed with panoply of intracellular signaling pathways, we undertake that not all of the toxic metabolic effects prompted by Aβ•CaSR signaling have been yet identified in human neural cells. Moreover, the interactions of Aβs with receptors other than the CaSR and/or Aβs-mediated non-receptorial mechanisms add other neurotoxic factors (e.g., proinflammatory cytokines, chemokines, ROS, etc.) which confound the picture. Collectively, these manifold metabolic responses reveal the deep involvement of astrocytes in LOAD's promotion. This view is strengthened by a recent report demonstrating that, while human neurons release only $A\beta_{1-42}$, human astrocytes secrete a remarkable amount of N-terminally truncated Aβs, including $A\beta_{3-42}$ moieties that are transformed into the utterly toxic pE-$A\beta_{3-42}$ (Gunn et al., 2010; Morawski et al., 2014; Oberstein et al., 2015). However, here one should not overlook that oligodendrocytes and microglia and the cellular components of cerebral vessels also express the CaSR. Therefore, toxic Aβ•CaSR interactions do also occur at the level of the latter cell types which may induce the

production and release of further amounts of $A\beta_{42}$-os thereby helping advance AD progression. This field is worth exploring further because white matter damage, neuroinflammation, and local hypoxia/ischemia/stroke play relevant roles in LOAD pathophysiology.

It is noteworthy that allosteric CaSR antagonists or calcilytics can suppress upstream all the downstream toxic consequences of Aβ•CaSR signaling in both human neurons and astrocytes, and likely might do the same in all other neural and vascular cell types of the CNS. These findings make us posit that calcilytics would effectively prevent LOAD amyloidosis from spreading. Moreover, by hindering $A\beta_{42}$-os accumulation and diffusion calcilytics would prevent also the ensuing appearance and spread of the p-Tau-os and their lethal cooperation with $A\beta_{42}$-os (Dal Prà et al., 2005, 2014a,b, 2015a; Armato et al., 2013a).

In conclusion, these findings attest the need to increase our understanding of CaSR's pathophysiology in all types of human untransformed neural cells. In fact, one cannot disregard that meanwhile LOAD is flaring up worldwide in an epidemic-like fashion. Therefore, it would be timely to validate the anti-LOAD effectiveness of calcilytics in clinical trials recruiting aMCI and/or early symptomatic patients.

AUTHOR CONTRIBUTIONS

AC, ID, and UA contributed equally to the manuscript's conception. DL made searches and helped with the manuscript.

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frontiers in Physiology

MINI REVIEW
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Calcium-Sensing Receptor Regulates Cytosolic [Ca^{2+}] and Plays a Major Role in the Development of Pulmonary Hypertension

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Pulmonary arterial hypertension (PAH) is a progressive disease characterized by elevated pulmonary vascular resistance (PVR) leading to right heart failure and premature death. The increased PVR results in part from pulmonary vascular remodeling and sustained pulmonary vasoconstriction. Excessive pulmonary vascular remodeling stems from increased pulmonary arterial smooth muscle cell (PASMC) proliferation and decreased PASMC apoptosis. A rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in PASMC is a major trigger for pulmonary vasoconstriction and a key stimulus for PASMC proliferation and migration, both contributing to the development of pulmonary vascular remodeling. PASMC from patients with idiopathic PAH (IPAH) have increased resting $[Ca^{2+}]_{cyt}$ and enhanced Ca^{2+} influx. Enhanced Ca^{2+} entry into PASMC due to upregulation of membrane receptors and/or Ca^{2+} channels may contribute to PASMC contraction and proliferation and to pulmonary vasoconstriction and pulmonary vascular remodeling. We have shown that the extracellular Ca^{2+}-sensing receptor (CaSR), which is a member of G protein-coupled receptor (GPCR) subfamily C, is upregulated, and the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ is enhanced in PASMC from patients with IPAH in comparison to PASMC from normal subjects. Pharmacologically blockade of CaSR significantly attenuate the development and progression of experimental pulmonary hypertension in animals. Additionally, we have demonstrated that dihydropyridine Ca^{2+} channel blockers (e.g., nifedipine), which are used to treat PAH patients but are only effective in 15–20% of patients, activate CaSR resulting in an increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC, but not normal PASMC. Our data indicate that CaSR functionally couples with transient receptor potential canonical (TRPC) channels to mediate extracellular Ca^{2+}-induced Ca^{2+} influx and increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC. Upregulated CaSR is necessary for the enhanced extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and the augmented proliferation of PASMC in patients with IPAH. This review will highlight the pathogenic role of CaSR in the development and progression of PAH.

Keywords: Ca^{2+}-sensing receptor, store-operated calcium channel, smooth muscle cells, G-protein coupled receptor, pulmonary artery, pulmonary arterial hypertension

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INTRODUCTION

Under normal physiological conditions, the pulmonary circulation is maintained in a high flow, low pressure, and low resistance state. During conditions of increased blood flow or cardiac output (e.g., during exercise), vasodilation and recruitment (opening of closed blood vessels) of pulmonary arteries are two important mechanisms for reducing pulmonary vascular resistance (PVR). However, under pathological conditions, increased PVR is a major cause for the development of pulmonary hypertension. Elevated PVR over time leads to right ventricular hypertrophy and right heart failure.

Pulmonary hypertension is defined by a mean pulmonary arterial pressure (PAP) $\geq$25 mmHg at rest, as measured by right heart catheterization. Pulmonary arterial hypertension (PAH) describes a subpopulation of patients with pulmonary hypertension, and is hemodynamically defined by a resting mean PAP $\geq$25 mmHg, pulmonary arterial wedge pressure $\leq$15 mmHg, and PVR >3 Wood units (equivalent to mmHg·min/l) (Hoeper et al., 2013). Under the current clinical classification system established at the fifth World Symposium on Pulmonary Hypertension held in Nice, France in 2013, PAH comprises a group of uncommon conditions characterized by obliterative vasculopathy of the small pulmonary arteries. PAH can be idiopathic (IPAH, when no etiological factors are identified), heritable, induced by drugs or toxins, or be related to conditions, such as connective tissue diseases, congenital heart diseases, portal hypertension, or HIV infection (Simonneau et al., 2013). The population prevalence of PAH is estimated 15–50 cases per million, and the incidence is 2–7 cases per million, making PAH a rare disease (Humbert et al., 2006; Peacock et al., 2007). According to the Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL Registry), an observational US disease registry providing current information about demographics, disease course, and management of patients with PAH, the mean age at diagnosis of PAH is 50.1 $\pm$ 14.4 years, with a female to male ratio of 3:1 and a 5-year survival rate from time of diagnosis of 57% (Mcgoon et al., 2008; Badesch et al., 2010; Farber et al., 2015).

PATHOLOGICAL CHANGES IN THE PULMONARY VASCULATURE IN PATIENTS AND ANIMALS WITH PULMONARY HYPERTENSION

Although the subcategories of PAH originate from different underlying causes, all forms of PAH can be characterized by a combination of sustained pulmonary vasoconstriction, excessive pulmonary vascular remodeling, *in situ* thrombosis, and arterial wall stiffening (Humbert et al., 2004; Schermuly et al., 2011). Patients with PAH have been shown to have reduced levels of vasodilatory mediators, such as prostaglandin I_2 and nitric oxide, and increased levels of the potent vasoconstrictors thromboxane, rho-kinase, and endothelin 1 (Christman et al., 1992; Steudel et al., 1997). Additionally, in patients with IPAH and animals with experimental pulmonary hypertension, abnormalities in K^+ and Ca^{2+} channels have been linked with pathological pulmonary vasoconstriction (Yuan et al., 1998; Yu et al., 2004).

Controlling the balance between cell proliferation and apoptosis of pulmonary arterial fibroblasts, pulmonary arterial smooth muscle cells (PASMC), and pulmonary arterial endothelial cells is essential for maintaining normal structural and functional integrity of the pulmonary vasculature. If the balance is tipped in favor of cell proliferation, thickening of the wall, luminal narrowing, and eventual obliteration can occur. These structural changes that lead to hypertrophy and/or luminal occlusion are referred to as pulmonary vascular remodeling (Yu et al., 2004; Masri et al., 2007). The cellular and molecular mechanisms that lead to vascular remodeling are extremely complex, however it is generally understood that K^+ channels play a pivot role in this process as they are regulators of vessel tone, cell proliferation and apoptosis. Downregulation of K^+ channels is linked to sustained depolarization due to enhanced Ca^{2+} entry and diminished K^+ efflux, which promotes cell proliferation and inhibits apoptosis, respectively. Increased proliferation and hypertrophy of PASMC have been implicated in the development of PAH and these processes, like vasoconstriction, relate in part to disturbed Ca^{2+} homeostasis (Kuhr et al., 2012). We have recently shown that the extracellular Ca^{2+}-sensing receptor (CaSR) is upregulated in PASMC and lung tissue from patients with IPAH (Yamamura et al., 2012).

CHARACTERISTICS OF CaSR AS A GPCR

CaSR is a G-protein coupled receptor (GPCR) and a member of family C of the GPCR acids. It has a large extracellular domain that is approximately 600 amino acids long. This large extracellular domain is composed of a bi-lobed Venus-flytrap-like domain which is connected to the seven-transmembrane-domain by a cysteine rich region. The intracellular domain of CaSR contains the COOH domain which is 216 amino acids long (Hendy et al., 2009). The CaSR functions as a dimer in which the venus-flytrap-like domains of each monomer interact. Ca^{2+} binds in the cleft of the venus-flytrap-like domain and causes a conformational change in CaSR which instigates cells signaling events (Hendy et al., 2009). The CaSR couples to G proteins to activate or inhibit multiple intracellular signaling pathways.

CaSR was originally discovered in the parathyroid gland were it maintains Ca^{2+} levels in the blood by adjusting release of parathyroid hormone. When CaSR senses changes in extracellular Ca^{2+} levels, parathyroid chief cells release parathyroid hormone which normalizes Ca^{2+} levels by its actions on kidneys, bones, and indirectly, intestines (Brown et al., 1993; Kurokawa, 1994; Saidak et al., 2009). The primary physiological functions of CaSR are to detect changes in extracellular Ca^{2+} levels, modulate release of parathyroid hormone, and maintain constant blood Ca^{2+} levels. CaSR

is widely expressed and mutations in CaSR have been associated with several diseases. Inactivating mutations in CaSR cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism, while activating mutations cause autosomal dominant hypocalcemia and hypocalcemic hypercalciuria (Hendy et al., 2009).

CaSR SIGNALING

As a member of the Class C family of GPCRs, CaSR signals to downstream pathways via three main groups of heterotrimeric G-proteins, $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$ (Conigrave and Ward, 2013; **Figure 1**). CaSR-mediated activation of $G_{q/11}$ leads to activation of phospholipase Cβ (PLCβ) resulting in conversion of phosphatidylinositol 4,5 bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Kifor et al., 2001; Thomsen et al., 2012). This results in an increase in the intracellular Ca^{2+} concentration and phosphorylation of protein kinase C (PKC), which then activates the mitogen-activated protein kinase (MAPK) signaling cascade ultimately resulting in phosphorylation and activation of ERK1/2 (Kifor et al., 2001; Thomsen et al., 2012). CaSR-mediated activation of $G_{i/o}$ results in inhibition of adenylate cyclase (AC) leading to decreased conversion of ATP to cAMP and decreased protein kinase A activity (Kifor et al., 2001; Thomsen et al., 2012). The β/γ subunits of $G_{i/o}$ activate Ras leading to MAPK activation and ERK1/2 phosphorylation. Upon activation of CaSR, the GTP-bound α subunit of $G_{12/13}$ causes RhoGEF to translocate to the plasma membrane and activates its guanine nucleotide exchange factor (GEF) activity. RhoGEF then activates RhoA by catalyzing the exchange of GDP for GTP (Siehler, 2009). Downstream targets of RhoA include ROCK which mediates cell contraction by inhibiting myosin light chain phosphatase activity, resulting in increased myosin light chain phosphorylation. ROCK also plays a critical role in the polymerization and stabilization of actin filaments by direct activation of mDia (mammalian homolog of Drosophila diaphanous) and indirect phosphorylation of the depolymerizing factor cofilin, respectively (Jernigan and Resta, 2014).

CaSR-mediated activation of downstream signaling pathways results in increased intracellular Ca^{2+} concentration, increased Ca^{2+} sensitivity, cell contraction, and cell proliferation (**Figure 2**). Increased synthesis of IP_3 and DAG results in the binding of IP_3 to the IP_3 receptor (IP_3R) on the sarcoplasmic reticulum (SR) releasing Ca^{2+} from the SR to the cytosol. In PASMC, depletion of Ca^{2+} from the SR induces store-operated Ca^{2+} entry (SOCE) through store-operated Ca^{2+} channels (SOC). DAG activates receptor-operated Ca^{2+} channels (ROC) in the plasma membrane resulting in receptor-operated Ca^{2+} entry (ROCE). A rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in PASMC is a major trigger for pulmonary vasoconstriction and a key stimulus for PASMC proliferation. Several studies have demonstrated that resting $[Ca^{2+}]_{cyt}$, SOCE, and ROCE are all increased in PASMC isolated from IPAH patients (Yu et al., 2004; Zhang et al., 2007; Song et al., 2011).

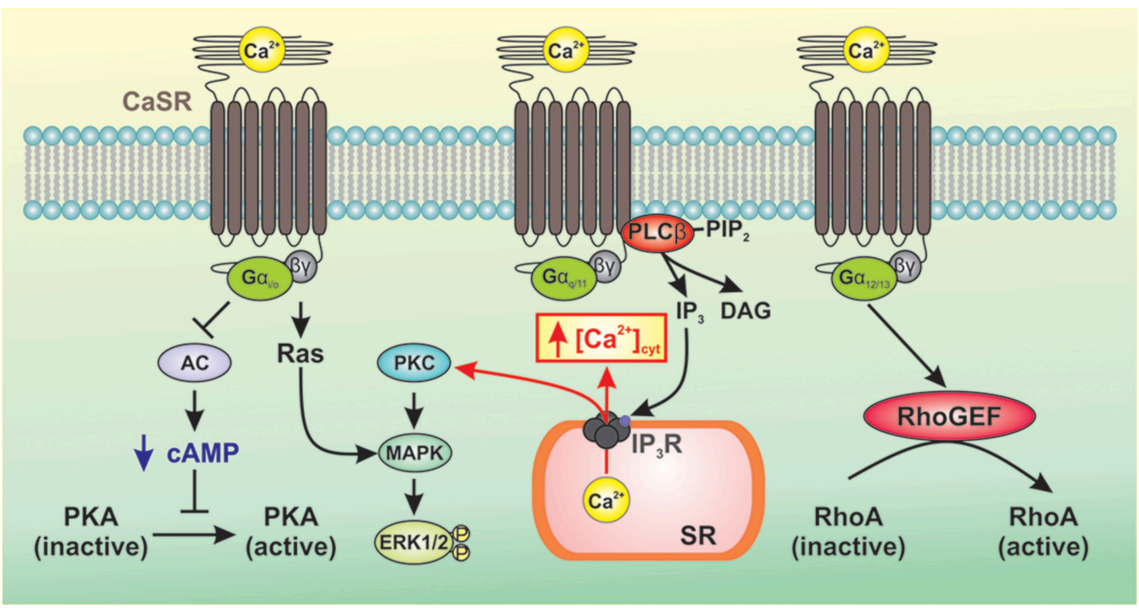

FIGURE 1 | Generalized representation of CaSR-mediated signaling pathways. CaSR signals to downstream pathways via three main groups of heterotrimeric G-proteins, $G_{q/11}$, $G_{i/o}$, and $G_{12/13}$. CaSR-mediated activation of $G_{q/11}$ leads to activation of PLCβ resulting in production of IP_3 which mobilizes cytosolic Ca^{2+} from intracellular Ca^{2+} stores and phosphorylation of PKC, thereby activating MAPK and subsequent phosphorylation and activation of ERK1/2. CaSR-mediated activation of $G_{i/o}$ inhibits AC, which reduces levels of cAMP and PKA activity. The β/γ subunits of $G_{i/o}$ activate Ras leading to MAPK activation and ERK1/2 phosphorylation. Activation of $G_{12/13}$ causes RhoGEF to translocate to the plasma membrane where it activates GEF. RhoGEF then activates RhoA by catalyzing the exchange of GDP for GTP.

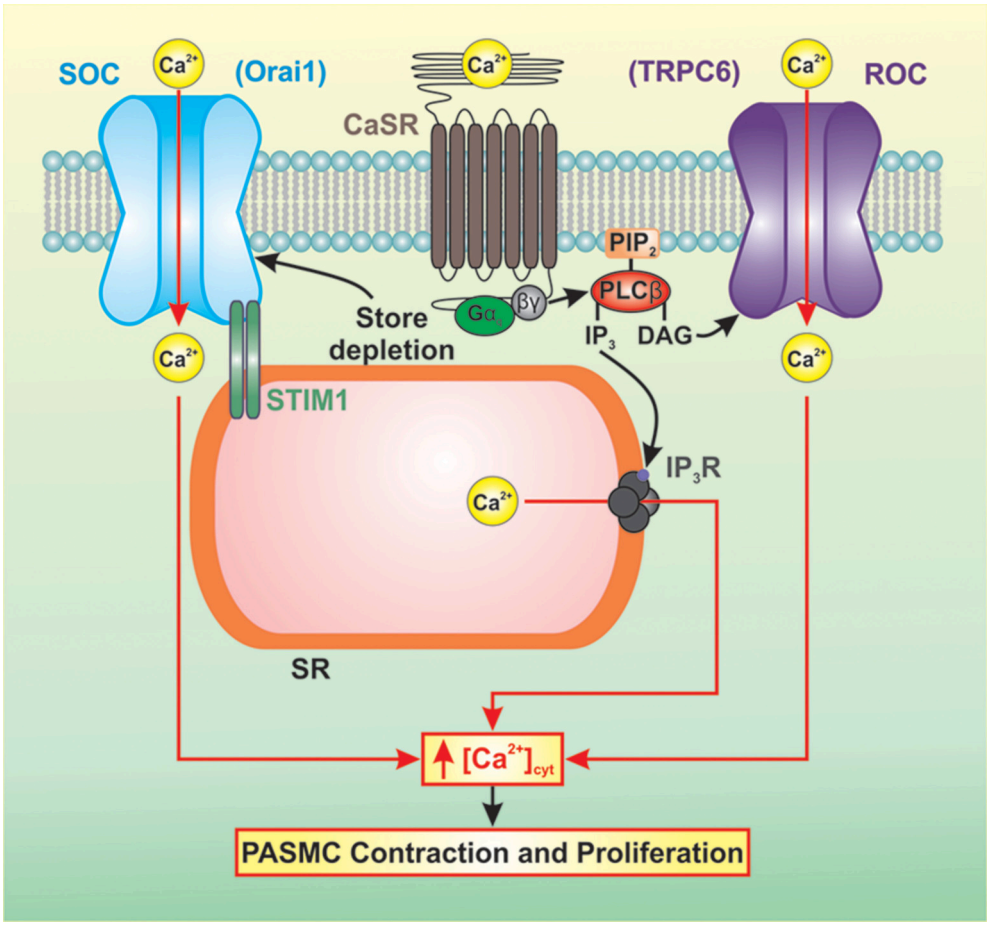

FIGURE 2 | CaSR-mediated activation of downstream signaling pathways results in increased intracellular Ca^{2+} concentration, increased Ca^{2+} sensitivity, cell contraction, and cell proliferation. Increased synthesis of IP_3 and DAG results in elevated $[Ca^{2+}]_{cyt}$ via Ca^{2+} release and Ca^{2+} entry. IP_3 binds to the IP_3R on the SR releasing Ca^{2+} from the SR to the cytosol. Depletion of Ca^{2+} from the SR induces SOCE through SOCs. DAG activates ROCs in the plasma membrane resulting in ROCE. This rise in $[Ca^{2+}]_{cyt}$ in PASMC is a major trigger for pulmonary vasoconstriction and a key stimulus for PASMC proliferation.

UPREGULATED EXPRESSION AND ENHANCED FUNCTION OF CaSR IN PATIENTS WITH IPAH AND ANIMALS WITH EXPERIMENTAL PH

Our studies have demonstrated that application of extracellular Ca^{2+} increased $[Ca^{2+}]_{cyt}$ in PASMC isolated from IPAH patients compared to PASMC from control patients and patients with chronic thromboembolic pulmonary hypertension (Yamamura et al., 2012). We found that this is due to increased expression of CaSR in PASMC and lung tissues from patients with IPAH. The extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in IPAH PASMC is dependent on PLC and IP_3R. Downregulation of CaSR in IPAH PASMC via siRNA reversed the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and attenuated the enhanced proliferation of IPAH PASMC (Yamamura et al., 2012). Additionally, overexpression of CaSR augmented the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and enhanced proliferation in normal PASMC (Yamamura et al., 2012). These data demonstrate that increased expression of CaSR and subsequently enhanced CaSR-mediated $[Ca^{2+}]_{cyt}$ increase contribute to the enhanced Ca^{2+} signaling and proliferation of PASMC in patients with IPAH.

Using two experimental models of PH, we confirmed the role of CaSR in the development of PH. CaSR mRNA and protein levels are increased in PASMC and small pulmonary arteries in the rat model of monocrotoline-induced pulmonary hypertension (MCT-PH) compared to vehicle injected control rats. Additionally, resting $[Ca^{2+}]_{cyt}$ and extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ were both enhanced in PASMC from MCT-PH rats, implying that functional upregulation of CaSR in PASMC contributes to the development of pulmonary hypertension in rats injected with MCT (Yamamura et al., 2012). Similar results were seen in the mouse model of hypoxia-induced pulmonary hypertension (HPH). Expression of CaSR mRNA and protein was significantly increased in HPH mice than in normoxic control mice, and extracellular Ca^{2+}-induced increase

in $[Ca^{2+}]_{cyt}$ was significantly enhanced in PASMC isolated from HPH mice (Yamamura et al., 2012). These data suggest that CaSR is functionally upregulated in PASMC from HPH mice. Pharmacological blockade of CaSR with NPS 2143, a CaSR antagonist, significantly attenuated the development of PH in both the rat MCT-PH model and the mouse HPH model. Treatment with NPS 2143 significantly inhibited the increase in right ventricular systolic pressure (RVSP, an indicator of pulmonary arterial pressure) and inhibited the ratio of the weight of the right ventricle to left ventricle plus septum (Fulton's index, an indicator of right ventricular hypertrophy) in MCT-PH rats and HPH mice (Yamamura et al., 2012). Collectively, these data indicate that increased expression of CaSR may play a pathogenic role in the development of PH and that antagonists of CaSR may therapeutic potential for patients with PH.

CaSR FUNCTIONALLY COUPLES TO TRPC CHANNELS TO REGULATE $[Ca^{2+}]_{CYT}$ IN PATIENTS WITH IPAH AND ANIMALS WITH EXPERIMENTAL PH

Upon activation by extracellular stimuli, CaSR induces activation of downstream signaling pathways which result in increased $[Ca^{2+}]_{cyt}$. The increased $[Ca^{2+}]_{cyt}$ results from Ca^{2+} release from intracellular stores and Ca^{2+} influx through ROC and SOC. Transient receptor potential conical (TRPC) channels have been shown to function as both ROC and SOC in PASMC (Yu et al., 2003, 2004). We recently discovered that CaSR functionally interacts with TRPC6 channels in PASMC from IPAH patients and animals with experimental PH and may play important role in the development and progression of sustained pulmonary vasoconstriction and pulmonary vascular remodeling (Tang et al., 2016). As discussed earlier, extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ is enhanced in IPAH-PASMC due to increased expression of CaSR (Yamamura et al., 2012). Blockade of TRPC6 channels significantly inhibits extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC (Tang et al., 2016). Overexpression of CaSR or TPRC6 in normal PASMC results in enhanced extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$, however, overexpression of both CaSR and TRPC6 dramatically increases extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ compared to either alone (Tang et al., 2016). These data strongly suggest that CaSR is functionally coupled to TRPC6 channels in IPAH-PASMC and that receptor—and store-operated Ca^{2+} entry via CaSR-mediated activation of TRPC6 is an important signaling cascade which leads to PASMC contraction, proliferation, and migration in patients with IPAH.

Studies in experimental models of PH revealed that CaSR plays a role in the development of PH. Using CaSR and parathyroid hormone double knockout mice ($casr^{-/-}/pth^{-/-}$; $casr^{-/-}$ mice are embryonic lethal) (Ho et al., 1995; Kos et al., 2003), we demonstrated that deletion of CaSR inhibited the development of PH in mice exposed to chronic hypoxia. Exposure of $casr^{-/-}$ mice (i.e., $casr^{-/-}/pth^{-/-}$ mice) to chronic hypoxia results in attenuated RVSP, Fulton's Index, and pulmonary arterial wall thickness compared to chronically hypoxic wildtype mice (Tang et al., 2016).

TARGET CaSR TO DEVELOP NOVEL THERAPEUTIC APPROACH FOR PAH

Calcium channel blockers (CCBs) have been successfully administered as a long term therapy to a number of PAH patients who respond positively to acute vasodilator challenge (Puri et al., 2007). Unfortunately only 10–15% of PAH patients actually meet the criteria, and within this subgroup only about half the patients exhibit any sustained benefit from using CCBs (Sitbon et al., 2005). Due to advances in the understanding of the pathological mechanisms of PAH, drug therapy for PAH has progressed in recent years via the development of several specific drugs that offer an effective alternative to CCBs, such as nifedipine and diltiazem. Most recently, efforts have turned toward the use of CaSR antagonists, also known as calcilytics, as potential drug candidates for treatment of PAH. Two structurally distinct calcilytics NPS2143 and Calhex 231 were shown to suppress excessive cell proliferation of IPAH-PASMCs, but not in normal or CTEPH (chronic thromboembolic pulmonary hypertension) PASMCs, whereas R568, an activator of CaSR, significantly enhanced the proliferation of IPAH-PASMCs (Yamamura et al., 2015). Additionally, NPS2143 but not R568 was shown to attenuate, the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ rise in IPAH-PASMC. Furthermore, intraperitoneal injection of NPS2143 prevented the development of pulmonary hypertension and right ventricular hypertrophy in MCT-PH rats and CH-PH mice (Yamamura et al., 2012). Phosphodiesterase type 5 (PDE5) inhibitors are widely used to treat IPAH patients. Sildenafil inhibits excessive cell proliferation of IPAH-PASMC, but does not affect the cell growth of PASMC from normal subjects and CTEPH patients. In combination with NPS2143 or Calhex 231, the antiproliferative effect induced by sildenafil was significantly enhanced in IPAH-PASMC (Yamamura et al., 2016). These findings reveal that CaSR antagonists and PDE5 inhibitors work together to additively suppress the excessive cell proliferation of IPAH-PASMC, suggesting that a combination therapy of a PDE5 inhibitor with a calcilytic may be useful as a novel therapeutic approach for IPAH.

SUMMARY AND CONCLUSION

An atypical rise in $[Ca^{2+}]_{cyt}$ is a major cause for sustained pulmonary vasoconstriction, and excessive pulmonary vascular remodeling observed in patients of IPAH. Our group has previously shown that expression of CaSR, a member of the GPCR family, is upregulated in PASMC isolated from IPAH patients and in animal models of pulmonary hypertension. Additionally, CaSR has been shown to be necessary for the increased extracellular Ca^{2+} influx and subsequent elevation of $[Ca^{2+}]_{cyt}$, that in turn leads to enhancement of cellular proliferation, contraction, and migration in IPAH-PASMC. Therefore, it is feasible to say that upregulated CaSR in PASMC presents a novel pathogenic mechanism

contributing to sustained vasoconstriction and excessive vascular remodeling seen in IPAH patients. Our group has also shown that CaSR regulates ROCE and SOCE via activation of TRPC6 in IPAH-PASMC from patients and animals with experimental pulmonary hypertension suggesting a functional coupling between CaSR and TRPC6. Pharmacological blockade and targeted deletion of CaSR has been shown to inhibit the CaSR-mediated increase in $[Ca^{2+}]_{cyt}$ and attenuates the development of experimental-induced pulmonary hypertension in animal models of the disease. Thus, pharmacological targets of CaSR may reveal novel therapeutic strategies for controlling aberrant Ca^{2+} signaling observed in PAH patients.

FUTURE RESEARCH DIRECTION

While it has been demonstrated that both CaSR and TRPC6 are upregulated in PASMC from IPAH patients and animals with experimental pulmonary hypertension and play a critical role in the pathogenesis of PAH, it remains unknown how this is achieved. The human CaSR gene, CASR, can be transcriptionally regulated by transcription factors, post-transcriptionally regulated by microRNAs and epigenetically regulated by DNA methylation (Hendy et al., 2009). The promoter region of CASR contains numerous binding sites of transcription factors that are associated with cell proliferation (Fantozzi et al., 2003; Firth et al., 2010; Crosswhite and Sun, 2014). Not surprisingly, several of these transcription factors have been demonstrated to be upregulated in PASMC from patients with IPAH and animals with experimental PH (Yu et al., 2003; Firth et al., 2010; Crosswhite and Sun, 2014; Zabini et al., 2015). It is likely these upregulated transcription factors bind directly to the promoter of CASR and activate CASR transcription. MicroRNAs (miRNA or miR) are another area of interest as they can potentially regulate the CaSR mRNA and protein level by directly binding to the 3′-UTR of CaSR mRNA thereby decreasing expression levels via inhibition of protein translation or increase the rate of mRNA degradation. Using the *in silico* prediction approach (provided by microrna.org), we have identified several miRNAs that may potentially target CASR. Further efforts should focus on *in vitro* and *in vivo* approaches to examine the potential involvement of upregulated/downregulated miRNAs in the upregulation of CASR shown in PASMC from IPAH patients and animals with experimental PH.

AUTHOR CONTRIBUTIONS

KS: Drafted Manuscript. KS, RA, HT, AM, JY: Edited Manuscript. KS, RA, HT, AM, JY: Approved Final Submission.

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REVIEW
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Calcium-Sensing Receptor in Breast Physiology and Cancer

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The calcium-sensing receptor (CaSR) is expressed in normal breast epithelial cells and in breast cancer cells. During lactation, activation of the CaSR in mammary epithelial cells increases calcium transport into milk and inhibits parathyroid hormone-related protein (PTHrP) secretion into milk and into the circulation. The ability to sense changes in extracellular calcium allows the lactating breast to actively participate in the regulation of systemic calcium and bone metabolism, and to coordinate calcium usage with calcium availability during milk production. Interestingly, as compared to normal breast cells, in breast cancer cells, the regulation of PTHrP secretion by the CaSR becomes rewired due to a switch in its G-protein usage such that activation of the CaSR increases instead of decreases PTHrP production. In normal cells the CaSR couples to $G\alpha_i$ to inhibit cAMP and PTHrP production, whereas in breast cancer cells, it couples to $G\alpha_s$ to stimulate cAMP and PTHrP production. Activation of the CaSR on breast cancer cells regulates breast cancer cell proliferation, death and migration, in part, by stimulating PTHrP production. In this article, we discuss the biology of the CaSR in the normal breast and in breast cancer, and review recent findings suggesting that the CaSR activates a nuclear pathway of PTHrP action that stimulates cellular proliferation and inhibits cell death, helping cancer cells adapt to elevated extracellular calcium levels. Understanding the diverse actions mediated by the CaSR may help us better understand lactation physiology, breast cancer progression and osteolytic bone metastases.

Keywords: calcium-sensing receptor, breast cancer, parathyroid hormone-related protein, mammary gland, lactation

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INTRODUCTION

The extracellular calcium-sensing receptor (CaSR) is a G-protein-coupled receptor (GPCR) that was first identified because of its ability to regulate parathyroid hormone (PTH) secretion in response to changes in extracellular free calcium (Ca^{2+}) (Brown et al., 1993; Brown, 2013). It belongs to class C of the GPCR superfamily, which also includes metabotropic glutamate receptors (mGluRs), GABA B receptors, taste receptors and pheromone receptors (Pi et al., 2005; Brauner-Osborne et al., 2007). The CaSR has been shown to bind and signal in response to extracellular Ca^{2+} concentrations within the physiologic range (Brown and MacLeod, 2001). It can also be activated by a variety of other cations and cationic compounds such as gadolinium, amino acids, spermine and certain antibiotics (Brown and MacLeod, 2001; Ward et al., 2002). The receptor appears to act as a homodimer, or perhaps a tetramer, and has also been shown to heterodimerize to form hybrid, signaling complexes with other class C GPCRs (Bai et al., 1998, 1999; Gama et al., 2001; Chang et al., 2007). The CaSR complex is sensitive to

allosteric modulation that can alter its sensitivity to direct agonists such as extracellular Ca^{2+}, an aspect of its biology that has been exploited to develop therapeutic agents such as Cinacalcet (Brown and MacLeod, 2001; Filopanti et al., 2013).

The CaSR is highly expressed by parathyroid glands, bone cells and the kidney where it acts to regulate PTH secretion, bone turnover and renal calcium handling in order to coordinate systemic calcium metabolism and to maintain a stable Ca^{2+} concentration in the extracellular fluid. In addition to its role as a master regulator of calcium metabolism, a large body of evidence also supports its involvement in the regulation of diverse processes, such as cellular proliferation, differentiation, apoptosis, hormone secretion, gene expression and ion transport in many different organs (Brown and MacLeod, 2001; Hofer and Brown, 2003; Brennan and Conigrave, 2009; Breitwieser, 2012; Chakravarti et al., 2012; Brennan et al., 2013). Interestingly, at sites other than the parathyroid gland, the CaSR often regulates the secretion of parathyroid hormone-related protein (PTHrP) (Wysolmerski, 2012), a locally produced cytokine growth factor that is evolutionarily related to PTH and uses the same Type 1 PTH/PTHrP receptor (PTH1R). In this review we will focus on the functions of the CaSR in mammary gland biology, its regulation of mammary gland PTHrP production and its contribution to the development and progression of breast cancer.

THE CASR IN THE NORMAL BREAST

CaSR Expression in Normal Mammary Epithelial Cells

The first documentation of CaSR expression in normal breast tissue was reported by Cheng et al. (1998). They demonstrated both CaSR mRNA and protein expression in ductal epithelial cells in the human breast. Subsequent studies in mice confirmed these observations and showed that CaSR mRNA levels in the mouse mammary gland are low during ductal development and early to mid-pregnancy but subsequently increase to a peak during lactation (VanHouten et al., 2004). It is not entirely clear whether these changes represent alterations in the proportion of the gland represented by epithelial cells at these different time points, an actual increase in the expression of CaSR mRNA in individual epithelial cells, or both. Either way, the expression of the CaSR is rapidly reduced after weaning of the pups and the cessation of lactation. Cheng and colleagues demonstrated CaSR staining on epithelial cells in human breast samples (Cheng et al., 1998). Likewise, in mice, CaSR immunofluorescence staining demonstrated that, during lactation, the receptor is expressed primarily on the basolateral surface of ductal and alveolar cells as well as within the intracellular compartment of these same cells (VanHouten et al., 2004, 2007). Although these studies do not rule out low levels of receptor in stromal cells, it appears that the CaSR is located predominantly on epithelial cells within the breast.

CaSR Function in the Normal Mammary Gland

Our laboratory has disrupted the CaSR selectively in mammary epithelial cells, using both MMTV-Cre and BLG-Cre mice. In MMTV-Cre;$CaSR^{lox/lox}$ mice, the CaSR gene is disrupted in mammary epithelial cells soon after weaning. This caused no apparent abnormalities in ductal development during puberty or alveolar development during pregnancy (Kim et al., 2016). In contrast, in BLG-Cre;$CaSR^{lox/lox}$ mice, the CaSR gene is disrupted in mammary epithelial cells at the transition from pregnancy to lactation. While this did not affect secretory differentiation of the epithelial cells, it did have consequences for calcium transport, PTHrP production and systemic calcium metabolism during lactation, confirming previous pharmacologic and genetic studies (VanHouten et al., 2004, 2007; Ardeshirpour et al., 2006; Mamillapalli et al., 2013). These studies suggest that, despite its effects on proliferation and apoptosis in breast cancer cells (see below), the CaSR does not have a dominant role in regulating morphological development or differentiation in the normal mammary gland. Therefore, we will limit further discussion to the role of the CaSR in mammary gland physiology during lactation.

Regulation of PTHrP Production

Parathyroid hormone-related protein (PTHrP, gene symbol *PTHLH*) was discovered as the cause of humoral hypercalcemia of malignancy (HHM) a common paraneoplastic syndrome (Wysolmerski, 2012). It has important functions during mammary gland development and also participates in the regulation of systemic calcium metabolism during lactation. PTHrP is produced by mammary epithelial cells and is secreted both into milk and into the systemic circulation. Circulating PTHrP activates bone resorption during lactation in order to liberate maternal skeletal calcium stores that are used by the mammary gland for milk production (Wysolmerski, 2012). PTHrP in milk modulates neonatal calcium accrual through unknown mechanisms (Mamillapalli et al., 2013). The expression of *Pthlh* mRNA by the lactating murine mammary gland and the secretion of PTHrP into the maternal circulation and into milk are regulated by the CaSR so that PTHrP production by mammary epithelial cells is ultimately responsive to the availability of calcium for milk synthesis (VanHouten et al., 2004; Ardeshirpour et al., 2006; VanHouten and Wysolmerski, 2007; Mamillapalli et al., 2013; VanHouten and Wysolmerski, 2013).

As noted in the Introduction, the CaSR has been shown to regulate PTHrP production by several cell types, such as astrocytes, ovarian epithelial cells, cytotrophoblasts, hepatocytes, osteoblasts, prostate cancer cells, CaSR transfected HEK293 cells, and breast cancer cells (Brown and MacLeod, 2001; Chattopadhyay, 2006; Reyes-Ibarra et al., 2007; Wysolmerski, 2012; Organista-Juarez et al., 2013). In most cell types studied, activation of the CaSR stimulates PTHrP production. However, in normal mammary epithelial cells, activation of the CaSR suppresses PTHrP production (Sanders et al., 2000; VanHouten et al., 2004). Our laboratory, as well as others, has demonstrated this effect in the intact mammary gland as well as in isolated mammary epithelial cells in cell culture using both genetic and pharmacologic approaches. For example, reducing dietary calcium intake in lactating wild-type mice increases *Pthlh* gene transcription and PTHrP secretion into milk. Increasing dietary calcium intake in lactating WT, $PTH^{-/-}$, $1\alpha(OH)ase^{+/-}$ or

$1\alpha(OH)ase^{-/-}$ mice did the opposite and decreased PTHrP concentrations, demonstrating a consistent inverse relationship between circulating calcium concentrations and mammary PTHrP levels in lactating mice (Cao et al., 2009; Ji et al., 2011; VanHouten and Wysolmerski, 2013). Infusion of the calcimimetic compound NPS-R467, an allosteric activator of the CaSR, in lactating mice fed a low calcium diet recovered PTHrP production to levels observed in control mice fed a normal calcium diet, demonstrating that these dietary manipulations regulate mammary PTHrP production through the CaSR (VanHouten et al., 2004). We have also observed similar findings in genetic models of CaSR deficiency. Homozygous disruption of the CaSR gene results in neonatal death, but the mammary glands of $CaSR^{+/-}$ mice produce more PTHrP during lactation than WT mice (Ardeshirpour et al., 2006). To circumvent the neonatal death of $CaSR^{-/-}$ mice, we used the BLG-Cre transgene to disrupt the floxed *Casr* gene during late pregnancy and lactation (Mamillapalli et al., 2013). Loss of the CaSR on mammary epithelial cells resulted in increased *Pthlh* mRNA expression, increased milk PTHrP levels and increased secretion of PTHrP into the maternal circulation. All together, these studies support the conclusion that activation of the CaSR on mammary epithelial cells suppresses PTHrP production and secretion into the circulation and milk (VanHouten and Wysolmerski, 2013). As discussed below, this defines a negative feedback loop between calcium delivery to the lactating mammary gland and PTHrP production by mammary epithelial cells.

The CaSR binds Ca^{2+} mainly through the large extracellular domain (ECD) and acts as a homodimer or heterodimer (Bai et al., 1998, 1999; Chakravarti et al., 2012). Upon ligand binding, the CaSR undergoes a conformational change, which promotes GDP dissociation from a Gα subunit of a heterotrimeric G-protein complex, causing it to dissociate from the Gβγ subunits. As with many GPCRs, the CaSR interacts with multiple Gα sub-types, and the downstream signaling pathways are highly divergent, and depend on the cellular context (Chakravarti et al., 2012). The CaSR has been shown to stimulate the PLC/PKC pathway downstream of $G\alpha_q$ and to decrease cAMP downstream of $G\alpha_i$ (Chakravarti et al., 2012). Both $G\alpha_q$ and $G\alpha_i$, as well as interaction with the scaffolding proteins filamin-A and caveolin-1, are thought to be involved in the inhibition of PTH secretion by the CaSR in parathyroid chief cells (Chakravarti et al., 2012). In several cell types, the CaSR has been suggested to regulate PTHrP secretion by modulating MAPK signaling (Tfelt-Hansen et al., 2003). However, in normal mammary epithelial cells, regulation of cAMP/PKA pathways appears to be more important for PTHrP production than changes in the MAPK/ERK pathway (Mamillapalli et al., 2008). Our laboratory has demonstrated that, in normal breast cells, the CaSR couples with $G\alpha_i$ to decrease cAMP production by adenylylcyclase, and subsequently PKA activation, without affecting phosphodiesterase activity (Mamillapalli et al., 2008). Lower cAMP production results in the inhibition of *Pthlh* gene expression and PTHrP secretion, an effect that can be mimicked by inhibition of PKA, or reversed with forskolin or dibutyryl-cAMP (Mamillapalli et al., 2008).

Regulation of Calcium Transport into Milk

Milk production requires the transport of large amounts of calcium from the maternal circulation into milk. There is little paracellular transport in the lactating mammary gland and calcium must pass through the epithelial cells to enter the lumen (VanHouten and Wysolmerski, 2007). The plasma membrane calcium-ATPase 2 (PMCA2), a P-type, ion transport ATPase pump is expressed on the apical surface of mammary epithelial cells and transports 60–70% of milk calcium from the epithelial cells across the apical membrane and into the acinar lumen (Reinhardt et al., 2004; VanHouten et al., 2007). The remainder of the calcium in milk appears to be pumped into the endoplasmic reticulum or secretory granules and to be secreted bound to caseins via the exocytosis of casein-containing granules. The CaSR regulates transcellular calcium transport by altering the activity of PMCA2 in mammary epithelial cells (VanHouten et al., 2007; VanHouten and Wysolmerski, 2007). In this instance, delivery of calcium to the mammary gland activates the CaSR, which, in turn, stimulates PMCA2 activity and increases calcium transport into milk. The intracellular signal transduction pathways linking the CaSR to PMCA2 activation are unknown and require further study (VanHouten and Wysolmerski, 2013). It has been suggested that calcium enters mammary epithelial cells through interactions between another calcium pump, SPCA2, and the store-operated calcium channel, ORAI1 (Cross et al., 2013; Ross et al., 2013). At this juncture, it is also unknown whether CaSR signaling regulates this complex to increase calcium entry into the epithelial cells. However, the available data clearly demonstrate that the CaSR regulates the amount of calcium transported into milk in a positive feedback loop that is responsive to the availability of maternal calcium supplies (VanHouten and Wysolmerski, 2013).

Systemic Aspects of the CaSR-PTHrP and CaSR-PMCA2 Feedback Loops during Lactation

Current data suggest that the CaSR and PTHrP are involved in a negative feedback loop that regulates the supply of calcium to the mammary gland to support milk production (see **Figure 1**; VanHouten, 2005; VanHouten and Wysolmerski, 2007, 2013). The effects of the CaSR on PTHrP production in the lactating mammary gland mimic the relationship between Ca^{2+}, the CaSR and PTH production by the parathyroid glands. In essence, it can be argued that the lactating breast serves as an accessory parathyroid gland that secretes PTHrP into the maternal circulation in a calcium-sensitive manner in order to regulate osteoclastic bone resorption and the release of stored calcium from the maternal skeleton into the circulation to be available for uptake by the mammary gland and secretion into milk. In addition, the CaSR stimulates calcium transport into milk, allowing the lactating mammary gland to transport more calcium when it is available and to decrease its calcium usage when the mother's supply of calcium becomes limiting. Ultimately, we believe that this combined control of calcium mobilization and calcium usage allows the CaSR to fine tune maternal calcium metabolism to ensure a steady supply of calcium for milk production but to protect against maternal hypocalcemia. If calcium is readily available in the diet, more

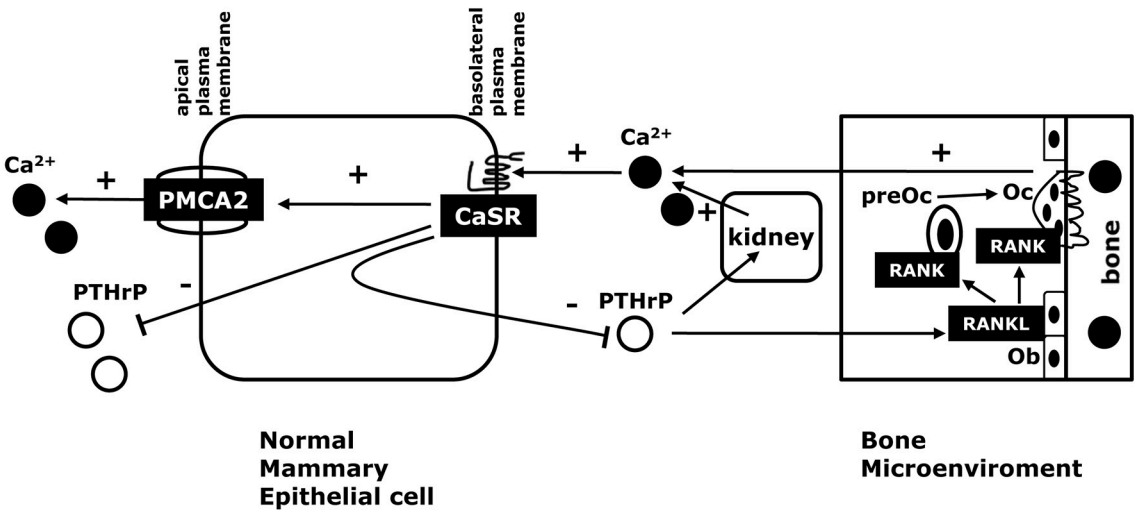

FIGURE 1 | The CaSR in normal lactation. Activation of the CaSR in the lactating breast promotes trans-epithelial calcium transport into milk by increasing the activity of the calcium pump, PMCA2. In addition, activation of the CaSR suppresses PTHrP release into milk and into the systemic circulation. Circulating PTHrP acts on osteoblasts (Ob) to increase osteoclast (Oc) development and activity through the RANK/RANKL pathway. This, in turn, stimulates bone resorption and liberates skeletal calcium stores. PTHrP also acts on the kidney to stimulate calcium reabsorption. If serum calcium is decreased, the CaSR decreases calcium transport into milk to decrease calcium utilization and increases PTHrP secretion into the maternal circulation to increase the supply of calcium from skeletal stores. In this way, the CaSR helps to maintain a steady supply of calcium for milk production and protects the mother from hypocalcemia if dietary calcium become limiting.

calcium is transported into milk but less PTHrP is produced and less calcium is liberated from skeletal stores. However, when dietary calcium is less abundant, the mammary gland reduces milk calcium content by slowing calcium transport and more PTHrP is produced, which increases bone resorption and liberates further calcium from skeletal stores. The importance of changes in milk PTHrP are less clear. However, recent studies have demonstrated that milk PTHrP content correlates inversely with milk calcium content and neonatal calcium accrual (Mamillapalli et al., 2013). We hypothesize that milk PTHrP may serve as a metabolic message that entrains maternal and neonatal bone and calcium metabolism. If less calcium is available to the mother, she reduces the calcium content of milk but increases milk PTHrP secretion, which, in turn, reduces neonatal calcium usage. If calcium is abundant, then milk calcium increases and milk PTHrP decreases, allowing increased neonatal calcium accrual. While the initial data support this model, much more work is needed to fully understand how milk calcium and PTHrP content regulate neonatal calcium and bone metabolism.

THE CASR IN BREAST CANCER

Breast cancer is a common malignancy in women that has been estimated to afflict one in eight women in the United States. The American Cancer Society estimates that, in 2016, 249,260 women in the United States will be diagnosed with breast cancer and approximately 40,890 will die from this disease (https://cancerstatisticscenter.cancer.org/). A particularly difficult problem in breast cancer is the frequent occurrence of bone metastases, which affect up to 65% to 80% of women with advanced disease (Weilbaecher et al., 2011). The ability of breast cancer cells to grow in bone has been shown to be related to their ability to recruit osteoclasts to resorb bone around the tumor deposit. This causes the release of growth factors from the bone matrix into the microenvironment around tumor cells, which increase their proliferation in response. The increased numbers of tumor cells then stimulate further osteolysis, which accommodates the growth of the tumor within the bone and releases even more growth factors to further feed tumor growth (Chen et al., 2010; Patel et al., 2011; Weilbaecher et al., 2011). This positive-feedback relationship between breast cancer cells and bone is referred to as a vicious cycle of osteolysis. This vicious cycle involves complex interactions between breast cancer cells, immune cells, osteoblasts and osteoclasts, mediated by a variety of biochemical signaling pathways and non-coding RNAs (Keklikoglou et al., 2012; Taipaleenmaki et al., 2015; Martin, 2016). Investigators have implicated both the CaSR and PTHrP as among the molecules contributing to the pathophysiology of osteolytic bone metastases (Yin et al., 1999; Mihai et al., 2006). In this Section, we will review current evidence suggesting that the CaSR regulates PTHrP production and tumor growth in breast cancer as well as recent findings suggesting that a CaSR-nuclear PTHrP signaling pathway may contribute to the proliferation and survival of breast cancer cells in high-calcium environments, such as bone.

CaSR Expression in Breast Cancer

Several studies have shown that the CaSR is expressed in breast carcinomas from patients and in breast cancer cell lines. CaSR expression was first demonstrated on an mRNA and protein level in breast tissue samples from 6 different women: 2 samples of normal breast tissue, 2 samples of fibrocystic disease, and 2 samples of ductal carcinomas (Cheng et al., 1998). Subsequent reports documented CaSR expression in standard human breast cancer cell lines, such as MCF-7 and MDA-MB-231 cells (Sanders et al., 2000). Some studies have shown that fully transformed MCF-7 and MDA-MB-231 breast cancer cell lines had higher CaSR levels than the nonmalignant breast cell lines, Hs578Bst and MCF-10A cells (Huang et al., 2009; VanHouten and Wysolmerski, 2013). In addition, we examined data from a study of rat mammary tumors induced by the chemical carcinogen N-Nitroso-N-methylurea (NMU) (Chan et al., 2005) and found that *Casr* mRNA levels were significantly higher in tumors as compared to normal mammary tissue (Kim et al., 2016). In contrast, recent studies from the Yang laboratory have suggested that human breast cancers may express lower levels of CaSR than normal breast tissue (Li et al., 2014a,b). In addition, they found that the "AG" or "GG" genotype at SNP rs17251221 was associated with reduced CaSR protein levels in 15 tumors and an increase in the risk of breast cancer in a case-control study of Chinese patients with ($n = 217$) or without ($n = 231$) breast cancer (Li et al., 2014a). Interestingly this same intronic polymorphism has been associated with prostate and ovarian cancer risk as well (Jorde et al., 2013; Jeong et al., 2016). A different study in 3663 cases and 4687 controls of women of African American descent did not replicate these associations but did find that another CaSR SNP (rs112594756) was associated with estrogen receptor status in breast tumors (Yao et al., 2016). A study that looked at CaSR immunohistochemistry in histological sections of primary tumors from 65 patients with metastatic breast cancer showed that the levels of CaSR expression varied among the different tumors and that these differences correlated with the biological behavior of the tumors. Primary tumors with high levels of CaSR expression were associated with bone metastases while those with lower levels of CaSR expression were not (Mihai et al., 2006). Finally, we examined CaSR protein expression using the semi-automated immunofluorescence AQUA platform in a tissue microarray consisting of 652 breast tumors with a median clinical follow up of 8.9 years (YTMA49) (Camp et al., 2002; VanHouten et al., 2010). In YTMA49, CaSR levels correlated inversely with pathological progesterone receptor positive status and positively with node-positive status. CaSR levels above the median value were associated with significantly shorter survival than those below the median, although, when considered as a continuous variable, CaSR levels did not significantly predict survival (Kim et al., 2016).

Other than to say that the CaSR is expressed in breast cancers and that there may be associations between CaSR expression and different aspects of breast cancer behavior, it is difficult to synthesize the above data into a coherent picture. One possibility is that decreased expression of the CaSR may be associated with an increased risk of developing breast cancer but, once breast cancer is established, tumor levels of CaSR may alter behaviors such as the ability to grow in bone. However, the results from the above case-control studies are based on relatively small numbers and larger studies will be required to determine whether increased or decreased levels of CaSR expression alter breast cancer risk. Furthermore, we did not find that ablation of the *Casr* gene in MMTV-PyMT transgenic mice increased the incidence of mammary tumors (see below) (Kim et al., 2016). It should also be noted that SNPs in the *CASR* gene locus have not been associated with breast cancer risk in GWAS studies (Fachal and Dunning, 2015). Very little is known about the regulation of CaSR expression in breast cancer cells and, therefore, we do not yet understand the mechanisms determining the level of CaSR expression in individual cancers. One study showed that BRCA1 regulates the expression of CaSR in MCF-7 and MDA-MB-231 cells but it is not known whether breast tumors in patients with BRCA1 mutations have different levels of CaSR expression than non-BRCA1-associated tumors (Promkan et al., 2011). Clearly, given the suggestion that CaSR levels might influence the likelihood of breast cancer risk, the estrogen-receptor status of tumors and/or the growth of bone metastasis, these issues need to be explored in larger studies with sufficient power to examine the different genetic sub-types of breast cancer individually.

Function of the CaSR in Breast Cancer

Proliferation and Cell Death

Studies examining the effects of CaSR signaling on proliferation and cell death in breast cancer cells have reported contradictory results. A majority of studies have focused on cell proliferation and different labs have reported opposite results sometimes using the same cell lines. In one study, Ca^{2+} concentrations between 1.4 and 5.0 mM stimulated the proliferation of MCF-7 cells by 25 $\pm$ 3% as assessed by measuring cell accumulation (El Hiani et al., 2009a). These authors described a pathway by which stimulation of the CaSR activated membrane metalloproteinases (MMPs), which led to shedding of membrane-bound EGF-family growth factors, stimulation of the EGFR, ERK1/2 phosphorylation, upregulation of the transient receptor potential channel 1 (TRPC1) and the stimulation of cell proliferation (El Hiani et al., 2009b). It has been well documented that GPCRs can transactivate EGFRs both in ligand-dependent and ligand-independent signaling pathways (Bhola and Grandis, 2008), and the CaSR has been reported to activate the EGFR in this manner in leydig cancer cells, fibroblasts, and prostate cancer cells suggesting that this could be a commonly utilized signaling mechanism (Yano et al., 2004; Tfelt-Hansen et al., 2005; Tomlins et al., 2005). Activation of the CaSR in MCF-7 and MDA-MB-231 breast cancer cells has also been shown to increase the production of phosphocholine and the expression of choline kinase through activation of $G\alpha_{12}$ and Rho (Huang et al., 2009). Furthermore, overexpression of choline kinase in MCF-7 breast cancer cell has been shown to increase their invasiveness and drug resistance (Shah et al., 2010). The regulation of phosphocholine synthesis seems to be operative in human tumors *in vivo* as Baio and colleagues have used MRI spectroscopy in 23 patients to demonstrate a positive correlation

between the pre-operative choline content of breast cancers and the staining intensity of CaSR immunohistochemistry on pathological specimens after surgery (Baio et al., 2015). Elevated levels of phosphocholine and total choline-containing compounds have been observed in almost every cancer type studied and may be associated with tumor progression (Glunde et al., 2011). In the aggregate, these data support that notion that the CaSR promotes increased proliferation of breast cancer cells and suggest two interesting signaling pathways that may mediate these effects.

In contrast to the above studies, other reports have suggested that CaSR signaling has no effect, or reduces proliferation and decreases the malignant behavior of breast cancer cells (Liu et al., 2009; Promkan et al., 2011). Sanders et al. examined responses to varying concentrations of Ca^{2+} between 0.5 and 10 mM and found that changing extracellular calcium had no effect on cell proliferation in MCF-7 or MDA-MB-231 cells as assessed by cell accumulation (Sanders et al., 2000). In a different study, Liu and colleagues found that exposing MCF-7 cells to physiological concentrations of Ca^{2+} (1.4 mM) reduced proliferation (as assessed by changes in cell numbers) as compared to low concentrations of Ca^{2+} (0.175–0.2 mM) (Liu et al., 2009). These investigators have also reported that activation of the CaSR increased the sensitivity of MCF-7 and MDA-MB-231 breast cancer cells to cell death in response to paclitaxel (Liu et al., 2009; Promkan et al., 2011). Similar findings have been reported in colon cancer, where it has been proposed that activation of the CaSR inhibits tumor progression and enhances chemotherapeutic responses (Rogers et al., 2012). The growth inhibitory effects of the CaSR in breast cancer cells have been reported to be mediated by the downregulation of the expression of survivin, a well described anti-apoptotic factor in breast and other cancers (Liu et al., 2009; Promkan et al., 2011). Interestingly, in these studies, wild-type BRCA1 was shown to inhibit survivin expression in a CaSR-dependent manner (Promkan et al., 2011). Data from these several studies suggest that the CaSR inhibits proliferation and promotes cell death in breast cancer cell lines, implying that it may inhibit breast cancer development and/or progression.

It is unclear how to interpret the contradictory findings suggesting that the CaSR both opposes and promotes proliferation and/or cell death, especially when groups reporting opposite findings have used the same cell lines. In essence, one can find data showing that high Ca^{2+} stimulates, inhibits or has no effect on the proliferation of MCF-7 or MDA-MB-231 cells. The reported differences might be explained by differences in the Ca^{2+} concentration used by the different groups (El Hiani et al., 2009a,b; Liu et al., 2009; Promkan et al., 2011) or they may be the consequence of complex patterns of downstream signaling that might be very context dependent (Mamillapalli et al., 2008; Promkan et al., 2011; Breitwieser, 2012; Chakravarti et al., 2012).

Regulation of Cell Migration

The development of distant metastases is perhaps the most significant cause of cancer mortality. Metastasis is a multi-step process, which requires cells to migrate in order for them to successfully invade surrounding tissues, to disseminate widely and to colonize other tissue and form metastases. Several studies have suggested that the CaSR can promote cell migration and therefore may promote the development of metastases. Using the Boyden Chamber and Scratch Wound migration assays, Saidak et al. showed that stimulation of the CaSR with extracellular calcium concentrations between 1.8 and 5.0 mM stimulated the migration of MCF7, T47D and MDA-MB-231 breast cancer cells (Saidak et al., 2009). Interestingly, of these 3 cell lines, the highly bone metastatic MDA-MB-231 cells showed the most vigorous cell migration response. Knocking down CaSR expression inhibited cell migration in response to Ca^{2+} in all three, cell lines demonstrating that the CaSR mediated the migratory effect of extracellular calcium (Saidak et al., 2009). Activation of the CaSR appeared to enhance migration in these cells by activating the ERK1/2, MAPK and phospholipase Cβ (PLCβ) pathways. Activation of the CaSR has also been shown to induce the secretion of a series of pro-angiogenic cytokines by MDA-MB-231 cells, at least in part, through activation of EGFR signaling (Hernandez-Bedolla et al., 2015). In this instance the CaSR appears to indirectly regulate the migration and morphogenesis of endothelial cells and may contribute to tumor neovascularization. The CaSR has also been shown to affect migration in other cell types. In medullary thyroid carcinoma cells, the CaSR interacts with β1 integrin, modulating cellular adhesion and inducing migration, through a PLC/intracellular calcium-mediated pathway (Tharmalingam et al., 2011). In keratinocytes, the CaSR physically interacts with Trio, Rho, and filamin A to form a signaling complex that regulates E-cadherin-mediated cell-cell adhesion and differentiation (Tu and You, 2014). Finally, the CaSR also has been shown to modulate E-cadherin's membrane localization and binding with β-catenin in colon carcinoma cells (Wang et al., 2010). In the aggregate, these findings suggest that the CaSR may regulate cell migration in breast cancer cells, however, much more work must be done in this area to determine how important these observations *in vitro* are to the behavior of breast cancers *in vivo*.

Regulation of PTHrP Production

Activation of the CaSR in normal mammary epithelial cells reduces PTHrP mRNA expression and PTHrP secretion (VanHouten et al., 2004; Ardeshirpour et al., 2006; Mamillapalli et al., 2008; 2013). However, just the opposite has been observed for breast cancer cells. In immortalized or malignant breast cells, such as Comma D, MCF-7, BT474 and MDA-MB-231 cells, activation of the CaSR with Ca^{2+}, spermine, aminoglycoside antibiotics, or allosteric (type-II) calcimimetics has been shown to stimulate PTHrP secretion (Sanders et al., 2000; Mamillapalli et al., 2008; Kim et al., 2016). Interestingly, activation of the CaSR can act synergistically with TGFβ to increase PTHrP secretion in hepatocytes as well as MCF-7 and MDA-MB-231 breast cancer cells, a characteristic that may contribute to the pathogenesis of bone metastasis (Sanders et al., 2000; Organista-Juarez et al., 2013). Our laboratory has been interested in understanding the mechanisms by which malignant transformation of mammary epithelial cells causes CaSR activation to switch from inhibiting PTHrP production to stimulating PTHrP production. Initial

studies demonstrated that divergent cAMP production, but not alterations in MAPK or PLC signaling correlated with the changes in PTHrP production (Mamillapalli et al., 2008). Activation of the CaSR decreases cAMP levels in normal breast cells, but increases cAMP response in breast cancer cells (Mamillapalli et al., 2008; Kim et al., 2016). We found that in normal breast cells, the CaSR couples to $G\alpha_i$ and inhibits adenylyl cyclase while, in breast cancer cells, the CaSR couples to $G\alpha_s$ and stimulates cAMP production. The *Pthlh* gene is known to be regulated by a cAMP-response element located within exon 4 of the human gene, and the effects of CaSR activation on PTHrP production in these different cell types could be mimicked by manipulating cAMP levels independently from CaSR activation (Chilco et al., 1998; Mamillapalli et al., 2008). Therefore, it appears that during the transformation of normal breast cells into malignant breast cancer cells, the CaSR switches its G-protein preference, which, in turn, leads to completely opposite effects on PTHrP production. Ongoing studies are attempting to define the molecular mechanisms that underlie the G-protein switching that occurs in response to malignant transformation.

A CaSR-nuclear PTHrP Pathway Regulates Cell Proliferation and Survival

Given that the CaSR upregulates PTHrP and given that PTHrP has been shown to regulate breast cancer progression, we were interested in determining whether PTHrP might mediate some of the effects of the CaSR on breast cancer cells *in vitro* and *in vivo*. In order to examine this question, we first asked whether there was an association between CaSR and PTHrP levels in breast cancers. In NMU-induced mammary tumors in rats, there was a clear positive correlation between *Casr* and *Pthlh* mRNA levels. This was also true in human breast cancers at both an mRNA and protein level; *CASR* gene expression correlated directly with *PTHLH* gene expression in a gene array study of 204 human breast tumors (Mu et al., 2012; Kim et al., 2016) and CaSR and PTHrP protein expression showed a positive correlation in the YTMA49 tissue array of 652 breast tumors discussed previously (Camp et al., 2002; VanHouten et al., 2010; Kim et al., 2016). Finally, when we disrupted the *Casr* gene in mammary tumors in MMTV-Cre; $CaSR^{lox/lox}$; MMTV-PyMT mice, tumor PTHrP mRNA levels were significantly reduced (Kim et al., 2016). Together, these data demonstrate that activation of the CaSR increases PTHrP production by breast cancer cells in rodents and in humans, both *in vitro* and *in vivo*.

Next, we found that knocking down either the CaSR or PTHrP in BT474 cells and MDA-MB-231 cells both inhibited proliferation and promoted cell death in response to high extracellular calcium levels. Stimulation of the CaSR promoted proliferation by reducing $p27^{kip1}$ levels and increasing CDK2 activity while it promoted cell survival by inhibiting nuclear accumulation of apoptosis-inducing factor (AIF) (**Figure 2**). We also found that knocking out the CaSR inhibited the proliferation of mammary tumor cells in MMTV-Cre; $CaSR^{lox/lox}$; MMTV-PyMT mice *in vivo*, slowing the growth of tumors. Furthermore, disrupting the CaSR inhibited growth and increased apoptosis in MMTV-PyMT tumor cells grown *ex vivo*, again by altering

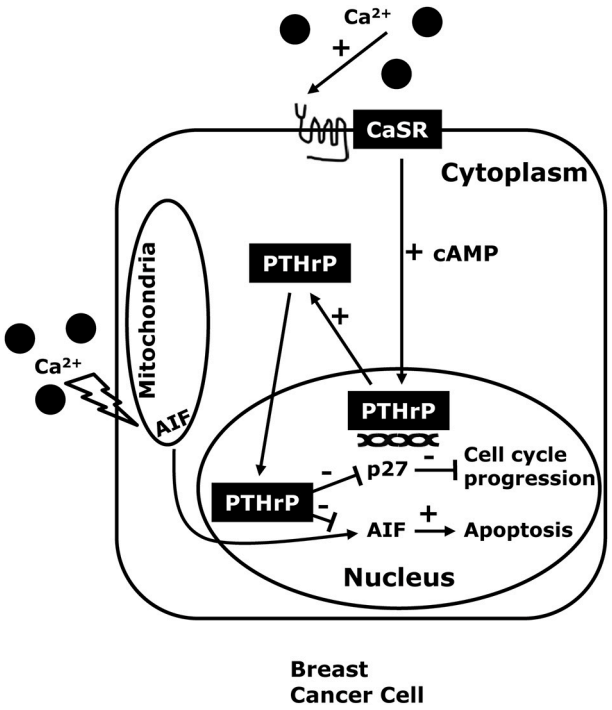

FIGURE 2 | The CaSR-nuclear PTHrP pathway. CaSR activation in breast cancer cells, stimulates PTHrP production through increased intracellular cAMP levels and also promotes the proliferation and inhibits cell death in high extracellular calcium concentrations. The effects of the CaSR on tumor cell growth appear to be mediated by nuclear actions of PTHrP that decrease expression of the cell cycle inhibitor $p27^{kip1}$ and that prevent nuclear accumulation of apoptosis-inducing factor (AIF) which activates apoptotic cell death.

$p27^{kip1}$ levels and nuclear AIF accumulation respectively. Interesting, although disruption of the PTHrP gene mirrored the effects of targeting the *Casr* gene, disruption of the Type 1 PTH/PTHrP receptor (PTH1R) did not (Kremer et al., 2011; Li et al., 2011; Kim et al., 2016). In addition, adding PTHrP to the media of cells could not rescue the loss of the CaSR, but transducing the same cells with a viral expression plasmid for wtPTHrP did rescue both the defects in proliferation and cell survival. By contrast, transducing the cells with a mutant form of PTHrP lacking the ability to translocate into the nucleus (ΔNLS-PTHrP) did not rescue the phenotytes caused by loss of the CaSR (Kim et al., 2016). In the aggregate, these findings clearly suggest that the CaSR affects breast cancer cell proliferation and apoptosis, at least in part, by stimulating the production of PTHrP, which, in turn, acts in the nucleus to regulate $p27^{kip1}$ and AIF levels (see **Figure 3**).

Does the CaSR Contribute to Bone Metastases?

As discussed previously, Mihai et al. (2006) reported that the levels of tumor CaSR expression predicted the development of bone metastases. Larger clinical studies are needed to confirm these findings, but, in light of our recent findings, it is interesting

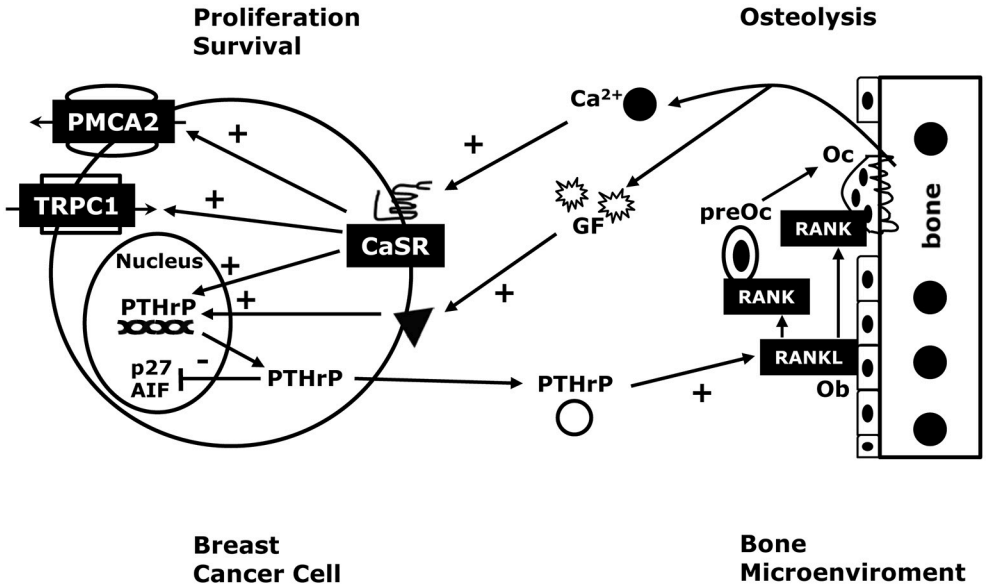

FIGURE 3 | The CaSR in breast cancer. In breast cancer cells, stimulation of the CaSR increases PTHrP production. Therefore, the high local calcium concentrations surrounding bone metastases would be expected to stimulate PTHrP secretion, which would stimulate osteoblasts (Ob) to produce more RANKL, thereby driving more osteolysis and releasing growth factors (GF) from the bone matrix that stimulate tumor cell growth. As a result, activation of the CaSR facilitates a feed-forward, vicious cycle of bone resorption, tumor growth and osteolysis. In addition, the CaSR increase the PMCA2 calcium pump activity to protect the breast cancer cells from calcium-mediated apoptosis, and regulates the TRPC1 calcium channel, which can stimulate proliferation. Finally, as outlined in **Figure 2**, activation of the CaSR promotes breast cancer cell proliferation and enhances cell survival by activating a nuclear PTHrP signaling pathway that inhibits $p27^{kip1}$ and AIF respectively. These findings suggest that extracellular calcium may promote tumor cell growth by activating the CaSR, which, in turn, can activate PTHrP-dependent and PTHrP-independent signaling pathways that stimulate cancer cell proliferation, inhibit cancer cell death and promote osteoclastic bone resorption.

to speculate on mechanisms by which the CaSR might promote the development of osteolytic bone metastases. In order to metastasize to the bone and grow in size, cancer cells must resorb the surrounding mineralized tissue, which requires them to stimulate osteoclast development and/or activity. Accelerated osteoclastic bone resorption, in turn, releases high levels of Ca^{2+} into the local microenvironment (Akhtari et al., 2008; Patel et al., 2011; Theriault and Theriault, 2012). For instance, some studies suggest that Ca^{2+} concentrations may increase to levels as high as 40 mM in the microenvironment surrounding actively resorbing osteoclasts (Silver et al., 1988; Berger et al., 2001). Therefore, it would be advantageous for a tumor cell to respond to Ca^{2+} in ways that would stimulate cell growth and further promote osteolysis. Given that PTHrP stimulates osteoclast differentiation and activity (Yin et al., 1999; Akhtari et al., 2008; Patel et al., 2011; Theriault and Theriault, 2012), increased PTHrP secretion in response to high Ca^{2+} levels would be expected to act in a paracrine fashion and contribute to accelerated osteolysis (Mamillapalli et al., 2008; **Figure 3**). Our findings also suggest that CaSR signaling can increase intracrine/nuclear signaling by PTHrP and directly stimulate tumor cell proliferation and enhance the ability of the cells to survive in the face of the elevated extracellular calcium concentrations resulting from active bone resorption (**Figure 3**). As discussed previously, increased bone resorption releases bone matrix-derived growth factors such as TGF-β, IGFs and FGFs, all of which can stimulate tumor cell growth and/or increase PTHrP production, defining a vicious cycle of osteolysis (Chirgwin and Guise, 2000; Mundy, 2002; Kremer et al., 2011). The majority of breast cancers that metastasize to bone are estrogen receptor (ER)-positive so it is also interesting that activation of the CaSR has been shown to increase ER transcriptional activity and enhance the effects of estradiol in MCF-7 cells (Journe et al., 2004; Leclercq, 2012). All of these observations demonstrate that CaSR signaling can exert proliferative, pro-survival and bone-resorbing activities that would be expected to enhance the progression of bone metastases (**Figure 3**). Therefore, although much research will be required to validate the above hypothesis, the CaSR-PTHrP axis may present new therapeutic opportunities to develop treatments for bone metastases from breast cancers.

CONCLUSIONS

The CaSR has a central role in orchestrating systemic calcium homeostasis. However, it is also expressed in a variety of different organs such as the breast, where it may contribute to the regulation of cell proliferation, cell differentiation and cell migration in response to alterations in the extracellular environment. By regulating PTHrP production in the lactating mammary gland, the CaSR allows mammary epithelial cells to actively participate in reordering systemic calcium and bone metabolism to provide calcium for milk production. In a similar fashion, the CaSR appears to regulate PTHrP production by

breast cancer cells, which causes osteolysis and stimulates the growth of tumor cells, contributing to the pathophysiology of osteolytic bone metastases. It is unlikely that PTHrP mediates all of the effects of the CaSR on breast cancers but, at this point, relatively little is known about the direct effects of CaSR signaling on breast cancer cell behavior, and many of the initial observation have been contradictory. Thus, more work will be required to fully elucidate the role of the CaSR in both normal and malignant breast cells and to determine whether targeting the CaSR-PTHrP axis would be an effective strategy against breast cancers.

AUTHOR CONTRIBUTIONS

WK and JW both contributed intellectually to the development of this review, including drafting and revising the manuscript. Both approved the final version to be published.

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REVIEW
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The Extracellular Calcium-Sensing Receptor in the Intestine: Evidence for Regulation of Colonic Absorption, Secretion, Motility, and Immunity

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Different from other epithelia, the intestinal epithelium has the complex task of providing a barrier impeding the entry of toxins, food antigens, and microbes, while at the same time allowing for the transfer of nutrients, electrolytes, water, and microbial metabolites. These molecules/organisms are transported either transcellularly, crossing the apical and basolateral membranes of enterocytes, or paracellularly, passing through the space between enterocytes. Accordingly, the intestinal epithelium can affect energy metabolism, fluid balance, as well as immune response and tolerance. To help accomplish these complex tasks, the intestinal epithelium has evolved many sensing receptor mechanisms. Yet, their roles and functions are only now beginning to be elucidated. This article explores one such sensing receptor mechanism, carried out by the extracellular calcium-sensing receptor (CaSR). In addition to its established function as a nutrient sensor, coordinating food digestion, nutrient absorption, and regulating energy metabolism, we present evidence for the emerging role of CaSR in the control of intestinal fluid homeostasis and immune balance. An additional role in the modulation of the enteric nerve activity and motility is also discussed. Clearly, CaSR has profound effects on many aspects of intestinal function. Nevertheless, more work is needed to fully understand all functions of CaSR in the intestine, including detailed mechanisms of action and specific pathways involved. Considering the essential roles CaSR plays in gastrointestinal physiology and immunology, research may lead to a translational opportunity for the development of novel therapies that are based on CaSR's unique property of using simple nutrients such as calcium, polyamines, and certain amino acids/oligopeptides as activators. It is possible that, through targeting of intestinal CaSR with a combination of specific nutrients, oral solutions that are both inexpensive and practical may be developed to help in conditioning the gut microenvironment and in maintaining digestive health.

Keywords: intestinal barrier function, enteric nervous system, gut immunity, secretory diarrhea, motility, irritable bowel syndrome, inflammatory bowel disease, calcium-sensing receptor

INTRODUCTION

The intestinal epithelium is faced with the complex task of providing a barrier impeding the entry of noxious substances and microbes, while concurrently allowing for nutrient and water absorption and secretion. The primary function of the gastrointestinal (GI) tract is to digest food and absorb nutrients. To aid in digestion, the GI tract secretes a large amount of fluid to mix the food components and to lubricate the surface of the lumen. It is estimated that in a 24-h period, an average of 7.0 L of digestive juices are secreted upon food ingestion. These include 1.5 L from the salivary glands, 2.5 L from the stomach, 0.5 L from the biliary system, 1.5 L from the pancreas, and 1.0 L from the intestine (Boron and Boupaep, 2008). Upon completion of digestion and extraction of nutrients, these secretions must be reabsorbed along with released nutrients while also ensuring that further post-digestive secretions do not occur. These processes are highly regulated and coordinated; failure to do so may result in diseases such as mal-digestion, mal-absorption, constipation, or diarrhea.

The mammalian gut is considered the largest immune organ in the body. It is estimated that 65–80% of the body's immune cells (e.g., macrophages, dendritic cells, T cells, and B cells) and over 90% of immunoglobulin-producing cells are found in the gut, residing in ∼100,000 isolated lymphoid follicles in the sub-epithelium lamina propria layer of the mucosa (Brandtzaeg et al., 1989). Adjacent to the epithelium is the lumen of the intestine, where, in addition to food and nutrients, hundreds of trillions of microorganisms reside. These include microbes that benefit the host, as well as those that cause disease. Additionally, during food ingestion, foreign antigens are being continuously introduced into the GI tract, and these molecules may also pose threats to the body. Accordingly, the GI tract must maintain an intact epithelial barrier and reliable immunity. The GI tract has the innate ability to control the flow of nutrients across the epithelium where the nutrients are absorbed, while also restricting microbes and food antigens to the lumen. In this manner, the immune cells of the gut are prevented from over-activation, and consequently, no inflammation, allergic reaction, or hypersensitivity results.

Notably, the mammalian epithelium lining the GI tract is a specially adapted tissue equipped with sensing receptor mechanisms. These sensing receptors are constantly detecting and responding to changes in the composition of local nutrient milieus and microbial environment. This constant monitoring by receptors ensures that the gut absorbs and secretes according to the state of digestion and nutrient availability, and that it alters intestinal permeability and immunity in accordance with the status of flora. One such sensing mechanism is the extracellular calcium-sensing receptor (CaSR), a multifaceted heptahelical guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR; Brown et al., 1993).

In this article, after briefly exploring the function of CaSR as a nutrient sensor along the mammalian GI tract, we discuss the emerging roles this receptor may play in regulating colonic secretion, absorption, motility, epithelial integrity, and immunity. The role of CaSR in the regulation of colonic epithelial proliferation/tumorigenesis, differentiation, and stem cell growth and renewal has been reviewed elsewhere (see references Whitfield, 2009; Ghevariya and Anand, 2011; Macleod, 2013a; Singh et al., 2013; Tennakoon et al., 2016; also in the article by Kallay and colleagues and the discussion by MacLeod, et al in this issue) and is therefore not discussed here, even though it is very important for maintenance of intestinal barrier integrity/immunity. A general approach that was used to verify the role of CaSR in the studies presented here is either pharmacological, using the specific CaSR inhibitors (e.g., NPS 2143, calhex 231) or activators (e.g., R568, cinacalcet), or genetic, by comparing the behavior difference between mice that lack CaSR *vs.* their wild type controls. Both global CaSR exon 5 null mice [e.g., $Casr^{-/-}PTH^{-/-}$ (Kos et al., 2003) and $Casr^{-/-}Gcm2^{-/-}$ (Tu et al., 2003)] and intestine-specific CaSR exon 7 null mice [e.g., $^{villin}Cre/Casr^{flox/flox}$ Rey O et al., 2012] are generated and successfully used. Double global CaSR knockout mice are used because deletion of the CaSR gene alone results in early death from the toxic effects of unregulated release of parathyroid hormone (PTH) from parathyroid chief cells as well as from the pathological effects of the consequent hypercalcemia (Ho et al., 1995). As a result, double knockouts with simultaneous ablation of additional PTH gene (as in $Casr^{-/-}PTH^{-/-}$) or gene that regulates PTH (e.g., Gcm2 as in $Casr^{-/-}Gcm2^{-/-}$) are generated to "rescue" the lethal CaSR-deficient phenotype.

CaSR AND NUTRIENT-SENSING

CaSR is a well-conserved ancient GPCR, originally cloned from bovine parathyroid glands (Brown et al., 1993) and then found in rat kidney (Riccardi et al., 1995). There, it acts as an extracellular calcium ion (Ca_o^{2+}) sensor and provides a key negative feedback mechanism for Ca_o^{2+} to regulate parathyroid hormone secretion and urinary Ca_o^{2+} excretion, thereby maintaining systemic Ca_o^{2+} homeostasis (Brown and MacLeod, 2001; Hofer and Brown, 2003). It was subsequently found in other tissues of diverse species that are not typically associated with Ca_o^{2+} homeostasis, thereby suggesting that this receptor may subserve other roles and functions beyond systemic Ca_o^{2+} homeostasis.

Importantly, CaSR is a member of the class C GPCR that uses nutrients as agonists. This receptor, therefore, not only senses ions, but may also recognize and respond to nutrients in the milieus. Like other members in this family of GPCRs, such as metabolic glutamate receptors, gamma amino butyrate B receptors, sweet, and umami taste receptors, and pheromone receptors, CaSR is structurally equipped with an unusually large extracellular domain (∼50% of the receptor mass) called the Venus fly trap module. Studies suggested that this Venus fly trap domain is located outside of the cell and senses nutrients, specifically protein breakdown products [e.g., amino acids (Conigrave et al., 2000; Mun et al., 2004), peptides (Conigrave and Brown, 2006; Wang et al., 2006; Broadhead et al., 2011), and polyamines (Quinn et al., 1997)] as well as other environmental cues [e.g., ionic strength and pH (Quinn et al., 2004)]. Considering that these nutrients/conditions are routinely

encountered by cells/tissues in the GI tract, this nutrient sensor may play crucial roles in GI physiology.

Indeed, CaSR has been widely detected in tissues and cell types in the GI tract and its accessory organs that are implicated in nutrient sensing and/or nutrient handling. These include the taste cells in the taste buds of the tongue, the gastrin-secreting G cells, and the cholecystokinin (CCK)-secreting K cells in the stomach and duodenum, the nutrient-absorbing villous cells in the small intestine, the short chain fatty acid (SCFA)-absorbing surface cells in the large intestine, and the enteric nervous system (ENS; see summary in **Table 1**). In these cells and tissues, CaSR may act as a nutrient sensor, monitoring, and coordinating digestion, secretion and absorption. For example, in the mouth, which is the beginning of the sensory portion of the gut, CaSR may allow the taste cells to chemo-sense bitter taste (calcium), and kokumi taste (γ-glutamyl peptides; Ohsu et al., 2010; Maruyama et al., 2012), thus facilitating food ingestion. In the digestive gut (i.e., the stomach and duodenum), this nutrient sensor may enable the gastrin cells and the CCK cells to detect the arrival of food, stimulate digestive secretions, and initiate postprandial food digestion. In support of this notion, the gastric G cells in wild type mice or cells were found to release gastrin upon activation by luminal calcium, phenylalanine, peptone, spermine, or the calcimimetic Cinacalcet (Ray et al., 1997), but not those G cells in CaSR-pharmacologically inhibited or genetically ablated ($Casr^{-/-}PTH^{-/-}$) mice (Feng et al., 2010). Similar observations were made in the intestinal I cells, which responded to luminal nutrients, calcium, phenylalanine, tryptophan and peptides, and secreted CCK only in wild type mice or cells, but not in CaSR-null ($Casr^{-/-}PTH^{-/-}$) mice (Liou et al., 2011) or CaSR activity-inhibited cells isolated from wild type mice (Wang et al., 2011). These findings point to the significance of CaSR in nutrient sensing in the gut.

In the absorptive gut (i.e., the jejunum and ileum) where dietary nutrients are fully released and extracted from ingested food, CaSR may function to inform the villus cells of the availability of nutrients, to activate absorption, and to provide a mechanism to signal to the ENS, coined "the brain of the gut," to coordinate food delivery and gut motility in order to maximize nutrient absorption. In this latter part of the gut, CaSR has also been found to be present in a number of enteroendocrine cells, including the glucagon-like peptide-1 (GLP-1)-secreting L cells (Mace et al., 2012; Pais et al., 2016), the glucose-dependent insulinotropic peptide (GIP)-secreting K cells (Mace et al., 2012), and the insulin-secretion β cells of the pancreatic Islets of Langerhans (Leech and Habener, 2003). Since the main function of GLP-1 and GIP is to enhance glucose-induced insulin secretion from β cells, it is possible that CaSR may play a role in nutrient (glucose/SCFA) utilization and energy homeostasis, through regulating the postprandial secretion of GLP-1, GIP, and insulin. Indeed, oral or duodenal administration of CaSR peptide agonists to experimental animals reduced rapid elevation of plasma glucose in response to oral glucose challenge (Muramatsu et al., 2014). Further, evidence supporting the role of CaSR as a nutrient sensor and food metabolism regulator comes from the discovery that CaSR is found in tissues that regulate appetite and satiety. In addition to the duodenal I cells that secrete satiety-inducing CCK, there is evidence that gastric ghrelin cells, which secrete ghrelin (a hunger hormone), express CaSR, too (Engelstoft et al., 2013; Vancleef et al., 2015). Thus, CaSR may participate in the regulation of food intake. Taken together, it is tempting to speculate that modulations of intestinal CaSR expression and function by calcimimetics or nutritional receptor agonists may alter the behavior of food intake and energy metabolism thus providing a novel pathway for prevention and treatment of both obesity and type 2 diabetes mellitus.

CaSR AND INTESTINAL ABSORPTION AND SECRETION

In addition to digesting food, the GI tract moves a large amount of fluid and electrolytes. Accordingly, CaSR may also regulate intestinal fluid homeostasis and electrolyte balance. Normally, fluid moves *across* and *along* the intestine. While the fluid movement *across* the intestine [absorption (**Figure 1A**) or secretion (**Figure 1B**)] is driven by active epithelium transport of electrolytes (mainly Na^+, Cl^-, and HCO_3^-) and solutes (mainly glucose in the small intestine and SCFAs in the large intestine), the fluid moving *along* the intestine (anterograde or retrograde) is governed by gut motility (see **Figure 1D**). The ENS, the brain of the gut, controls both processes, with absorption/secretion regulated by the submucosal Meissner's plexus, and the motility under the control of the myenteric Auerbach's plexus (see **Figure 1D**). CaSR has been identified on the apical and basolateral membranes of fluid-absorbing villous/surface cells and fluid-secreting crypt cells of rat and human intestines (Chattopadhyay et al., 1998; Cheng et al., 2002), as well as on the Meissner's and Auerbach's plexuses of the ENS (Chattopadhyay et al., 1998; Cheng, 2012). Receptors in both membrane domains of these polarized epithelia, as well as the ENS, are functionally active (Chattopadhyay et al., 1998; Cheng et al., 2002; Cheng, 2012; Tang et al., 2015) and can be activated by Ca_o^{2+} (Cheng et al., 2002, 2004; Geibel et al., 2006), polyamines (Cheng et al., 2004), and the specific pharmacologic CaSR agonist R568 (a calcium mimetic drug; Geibel et al., 2006; Tang et al., 2015), suggesting its likely role in regulation of intestinal fluid metabolism.

Activation of CaSR Inhibits Anion Secretion

The first evidence that suggests the modulation of intestinal fluid metabolism by CaSR was made in perfused crypts isolated from rat colons (Cheng et al., 2002). In this study, the effect of increasing either luminal or basolateral Ca_o^{2+} on the direction and rate of net fluid movement ($^{net}J_v$) was determined in isolated rat distal colonic crypts in both basal and forskolin (cAMP)-stimulated states. Colonic crypt is a typical fluid-transporting epithelium that is able to move fluid in two directions. Depending on the presence or absence of secretagogues and/or anti secretagogues and their relative forces in the milieus, the fluid can be transported either from the luminal side to the vascular side, resulting in net fluid absorption or positive $^{net}J_v$, or from the blood side to the luminal side, resulting in net

TABLE 1 | CaSR and modulations of digestive functions.

Cell types that express	Activating nutrient(s)	Role and function	Type of evidence	References
CELLS THAT REGULATE INGESTION AND DIGESTION				
Tongue				
Taste buds	Ca_o^{2+}, γ-glutamyl peptide	↑bitter and kokumi taste perception	Pharmacological	Ohsu et al., 2010; Maruyama et al., 2012
Esophagus				
Epithelial cells (basal cells)		Inflammation-modulating (?)	Biochemical	Justinich et al., 2008; Abdulnour-Nakhoul et al., 2015
Stomach				
G cells (ap & bl)	Ca_o^{2+}, Phe, spermine, Peptide, cinacalcet	↑gastrin→↑gastric H^+ production	Pharmacogenetic	Ray et al., 1997; Buchan et al., 2001
D cells			Biochemical	Haid et al., 2012; Adriaenssens et al., 2015
Ghrelin cells (ap & bl)	Phe, Ala, peptide	↑and ↓ghrelin secretion	Pharmacological	Engelstoft et al., 2013; Vancleef et al., 2015
Parietal cells (bl)	Ca_o^{2+}, Mg^{2+}, amino acid	↑gastric H^+ secretion	Biochemical	Cheng et al., 1999; Caroppo et al., 2001; Geibel et al., 2001; Busque et al., 2005; Dufner et al., 2005
Mucous cells (ap & bl)	Ca_o^{2+}	↑mucous secretion	Biochemical	Rutten et al., 1999; Gilster et al., 2004
Duodenum				
I cells (ap & bl)	Ca_o^{2+}, Phe, Trp, peptide	↑CCK→↑pancreatic juice & bile	Pharmacogenetic	Sheinin et al., 2000; Hira et al., 2008; Nakajima et al., 2010, 2012; Liou et al., 2011; Wang et al., 2011
Pancreas				
Acinar cells		↑ secretion of pancreatic juice (?)	Biochemical	Bruce et al., 1999
Ductal cells (ap)	Ca_o^{2+}	↑pancreatic fluid flow & solubility	Biochemical	Bruce et al., 1999
α & β cells of Islet	Ca_o^{2+}, L-His	↑insulin→↑nutrient utilization	Biochemical	Bruce et al., 1999; Squires et al., 2000; Komoto et al., 2003; Leech and Habener, 2003
Liver				
Hepatocytes	Ca_o^{2+}, spermine	↑bile flow	Biochemical	Canaff et al., 2001
Cholangiocytes (?)				
CELLS THAT REGULATE ABSORPTION AND SECRETION				
Small intestine				
Villus cells (ap & bl)		↑absorption	Biochemical	Chattopadhyay et al., 1998
K cells	Ca_o^{2+}, amino acid	↑GIP→↑insulin/nutrient utilization	Pharmacological	Mace et al., 2012
L cells	Ca_o^{2+}, amino acid, peptide	↑GLP-1→↑insulin/nutrient utilization	Pharmacological	Leech and Habener, 2003; Mace et al., 2012; Diakogiannaki et al., 2013; Pais et al., 2016
Colon				
Surface & crypt cells (ap & bl)	Ca_o^{2+}, polyamine, R568	↑absorption; ↓secretion	Pharmacogenetic	Chattopadhyay et al., 1998; Cheng et al., 2002; Cheng, 2012
		↑barrier function; ↑gut immunity	Pharmacogenetic	Jouret et al., 2013; Macleod, 2013b; Cheng et al., 2014
		↓proliferation; ↑differentiation	Pharmacogenetic	Chakrabarty et al., 2003; Rey O et al., 2012; Aggarwal et al., 2015
		↓colon cancer	Pharmacogenetic	Kallay et al., 2000; Sheinin et al., 2000; Singh and Chakrabarty, 2013; Aggarwal et al., 2015; Singh et al., 2015
CELLS THAT REGULATE OTHER FUNCTIONS				
Myofibroblasts	Ca_o^{2+}	↑wnt5a and BMP-2 secretion	Biochemical	Peiris et al., 2007; Pacheco and Macleod, 2008
ENS	peptide, R568	↓secretion; ↓motility	Pharmacological	Chattopadhyay et al., 1998; Cheng, 2012; Muramatsu et al., 2014
Immune cells	Ca_o^{2+}	↓inflammation	Biochemical	Kelly et al., 2011

Ala, alanine; ap, apical membrane; bl, basolateral membrane; Ca_o^{2+}, extracellular calcium; CCK, cholecystokinin; ENS, enteric nervous system; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; Phe, phenylalanine; PYY, peptide tyrosine tyrosine; Trp, tryptophan.

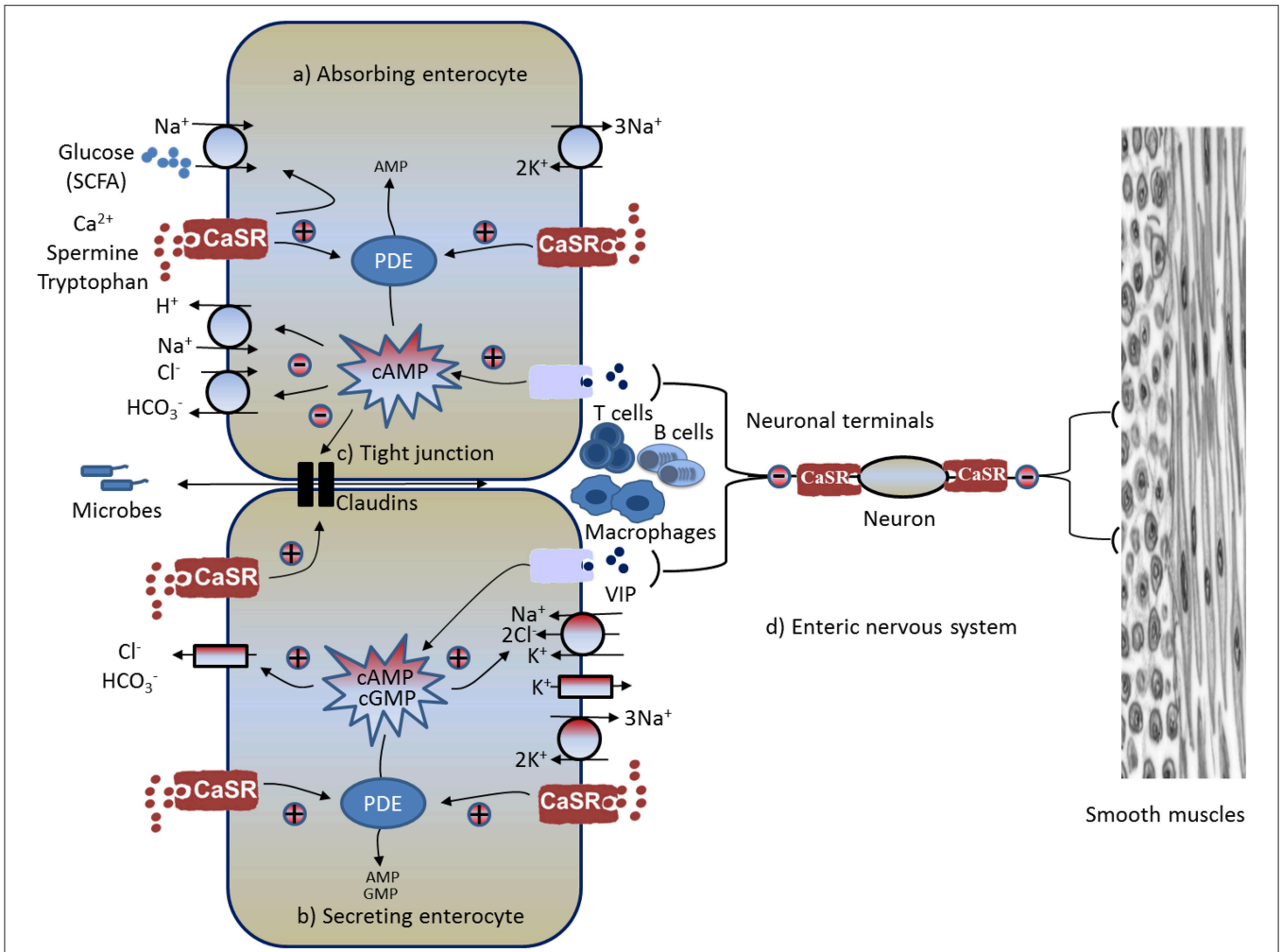

FIGURE 1 | Schematic diagram illustrating pathways and mechanisms through which CaSR-activating calcimimetics and agonists modulate GI physiology and immunophysiology. Known CaSR effects include: **(A)** increased absorption, **(B)** decreased secretion, **(C)** enhanced intestinal barrier and reduced inflammation, and **(D)** reduced enteric nerve activity and motility (see text for explanations). CaSR, calcium-sensing receptor; CFTR, cystic fibrosis transmembrane conductance regulator; PDE, phosphodiesterase; SCFA, short-chain fatty acid; VIP, vasoactive intestinal peptide; +, stimulation; –, inhibition.

fluid secretion or negative ${}^{net}J_v$. The study showed that, in the absence of the secretagogue, the colonic crypt exhibited a positive ${}^{net}J_v$, indicating net fluid absorption; exposure of crypts to the cAMP-elevating secretagogue forskolin induced net fluid secretion; increasing Ca_o^{2+} in either the luminal or vascular perfusate abolished the stimulatory effect of forskolin on net fluid secretion. In the presence of increased Ca_o^{2+}, net fluid secretion induced by forskolin was completely reversed, resulting in net fluid absorption (Cheng et al., 2002). Although the concentrations of Ca_o^{2+} used in this study (2 and 5 mM) were slightly higher than the Ca_o^{2+} concentrations in the blood and may be less physiological, the finding suggested a potential role for Ca_o^{2+} acting via CaSR in regulating intestinal fluid movement.

A subsequent study used this CaSR-modulated response to further assess the interactions of spermine and Ca_o^{2+} (Cheng et al., 2004). In this study, the effect of increasing luminal or basolateral polyamine on forskolin-stimulated ${}^{net}J_v$ was examined in the presence of a fixed dose of Ca_o^{2+}. Three doses of Ca_o^{2+} were tested: the threshold (0.1 mM), the near physiological (0.5 mM), and the physiological (1.0 mM). Similar to increasing Ca_o^{2+}, addition of the polyamine spermine to either the luminal or basolateral perfusate dose dependently reversed net fluid secretion to absorption; the extent to which spermine reduced the net fluid transport depended on the concentration of Ca_o^{2+}. Thus, at a threshold dose of Ca_o^{2+}, millimolar or sub-millimolar concentrations of spermine were required to reverse the secretory ${}^{net}J_v$; on the other hand, when a physiological Ca_o^{2+} was present, a much lower concentration (low micromolar) of spermine was needed to reverse the direction of forskolin-stimulated ${}^{net}J_v$ (Cheng et al., 2004). The latter is the polyamine concentration most often seen in breast milk (Pollack et al., 1992; Romain et al., 1992; Buts et al., 1995) but not in infant formulas [in which the polyamine concentration is at least 1 order of magnitude

lower than in breast milk and 2–3 orders of magnitude lower than the polyamine concentration in the lumen of the intestine shortly after ingestion of a typical adult human meal (see reviews Bardócz et al., 1995; Ralph et al., 1999; Milovic, 2001)]. Thus, it is conceivable that supplementation of oral rehydration solution or infant formulas with polyamines may be beneficial in treating children with diarrhea.

A definitive study that established the role for the colonic CaSR in regulating intestinal fluid secretion was the comparison of secretagogue-stimulated $^{net}J_v$ responses to Ca_o^{2+} and R568 in colonic crypts of CaSR null mice ($Casr^{-/-}Gcm2^{-/-}$) with the wild type controls ($Casr^{+/+}Gcm2^{+/+}$) (Geibel et al., 2006). It showed that CaSR, activated from either the mucosal or serosal side by Ca_o^{2+} or R568, reduced secretagogue-stimulated net fluid secretion in colonic crypts of wild type mice, but not in colonic crypts from CaSR null mice. In CaSR null mice, colons responded to cholera toxin or guanylin with stimulated fluid secretion but, unlike the wild type controls, failed to generate the inhibitory actions of Ca_o^{2+}or R-568 (Geibel et al., 2006). Speculating that CaSR may inhibit fluid secretion through inhibiting cyclic nucleotide accumulation or metabolism, levels of colonocyte cAMP, and cGMP were measured. As expected, stimulation of adenylyl cyclase with forskolin or cholera toxin increased cytosolic cAMP, and stimulation of guanylyl cyclase with guanylin or the *Escherichia coli* heat-stable enterotoxin STa increased cytosolic cGMP; increased Ca_o^{2+} or R568 abolished these effects (Geibel et al., 2006). Similar inhibitory effects were seen for spermine in isolated human colonic mucosa (Rogers et al., 2015). Interestingly, the CaSR-mediated inhibitory effects on cyclic nucleotides as well as on increased fluid secretion were prevented by the phosphodiesterase (PDE) inhibitor IBMX (Geibel et al., 2006). Similar preventative effects on CaSR were also produced by inhibition of phospholipase C (PLC) by U73122 (Geibel et al., 2006) or by depletion of inositol trisphosphate (IP_3)-sensitive intracellular Ca^{2+} stores by thapsigargin (Cheng et al., 2002). These *in vitro* studies strongly support the notion that activation of CaSR in the colon inhibits fluid secretion through receptor-mediated destruction of cyclic nucleotides by PDE using a signaling pathway that activates PLC-IP_3- Ca_i^{2+} (see **Figure 1B**).

Cl^- Secretion

Fluid secretion is driven primarily by transepithelial anion (e.g., Cl^-) secretion (Barrett and Keely, 2000; Kere and Höglund, 2000; Kunzelmann and Mall, 2002). The next question that was examined was whether CaSR activation inhibits transepithelial Cl^- secretion. The secretion of Cl^- into the lumen of the intestine requires two separate, but interconnected movements: Cl^- entry from the blood into the cell and Cl^- exit from the cell into the lumen (see **Figure 1B**). The egress of the anion is conducted through apical membrane anion channels primarily the cAMP-dependent, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB)/glibenclamide-sensitive cystic fibrosis transmembrane conductance regulator chloride channels (CFTR), whereas the entry of Cl^- is critically dependent on the activity of the basolateral membrane bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (NKCC1; Kunzelmann and Mall, 2002). Mice deficient in CFTR lack a secretory response to cholera toxin (Gabriel et al., 1994); similarly, mice lacking NKCC1 exhibit blunted secretion to cAMP or STa (cGMP) challenge (Flagella et al., 1999). By measuring short circuit current (I_{sc}) responses to pharmacological inhibitors of anion channels in the apical membrane of colonic mucosa mounted in Ussing chambers, it was shown that CaSR activation inhibited the cAMP-dependent, NPPB/glibenclamide-sensitive, apical anion channel activity (Tang et al., 2015). Likewise, by measuring Cl^--sensitive MQAE fluorescence responses in perfused colonic crypts, it was also demonstrated that secretagogue-induced, bumetanide-sensitive, basolateral membrane Cl^- entry into colonocytes was inhibited by either R568 or by increasing [Ca_o^{2+}] of the basolateral bath fluid, consistent with CaSR inhibition of NKCC1 (Geibel et al., 2006). Therefore, in colons CaSR inhibits both Cl^- entry and exit. Currently, it remains unknown whether CaSR directly inhibits these ion transporters or indirectly via the reversal of changes in cyclic nucleotide by the activation of the receptor.

HCO_3^- Secretion

In addition to Cl^- secretion, HCO_3^- secretion is increased upon secretagogue stimulation, and this stimulated HCO_3^- secretion contributes to alkaline deficit and metabolic acidosis, as well as to systemic volume depletion and dehydration seen in cholera and other diarrhea conditions (Fordtran, 1967; Powell et al., 1971). Regulated HCO_3^- secretion is also essential for mucosal defense against luminal acid (via neutralization) in the upper GI tract and bacteria (via stimulation of mucus secretion and maintenance of intestinal barrier function) in the lower GI tract; defects in HCO_3^- secretion have been shown to be a risk factor for peptic ulcer diseases (Isenberg et al., 1987; Flemstrom and Isenberg, 2001; Allen and Flemström, 2005) and intestinal inflammation (Garcia et al., 2009; Xiao et al., 2012, 2013). To assess if CaSR activation inhibits secretagogue-induced HCO_3^- secretion, colonic mucosa HCO_3^- secretory response to R568 was also studied recently (Tang et al., 2015). In this study, the cAMP-elevating secretagogue forskolin was employed to induce HCO_3^- secretion, and HCO_3^- secretion was monitored both electrophysiologically by recording HCO_3^- ion-dependent short-circuit current (I_{sc}) and chemically through measuring the rate of HCO_3^- secretion (J_{HCO3}) using a so-called "pH stat" technique. The latter measures the amount of exogenous acid (HCl or H_2SO_4) delivered per hour per cm^2 surface area to neutralize the secreted alkaline (HCO_3^-) in order to maintain a constant lumen fluid pH. It was found that forskolin stimulated both I_{sc} and J_{HCO3} in colonic mucosa of rats, wild type mice, and mice lacking the intestinal epithelium CaSR ($^{villin}Cre/Casr^{flox/flox}$; Tang et al., 2015), consistent with active control of HCO_3^- secretion in the intestine by cyclic nucleotide. However, subsequent activation of CaSR, either apically or basolaterally by R568, significantly reduced forskolin-induced HCO_3^- secretion in colonic mucosa of rats and wild type mice, but not $^{villin}Cre/Casr^{flox/flox}$ (Tang et al., 2015), suggesting the involvement of CaSR in the inhibition of HCO_3^- secretion. These studies established that CaSR regulates intestinal HCO_3^- secretion.

Activation of CaSR Enhances Salt and Solute Absorption

CaSR is also expressed in absorbing villus/surface cells (**Table 1**), suggesting critical roles in regulating intestinal absorption (see **Figure 1A**). To address this, colonic crypts or mucosa from rat and mice were isolated, and effects of CaSR agonists on absorption were examined (Geibel et al., 2006). Bidirectional fluid movements (absorption and secretion) take place in the same enterocytes. To minimize interference from secretion, in these studies tissues were first treated with basolateral bumetanide to block secretion before absorption was investigated. Without secretagogues, perfused crypts exhibited a positive net J_v ($^{net}J_v$), suggesting that under baseline conditions, colonic crypts are in a net fluid absorption mode. Addition of bumetanide to the basolateral bath fluid of perfused crypts slightly increased the absorptive $^{net}J_v$, consistent with inhibition of a small component of fluid secretion that remains under basal non-stimulated condition. This low basal fluid secretion is likely attributable to the low cyclic nucleotide level that remains even in the absence of secretagogues. Thus, in the presence of bumetanide, $^{net}J_v$ measurements represent the absorptive component of fluid transport. This absorptive fluid movement was substantially reduced by exposure to cAMP or forskolin. Activation of CaSR by either increasing Ca_o^{2+} and/or addition of R568 to the basolateral bath significantly reduced the cAMP-mediated reduction in absorptive $^{net}J_v$, demonstrating that activation of CaSR not only suppresses the stimulated fluid secretion but also reverses the reduced fluid absorption.

Na^+ Absorption

Solute absorption in the colon (and the small intestine) is driven primarily by parallel Na^+/H^+ (sodium-hydrogen exchanger, NHE) and Cl^-/HCO_3^- exchange located at the apical plasma membranes (see **Figure 1A**). It is known that this Na^+-dependent fluid absorption in the colon, as well as in the ileum, is reduced by cholera toxin and other cyclic nucleotide-elevating enterotoxins via inhibiting NHE activity and Cl^-/HCO_3^- exchange (Thiagarajah et al., 2015). It is also acknowledged that this event contributes to the severity of fluid and electrolyte losses in secretory diarrheas (Kunzelmann and Mall, 2002; Field, 2003; Thiagarajah et al., 2015). Similar to the events in secretory diarrheas, during normal digestion, vasoactive intestinal peptide (VIP) and other cyclic nucleotide-stimulating paracrines/autocrines/neurocrines are generated from enteroendocrine cells and/or the subepithelial ENS. These secretagogues stimulate intestinal secretion in distinct ion transport processes that not only increase the secretory component but also reduce the absorptive component of transepithelial fluid movement, leading to net digestive secretion (Barrett and Keely, 2000; Kunzelmann and Mall, 2002). This secretion helps to mix up food components and to lubricate the lumen surface of the intestine. To evaluate whether CaSR also modulates the NHE-mediated Na^+ absorptive process, isolated colonocytes were preloaded with BCECF (a fluorescent probe for H^+ or pH), and effects of CaSR agonists on Na^+-dependent proton extrusion from the apical membrane of colonocytes (a standard measure of NHE-mediated Na^+ absorptive activity) were studied (Geibel et al., 2006). Raising the basolateral bath Ca_o^{2+} to activate CaSR significantly increased the Na^+-dependent acid extrusion rate; the addition of R568 resulted in further elevation of this rate, demonstrating that CaSR stimulates Na^+ absorption mediated by NHE.

Cl^- Absorption

Does CaSR stimulate Cl^- absorption mediated by parallel Cl^-/HCO_3^- exchange so as to match the receptor stimulation of Na^+ absorption by the receptor (see **Figure 1A**)? To answer this question, in another study, transepithelial Cl^- absorption was measured (Tang et al., 2015). Colonic mucosa were isolated, mounted into Ussing chambers and perfused, and lumen Cl^--dependent HCO_3^- secretion (a measure of Cl^-/HCO_3^- exchange-mediated Cl^- absorptive activity) was recorded and compared in the presence and absence of R568 stimulation using the Ussing chamber-pH stat technique (Tang et al., 2015). Similar to the R568 effects on Na^+/H^+ exchange, lumen Cl^--dependent HCO_3^- secretion was found to be significantly stimulated by R568 in the colons of both rats and wild type mice, but not in *CaSR*$^{-/-}$ mice (Tang et al., 2015).

SCFA Absorption

Short-chain fatty acids (SCFAs) are the major anions in stool. SCFAs are produced in the colon by bacterial fermentation of unabsorbed carbohydrates. SCFA absorption stimulates Na^+, Cl^-, and water absorption and represents a major mechanism in the colon to conserve fluid and electrolytes (see **Figure 1A**). SCFA absorption occurs via a process involving apical membrane Na^+/H^+, Cl^-/HCO_3^-, and $SCFA/HCO_3^-$ exchanges (Ruppin et al., 1980; Binder, 2010), and Na^+/H^+ and Cl^-/HCO_3^- exchanges are stimulated by activation of CaSR. This combined knowledge led to a hypothesis that CaSR activation also stimulates $SCFA/HCO_3^-$ exchange and enhances SCFA absorption. To examine this, in a separate experiment using the same aforementioned Ussing chamber—pH stat technique, SCFA absorption mediated by $SCFA/HCO_3^-$ exchange was measured as lumen isobutyrate-dependent HCO_3^- secretion (Tang et al., 2015). Comparable to the R568 effects on Na^+/H^+ and Cl^-/HCO_3^- exchanges, isobutyrate-dependent HCO_3^- secretion was also found to be significantly stimulated by R568 in the colons of rats and wild type mice, but once again not *CaSR*$^{-/-}$ mice (Tang et al., 2015).

Jointly, these studies have established that CaSR is a regulator of NaCl and SCFA absorption in the colon. These findings are not surprising considering that CaSR has been shown to be used by chloride cells of gills, as well as by ion transporting cells of the kidney and the gut of marine and freshwater fish alike in order to pump Cl^- and other ions to direct water flow (Nearing et al., 2002); however, these new data provide compelling support for the notion that CaSR is an important regulator of intestinal fluid movement. In order to generate the large quantities of fluid needed for the digestion of ingested food, the gut has evolved a complex series of neuronal, hormonal, and/or paracrine/autocrine feedback regulation mechanisms that allow for the continued production of fluid during the phases of ingestion and digestion. Moreover, when digestion is complete,

the gut signals for the induced secretion to stop and for subsequent absorption to occur. The studies presented suggest that the nutrients released from food as a result of digestion may act as signals that activate CaSR in the gut epithelium, and probably also CaSR in the ENS (see below), thereby initiating and coordinating transition of these processes.

Besides the aforementioned transporters, apical Na^+ and K^+ channels, as well as basolateral K^+ channels and Na^+,K^+-ATPase (**Figure 1**), also play critical roles in epithelial absorption and secretion. For example, in Cl^- secreting epithelia, the basolateral K^+ channels facilitate basolateral Cl^- entry (via cycling back the K^+ for NKCC1), as well as apical Cl^- exit (by maintaining a favorable transepithelial electrical gradient), while Na^+,K^+-ATPase pumps the Na^+ entered by NKCC1 out of the cell. Whether CaSR also affects the activity and function of these transporters remains to be determined.

Also needed to be studied are CaSR effects *in vivo*. Most of the studies so far are based on observations from perfused crypts or use of Ussing chambers. Studying these effects in isolation can be artificial. Future studies should be directed at the organismal and systemic levels in order to better define the net effect of the absence of the gene. In this respect, studies that characterize the phenotype of $CaSR^{-/-}$ mice (Rey O et al., 2012; Macleod, 2013b; Cheng et al., 2014) or associate SNPs in CaSR for diarrheal conditions (Ho et al., 2010; Romero et al., 2015) would be useful. Nonetheless, preliminary data from animals (Bovee-Oudenhoven et al., 1996, 1997; Bovee Oudenhoven et al., 1997, 1999) and clinical trials on humans (Bovee-Oudenhoven et al., 2003; Cheng et al., 2013; Dadu et al., 2015) using calcium or other CaSR ligands indicate that activation of this nutrient sensing receptor *in vivo* does reduce symptoms of infectious diarrheas (also see recent review by Cheng, 2016).

With regards to the mechanisms for the effects of CaSR on ion transport, it has been postulated that CaSR agonists enhance cyclic nucleotide destruction via G protein-mediated activation of PDE, instead of inactivation of adenylyl cyclase found in non-intestinal cells (Geibel et al., 2006). Consistent with this notion, inhibition of net fluid secretion by CaSR agonists correlated with reductions in cyclic nucleotide accumulations, both of which were abolished by IBMX (Geibel et al., 2006). IBMX is a non-specific PDE inhibitor. It remains unclear which specific isoform of PDE in the intestine mediates the CaSR action. Also unknown is the mechanism by which CaSR activates PDE. There are 11 families of cyclic nucleotide-degrading PDEs, each exhibiting selective affinities for degrading cAMP and/or cGMP (Beavo, 1995; Jeon et al., 2005). Although little information is available on the specific PDE genes expressed in intestinal epithelial cells, PDE1-5 isoforms have been identified in human colon cancer cells (Soh et al., 2000; O'Grady et al., 2002), and each of these PDEs is sensitive to IBMX. Because inhibition of PLC signaling abolishes CaSR effects on cAMP and cGMP accumulation (Geibel et al., 2006), it is tempting to speculate that activation of Ca_i^{2+}/calmodulin-dependent PDE1 may be responsible. Future work is needed to determine whether PDE1 is activated by CaSR, and if so, how it is activated. Also needed to be elucidated are the mechanisms for how CaSR regulates and coordinates distinct ion channels and transporters in secretion and absorption, and whether CaSR regulates absorption by surface/villous cells using the same or different mechanism than CaSR regulates secretion by crypts. CaSR binds a plethora of ligands, interacts with multiple G protein subtypes, and regulates divergent downstream signaling pathways. It is possible that CaSR may use a completely different mechanism to regulate the same type of function in a different cellular context. For example, a recent study by Xie et al. (2014) showed that in the duodenum CaSR seems to employ an alternative intracellular signaling pathway to increase HCO_3^- secretion in this tissue. This alternative pathway involves Ca_i^{2+}-dependent activation of receptor-operated channel, intermediate conductance K^+ channels, and CFTR. Similarly, Brennan et al. (2016) recently reported CaSR stimulation of CFTR in developing human fetal lung. This stimulatory effect of CaSR appears to be mediated through a Ca_i^{2+}-dependent adenylyl cyclase.

CaSR AND ENS ACTIVITY AND MOTILITY

As previously mentioned, the ENS is the brain of the gut. The ENS comprises two plexuses—the submucosal Meissner's plexus and the myenteric Auerbach's plexus. While the former controls secretion, the latter regulates motility (see **Figure 1D**). CaSR is expressed in both plexuses (nerve fibers and somas; Chattopadhyay et al., 1998; Cheng et al., 1999; Cheng, 2012), suggesting its potential role in controlling the activity and functions of these enteric neurons.

CaSR Inhibits ENS Activity and ENS-Mediated Secretion

In mammals (humans and rodents), as much as 80–90% of the fluid secreted at basal condition, during digestion, and in diarrheas (cholera and rotavirus) are ENS-mediated (Burleigh and Borman, 1997; Lundgren et al., 2000; Lundgren, 2002; Field, 2003; Farthing, 2006; Lorrot and Vasseur, 2007). These estimates are based on the magnitude to which the fluid secreted or current evoked is inhibited by tetrodotoxin (TTX) or lidocaine. As selective inhibitors of voltage-gated Na^+ channels in neural tissues, TTX and lidocaine are used to inhibit neurotransmission, and subsequently ENS activity. Using this method, TTX-sensitive I_{sc} responses in freshly isolated, intact ENS-containing rat colon segments mounted onto Ussing chambers were measured, and effects in the presence versus absence of serosally added R568 were compared (Cheng, 2012; Cheng et al., 2012). In these experiments, TTX-sensitive I_{sc} was employed as a measure of ENS activity and ENS-mediated anion secretion, and serosal R568 was applied to activate CaSR in the ENS. Consistent with active regulation of secretion by the ENS, a large portion (∼70–75%) of I_{sc} in the proximal and distal colon was inhibited by TTX, both at basal and under cAMP (forskolin or cholera toxin)-stimulated conditions. Furthermore, the addition of forskolin or cholera toxin increased TTX-sensitive I_{sc}; subsequent addition of R568 to the serosal bath to activate CaSR in the ENS abolished the secretagogue-stimulated TTX-sensitive I_{sc}. Similar effects of R568 addition were also observed on basal TTX-sensitive I_{sc}. These results demonstrate that CaSR agonists

function as inhibitors of ENS activity, reducing ENS-mediated secretion. These findings also point to a dual model for regulating intestinal fluid transport, in which neuronal and non-neuronal secretagogue actions are modulated by the inhibitory effects of CaSR on the ENS—which is TTX/lidocaine-sensitive—as well as by CaSR on the epithelium—which is TTX/lidocaine-insensitive. Clearly, further studies are needed to address these possibilities. Also, future experiments will be required to understand which type of neuron(s) in the ENS expresses and is affected by CaSR and to determine what the intracellular second messenger mechanism(s) is involved in this process.

CaSR Inhibits Motility

CaSR is also present in the myenteric Auerbach's plexus that governs gut motility (Chattopadhyay et al., 1998; Cheng, 2012). Thus, CaSR may play a role in regulating motility as well. In support of this notion, people who take high calcium are often constipated (Prince et al., 2006), as are patients with hypercalcemia (Ragno et al., 2012). Likewise, animals receiving treatment of polyamines (e.g., spermine) or peptides (e.g., γ-glutamyl cysteine), both of which are classes of agonists for CaSR, display profound inhibition on gastric emptying and/or intestinal motor activity (Belair et al., 1981; Tansy et al., 1982; Muramatsu et al., 2014). Polyamine inhibition of GI transit is also noted in several rodent models of diarrhea-dominate forms of irritable bowel syndrome (Bergeron et al., 1996, 2001a,b). Together, these studies highlight the importance of this nutrient sensing receptor in modulating ENS activity and gut motility.

The physiological and pathophysiological significance of these findings remains to be determined. Since the enteric nerve network follows the nutrient-carrying blood and lymph vessel networks, CaSR may provide a mechanism for the ENS to sense nutrients and fine-tune its activity and function in accordance with the status of digestion and absorption. For example, upon food ingestion, mechanical signals activate the ENS, enhancing its functions in promoting secretion and motility to facilitate digestion; upon completion of digestion, the chemical signals (e.g., absorbed nutrients) carried in the parallel blood or lymph vessels may activate CaSR to deactivate the ENS, thereby slowing down secretion and motility in order to facilitate absorption. Considering its normal role in the gut, CaSR may represent an excellent candidate gene to be investigated in the pathophysiology of irritable bowel syndrome (IBS), a common clinical condition characterized by chronic diarrhea and/or constipation, in addition to abdominal pain and cramping. Given the key CaSR function in regulation and coordination of secretion and muscular contraction, it is possible that over activity of the CaSR may result in under-activation of the ENS, leading to the constipation-dominate form of IBS (IBS-C). Likewise, reduced activity of the CaSR may result in excessive activation of the ENS, leading to the diarrhea-dominate form of IBS (IBS-D). Clearly, more work is needed to verify these hypotheses in IBS patients, like the one that has recently been published (Romero et al., 2015) even if this latter study revealed no association between the common CaSR polymorphism rs1801725 and IBS.

CaSR AND INTESTINAL BARRIER INTEGRITY AND IMMUNITY

In addition to modulating the absorption of nutrients and secretion of electrolytes and fluids, the intestinal epithelial barrier, with its intercellular tight junctions, also controls the passage of gut microbes across the mucosa. This is to avoid over-activating the sub-epithelial immune cells, mainly dendritic cells (DCs), T cells, and B cells, thereby preventing local and systemic inflammation (Turner, 2006). More recently, our laboratory illustrated the role of CaSR and the consequence of its manipulation both locally and systemically using intestinal epithelium-specific *CaSR*$^{-/-}$ mice (^{villin}*Cre/Casr*$^{flox/flox}$; Cheng et al., 2014). We found that epithelial CaSR is also an important regulator of intestinal barrier integrity and immunity, as well as a key modulator of gut bacteria-sensing. Epithelial CaSR deficiency resulted in diminished intestinal barrier function, altered composition and distribution of the gut bacteria, and skewed immunity toward a pro-inflammatory response (Cheng et al., 2014). These observations strongly support the notion that the epithelial barrier plays an important role in triggering immune activation and gut inflammation.

CaSR Contributes to Intestinal Barrier Integrity

In contrast to transcellular transport, in which ions and solutes travel *through* the epithelial cell passing through both the apical and basolateral membranes, paracellular transport refers to the transfer of ions and substances across the intestinal epithelium by passing through the intercellular space *between* the cells. Situated between adjacent intestinal epithelial cells of the mucosa are structures called apical junctional complexes, such as tight junctions (TJs; **Figure 1C**). These intercellular structures, along with the layer of epithelium composing the intestinal mucosa, act as a barrier separating the luminal contents from the submucosal compartment, which is home to the gut immune system. TJs harbor complex interactions between ∼40 proteins, including the transmembrane proteins occludins and claudins. These proteins are anchored to the actin filaments and myosin light-chain through the zonula occludens family. Breaching of this dynamic barrier may result in excessive exposure of the gut immune system to luminal microbes and foreign antigens, leading to intestinal inflammation (Turner, 2006). Since it is well-known that Ca_o^{2+} is required for the development (Martinez-Palomo et al., 1980; Cereijido et al., 1981; Gonzalez-Mariscal et al., 1985) and the maintenance (Galli et al., 1976; Meldolesi et al., 1978; Palant et al., 1983) of stable epithelial TJs between epithelial cells, we hypothesized that CaSR may participate in regulating TJ assembly/formation and paracellular permeability.

Figure 2 shows the hypothesized mechanism for how CaSR produces its effects on the control of intestinal epithelial barrier permeability and inflammation. According to a current model of IBD (Turner, 2006), intestinal inflammation is induced by a self-amplifying pathway where a limited amount of luminal bacteria or bacteria-derived molecules cross the epithelium to activate

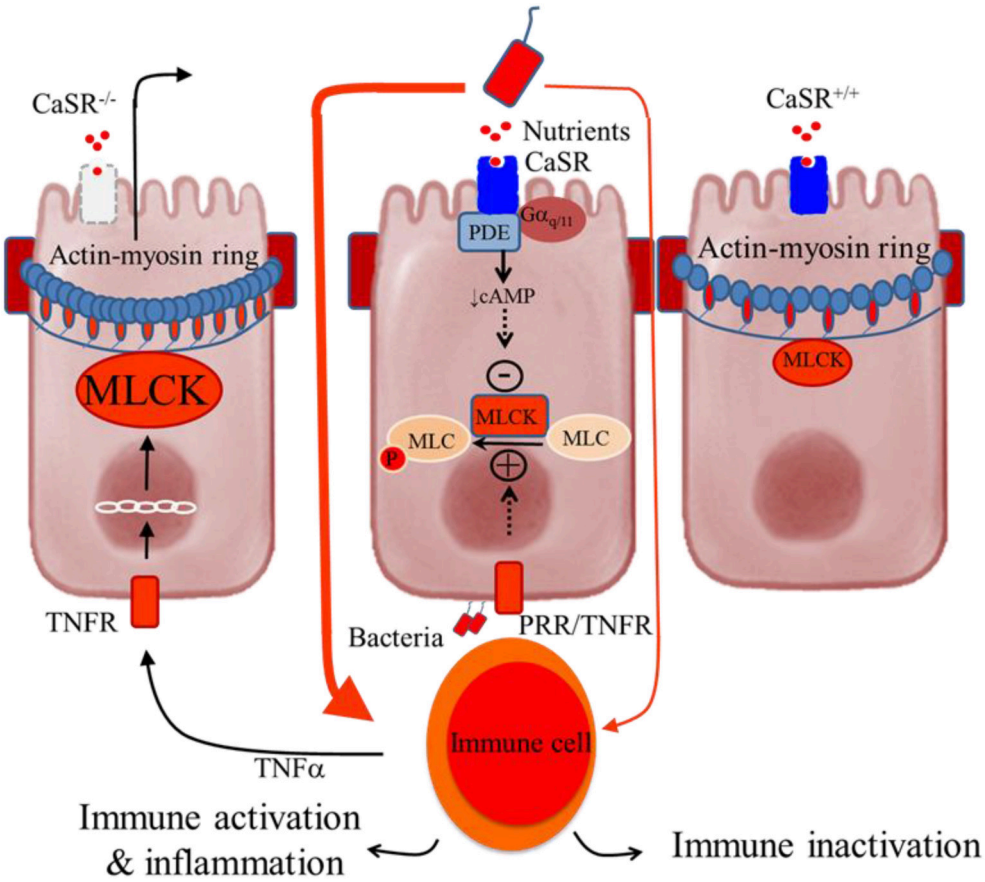

FIGURE 2 | Schematic representation of the colonocytes showing how deficiency in intestinal CaSR results in increased gut permeability and inflammation. Central panel, illustrates a current model of self-amplifying pathway for intestinal disease (Turner, 2006) where a small amount of luminal bacteria or bacteria-derived molecules pass across the epithelium to activate lamina propria immune cells, leading to secretion of proinflammatory cytokines (e.g., TNFα) and subsequent activation of their receptors (e.g., TNFR) in the epithelium. The latter increases MLCK transcription and activity and phosphorylation of myosin light chain (MLC), resulting in increased contractility of perijunctional actin-myosin ring and increased epithelial permeability. The consequences are greater access leakage of luminal materials, greater immune activation, and even greater barrier defects. The presence of CaSR ligands and signals limits amplifying of this cascade through activation of phosphodiesterase (PDE) (Geibel et al., 2006) and inhibition of MLCK (Cheng et al., 2014), leading to MLC dephosphorylation and barrier stabilization. Thus, in the presence of intact CaSR signaling, as in $CaSR^{+/+}$ mice, immune tolerance or only low-grade inflammation is seen **(Right panel)**. However, in the absence of CaSR signal, as in $CaSR^{-/-}$ mice, the limiting of this cascade amplification is lost, leading to immune activation and uncontrolled inflammation (Macleod, 2013b; Cheng et al., 2014) **(Left panel)**.

lamina propria immune cells. This leads to secretion of pro-inflammatory cytokines (e.g., TNFa) and subsequent activation of their receptors (e.g., TNFR) in the epithelium. The latter increases myosin light-chain kinase (MLCK) transcription via the IKKβ, IκBα, and NF-κB signalosome, and then phosphorylates the regulatory myosin light chain (MLC). The result of this is increased contraction of a band of actin and myosin (actin-myosin ring) located at the tight junctions, which subsequently promotes epithelial permeability. The consequences include greater access leakage of luminal materials, greater immune activation, and even greater barrier defects. CaSR limits the amplification of this cascade through receptor-mediated $G\alpha_{q/11}$-dependent activation of PDE (Geibel et al., 2006) and inhibition of NF-κB phosphorylation. As a result, MLCK is down-regulated, MLC phosphorylation inhibited, and epithelial barrier stabilized. Hence, in the presence of intact CaSR signaling, immune inactivation or tolerance is maintained. Conversely, in the absence of CaSR, ligands, or signals, as in $CaSR^{-/-}$ mice, this cascade is self-amplified, leading to immune activation and uncontrolled inflammation.

To test this hypothesis, we examined the intestinal permeability of *CaSR* null mice ($^{villin}Cre/Casr^{flox/flox}$) lacking CaSR in the intestinal epithelium *ex vivo* using the aforementioned Ussing chamber technique (Cheng et al., 2014). Compared to wild type controls, $CaSR^{-/-}$ mice displayed significantly lower transepithelial electrical resistance, higher conductance, and higher passive transport of FITC-conjugated dextran (Cheng et al., 2014). Surprisingly, significant alterations in I_{sc} and ion transporter transcript expressions, indicative of defective transcellular transport, were not observed in $CaSR^{-/-}$ mice (Cheng et al., 2014). This suggests that, unless in minute-to-minute regulations—as detailed in previous sections—in

the setting of long-term regulation, CaSR appears to affect the paracellular transport pathway exclusively.

Consistent with a defective intestinal barrier, $CaSR^{-/-}$ mice showed decreased colonic epithelial expression of TJ molecules, particularly *claudin-2*, a major component of TJs, whereas expression of MLCK-1 was found to be significantly increased (Cheng et al., 2014). Also increased was the expression of the TJ protein *Cingulin* in $CaSR^{-/-}$ mice compared to their wild type counterparts (Cheng et al., 2014). Although the exact function is unknown, *Cingulin* has been associated with the expression of occludin and claudins, and the epithelial specific transcription factors *Gata4*, *Gata6* and *Hif-1α*, which is a feature also embodied by $CaSR^{-/-}$ mice (Cheng et al., 2014).

CaSR Regulates Gut Bacteria Sensing and Balance

Besides handling dietary nutrients, the mammalian gut harbors a huge number (10^{13}–10^{14}) of microorganisms, collectively known as microbiota, especially in the colon. With the utilization of improved techniques measuring gut microbiota composition and function, such as 16S rDNA high-throughput sequencing, a growing number of research studies have shed light on the mutual relationship and bidirectional interactions between gut microbiota and nutrition (see recent reviews Flint et al., 2012; Maukonen and Saarela, 2015). For example, while energy extraction and metabolism are greatly influenced by these gut microbes, the composition and activity of these microbes are also substantially affected by the energy (diet and nutrients) one consumes. Multiple mechanisms are proposed to explain these interactions. We hypothesized that CaSR as a sensor for many nutrients may be involved in gut bacteria-sensing and ecosystem-balancing, regulating the nutrition-microbial-host interactions. To examine this, we analyzed and compared the microbiota of steady-state $CaSR^{-/-}$ and wild type mice using the 16S rDNA sequencing technique. We found that deficiency in epithelial CaSR altered the gut's microbe balance (Cheng et al., 2014). At the phylum level, an outgrowth in Deferribacteraceae was noted, which has previously been found to correlate with inflammatory responses in the colons of *Citrobacter rodentium*-infected mice (Hoffmann et al., 2009), a model of bacterial colitis. Concurrently, beneficial Lactobacilli and Clostridium were decreased in $CaSR^{-/-}$ mice (Cheng et al., 2014). Furthermore, the relative abundance and distribution of the gram-positive organism *Clostridium coccoides* was also significantly altered in $CaSR^{-/-}$ mice, with depletion noted in the lumen and enrichment in the sub-epithelial layer. Consistent with enhanced bacterial translocation and dissemination in host tissues, $CaSR^{-/-}$ mice had significantly decreased epithelial expression of *Reg3*β and *Reg3*γ, which encode secreted C-type lectins that bind and protect against translocation and dissemination of Gram-negative (van Ampting et al., 2012) and Gram-positive bacteria (Cash et al., 2006; Brandl et al., 2007), respectively.

In addition to detecting "health signals" (i.e., nutrients), CaSR has recently been shown to be involved in the detection of "danger signals" derived endogenously [e.g., Ca^{2+} released as a result of tissue injury (Lee et al., 2012)] or exogenously [e.g., chitin and chitosan (Huang et al., 2015; Muanprasat et al., 2015)]. Since these endogenous and exogenous danger signals are two types of molecules that belong to so-called damage-associated molecule patterns (DAMPs) and pathogen-associated molecule patterns (PAMPs), and are also ligands of the primitive pattern recognition receptors (PRRs), it is reasonable to speculate that CaSR and PRR—two ancient basic sensing mechanisms—may communicate in a way to detect an appropriate level of danger in the gut. Indeed, intestinal epithelial cells were found to express all PRRs that recognize different ligands. These include Toll-like receptors (TLRs), C-type lectins, nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs), and retinoic acid-inducible gene I-like receptors (RLRs) (Abraham and Medzhitov, 2011; Cheng et al., 2014). When CaSR function was lost, all the PRRs were up-regulated (Cheng et al., 2014). Thus, it is likely that CaSR may normally antagonize PRR signaling and keep the latter in check. Alternatively, PRRs may be up-regulated in $CaSR^{-/-}$ mice to compensate for the lost CaSR function. Similar studies that examine CaSR expression and function in PRR-deficient mice would help to address these possibilities.

Of the greatest relevance is probably the ability of the microbiota to produce polyamines, spermine (tetra-amine), spermidine (tri-amine), and their di-amine precursor putrescine. As alkaline molecules, these polycationic polyamines are fully protonated in physiologic solutions so that they resemble multivalent metal cations Ca^{2+} and Mg^{2+} and can thereby participate in many biological processes required for the maintenance of gut epithelium health (Dufour et al., 1988; Buts et al., 1993; Loser et al., 1999). In the gut, while virtually every microbiome has genes for polyamine synthesis, the major polyamine-producers are Bacteroides, Enterobacteria, and Fusobacterium (Noack et al., 1998; Sabater-Molina et al., 2011). Among the three, Bacteroides is the most predominant species of the gut flora, particularly in those individuals who consume protein and animal fat. Pectin, fructans, and other indigestible carbohydrates and dietary fiber (collectively called prebiotics) are known to be beneficial to the host. It has been postulated that this benefit is attributed to their fermentation end products short-chain fatty acids. New emerging evidence, however, suggests that prebiotics are also beneficial because they select polyamine-producing microflora (Noack et al., 1998; Delzenne et al., 2000; Sabater-Molina et al., 2011). In the colon, polyamines, specifically spermine, are potent positive allosteric modulators of CaSR (Cheng et al., 2004). In the presence of physiological or near physiological concentrations of Ca_o^{2+}, as low as a few nM of spermine added luminally or basolaterally was found to produce significant biological effects such as inhibition of cAMP-dependent fluid secretion by colonic crypts (Cheng et al., 2004). Thus, the bacterial polyamine-activated CaSR signaling may very well be involved in the cross-talk between the microbiome and the epithelium—an absolutely essential process required for the proper development of both innate and adaptive immune responses.

CaSR Regulates Intestinal Innate and Adaptive Immune Responses

The colon is in a constant state of inflammation, the magnitude of which is controlled largely by the integrity of the intestinal barrier and the microbiota. Speculating that dysbiosis may lead to pathogenic inflammatory immune responses locally, gene array analyses of the distal colon of wild type and *CaSR*$^{-/-}$ mice were performed (Cheng et al., 2014). As expected, the colon of *CaSR*$^{-/-}$ mice showed a marked increase in expression of an array of cytokine-encoding genes, including IL-1β, TNF-α, INF-γ, IL-6, IL-12, IL-17, IL-22, IL-23, NO synthase 2, and prostaglandin E synthase 3. These changes in cytokine expression are likely attributed to the CaSR null epithelium because similar changes in expression were noted *in vitro* in cultured colonic epithelial cells as well (Mine and Zhang, 2015; Zhang et al., 2015). Increased IL-1R expression was also seen in colonic DCs in *CaSR*$^{-/-}$ mice, as well as in colonic CD4^{+} and CD8^{+} T lymphocytes. As further evidence of chronic intestinal inflammation in *CaSR*$^{-/-}$ mice, higher IL-1R and programmed cell death protein 1 (PD-1) were significantly expressed in colonic CD4^{+} and CD8^{+} T cells, and increased number of B cells with higher levels of IgA expression was found to accumulate in the colon. Moreover, compared to their wild type littermate counterparts, *CaSR*$^{-/-}$ mice demonstrated more severe colitis in response to challenge by dextran sodium sulfate (DSS). Their recovery was also significantly delayed, both clinically, as assessed by changes in body weight, stool consistency and stool blood, and histologically, as demonstrated by alterations in colonic inflammation and mucosal damage.

Thus, our *CaSR*$^{-/-}$ mice studies demonstrated that a healthy epithelial CaSR signal is required for the maintenance of a functional epithelial barrier, a symbiotic microbial-host interaction, and a balanced immune system; deficiency in CaSR results in compromised barrier function and increased translocation of bacteria that trigger the immune system, thereby potentiating this cycle, ultimately leading to inflammation.

Still, many questions remain to be addressed. For example, CaSR is lost in colon cancer (Sheinin et al., 2000; Fetahu et al., 2014, 2016). What happens to CaSR in the colons of IBD patients and animal models of DSS or infectious diarrhea? Is the expression or function of the CaSR altered as a result of inflammation or due to responsiveness to nutrients or allosteric modulators, as recently demonstrated in transformed colonic cells (Fetahu et al., 2014)? In IBD, there are significant changes to the ENS and neuroplasticity (Lomax et al., 2005); is CaSR involved? If so, what are the consequences to motility, neurosecretion, epithelial, and immune function?

SUMMARY AND CONCLUSIONS

In summary, we have shown that gut epithelium CaSR, a fundamental mechanism for sensing and regulating ionic and nutrient compositions of extracellular milieu in the small and large intestine, controls digestion, absorption, and secretion. Consequently, during the digestion phase (i.e., when food is ingested into the stomach before nutrients are released), CaSR stimulates secretion to aid in food breakdown; in contrast, during the absorptive phase (i.e., when nutritional signals are fully extracted and have reached the absorptive gut of the small intestine), CaSR stimulates absorption and inhibits secretion so as to terminate digestion and complete the cycle. We have also provided evidence that this same receptor controls intestinal permeability and immunity. Thus, whereby disrupting or inhibiting this nutrient-sensing mechanism may be associated with over-activation of the immune system and loss of immune tolerance, activating CaSR expression and activity would be helpful, not only in the maintenance of a balanced immune system, but also in deactivating the over-activated immune responses and restoring immune homeostasis. These results suggest a new paradigm for regulation of intestinal physiology and immunology, in which both fluid metabolism and immune balance may be fine-tuned by CaSR in accordance with nutrient availability and the state of digestion and absorption. Most of the studies so far are conducted *in vitro* or based on observations made from *ex vivo* tissues. Future studies need to define CaSR effects *in vivo* in animal models and transgenic mice before CaSR agonist clinical trials are performed in diarrheal patients.

Acute infectious diarrhea remains the number one cause of death among young children, particularly in developing nations. It is estimated that 1.3 million children die each year, not due to infection *per se*, but as a result of the associated diarrhea and dehydration. Although oral rehydration solution is valuable for correcting dehydration, so far there is no cost-effective therapy to stop this ongoing loss of fluid and to reduce the duration of diarrhea. Likewise, on the other end of the spectrum, chronic inflammatory, and neurogenic diarrhea, such as those caused by IBD and IBS, is a major health problem in developed countries. It is especially prevalent among adolescents and young adults, affecting ∼5% of the population. Although an increased number of treatment options have become available, there is currently no cure for these conditions. Total enteral nutrition is an effective primary therapy for Crohn's disease, but the exact mechanism of action remains unspecified. The ability of CaSR agonists to reverse increased intestinal secretion and decreased absorption induced by bacterial enterotoxins, diminish overly active enteric nerve activity and motility, as well as restore compromised barrier function and imbalanced immune responses suggests that modulations of CaSR expression and activity using calcium mimetic or a combination of specific nutrients may provide a novel therapeutic approach for secretory diarrhea, IBS, and IBD.

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LT, CC, XS, AP, MM and SC contribute to the conception or design of the work, drafting or revising the manuscript, final approval of the version to be published, and agreement to be accountable for the content of the work.

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CORRECTION
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Corrigendum: The Extracellular Calcium-Sensing Receptor in the Intestine: Evidence for Regulation of Colonic Absorption, Secretion, Motility, and Immunity

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Keywords: intestinal barrier function, enteric nervous system, gut immunity, secretory diarrhea, motility, irritable bowel syndrome, inflammatory bowel disease, calcium-sensing receptor

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Due to an error in the original Review article, the name of the author Mansour Mohamadzadeh was incorrectly spelled as "Mansour Mahamadzadeh". The authors apologize for the mistake and this error does not change the scientific conclusions of the article in any way.

The original article has been updated.

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REVIEW
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Disruption of Vitamin D and Calcium Signaling in Keratinocytes Predisposes to Skin Cancer

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1,25 dihydroxyvitamin D (1,25$(OH)_2$D), the active metabolite of vitamin D, and calcium regulate epidermal differentiation. 1,25$(OH)_2$D exerts its effects through the vitamin D receptor (VDR), a transcription factor in the nuclear hormone receptor family, whereas calcium acts through the calcium sensing receptor (Casr), a membrane bound member of the G protein coupled receptor family. We have developed mouse models in which the *Vdr* and *Casr* have been deleted in the epidermis ($^{epid}Vdr^{-/-}$ and $^{epid}Casr^{-/-}$). Both genotypes show abnormalities in calcium induced epidermal differentiation *in vivo* and *in vitro*, associated with altered hedgehog (HH) and β–catenin signaling that when abnormally expressed lead to basal cell carcinomas (BCC) and trichofolliculomas, respectively. The $Vdr^{-/-}$ mice are susceptible to tumor formation following UVB or chemical carcinogen exposure. More recently we found that the keratinocytes from these mice over express long non-coding RNA (lncRNA) oncogenes such as H19 and under express lncRNA tumor suppressors such as lincRNA-21. Spontaneous tumors have not been observed in either the $^{epid}Vdr^{-/-}$ or $^{epid}Casr^{-/-}$. But in mice with epidermal specific deletion of both *Vdr* and *Casr* ($^{epid}Vdr^{-/-}/^{epid}Casr^{-/-}$ [DKO]) tumor formation occurs spontaneously when the DKO mice are placed on a low calcium diet. These results demonstrate important interactions between vitamin D and calcium signaling through their respective receptors that lead to cancer when these signals are disrupted. The roles of the β–catenin, hedgehog, and lncRNA pathways in predisposing the epidermis to tumor formation when vitamin D and calcium signaling are disrupted will be discussed.

Keywords: vitamin D receptor, calcium sensing receptor, squamous cell carcinoma, long non coding RNA, UVB, hedgehog, β–catenin

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INTRODUCTION

Skin cancer is the most common form of cancer with an incidence estimated to be over 5 million skin cancers per year in the United States and rising (American Cancer Society, data available at http://www.cancer.org.cancer, April, 2016 update). Most of these (80%) are basal cell carcinomas (BCC). Squamous cell carcinomas (SCC) make up another 16% and melanomas another 4%. Sunlight, in particular the UVB part of the spectrum (up to 5% of the UV light reaching earth depending on the zenith angle of the sun associated with time of day and season), is the major etiologic agent for these cancers. UVB (280–320 nm) is absorbed by DNA in the epidermis resulting in mutations identified as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts (6–4 PP) that lead to C to T or CC to TT mutations if not repaired. These are commonly referred to as the UVB "signature" lesion (Freeman et al., 1989; Hussein, 2005). On the other hand UVA (320–400 nm), comprising 95% of the UV light reaching earth,

is capable of penetrating into the dermis and does its DNA damage primarily by oxidative processes (e.g., 8 hydroxy 2′ deoxyguanosine production), but CPDs may be produced at higher levels (Besaratinia et al., 2005). On the other hand UVB is required for vitamin D_3 production converting 7-dehydrocholesterol levels in the skin to pre vitamin D_3, which then isomerizes to vitamin D_3. White males with class III pigmentation require 18 mJ/cm^2 UVB exposure for vitamin D_3 production, (Matsuoka et al., 1989), a dose not likely to lead to tumors in most individuals. However, the efficiency of vitamin D production by solar radiation is less than that of monochromatic UVB in part due to the UVA portion of solar radiation that influences the ratio of pre D_3 to lumisterol$_3$ produced and that may contribute to tumor formation over and above that of UVB (MacLaughlin et al., 1982; Agar et al., 2004). Vitamin D_3 production is not the only metabolic step of which the skin is capable. Both the further conversion to 25OHD_3 (via Cyp27A1 and Cyp2R1) and then to its active metabolite 1,25(OH)$_2D_3$ (via Cyp27b1) (Bikle et al., 1986) take place. This latter step is promoted by UVR (Muthusamy and Piva, 2009), perhaps by stimulation of the expression of cytokines such as TNF (Bikle et al., 1991). Moreover the vitamin D receptor (VDR) is expressed in both melanocytes (Colston et al., 1982) and keratinocytes (Pillai et al., 1988) and have been shown to respond to 1,25(OH)$_2D_3$ by a reduction in proliferation and promotion of differentiation (Colston et al., 1981; Bikle, 2012b). A key rationale for our studies in the protective role of calcium and vitamin D signaling in UVR induced skin cancer is that the 1,25(OH)$_2D_3$ produced in the skin under the influence of UVR provides protection against UVB and likely UVA induced tumors, a rationale supported by epidemiologic evidence that suggests benefit of low dose UVR. For example, modest increases in UVB appeared to be protective against skin cancer in a meta analysis of 10 US studies (Armstrong and Kricker, 2001), although higher doses of UVB were not protective. Moreover, no significant correlation in SCC incidence was observed in association with time spent outdoors in an Australian population study (English et al., 1998). In a multicenter European study Rosso et al. (1996) identified a threshold of 70,000 accumulated hours of sunshine below which an increase in SCC was not observed, although the threshold for BCC was lower. Thus, the clinical evidence is at least supportive if not definitive for a protective role of low dose UVB, mediated we submit via vitamin D production. Although a similar case has not been made for calcium in epidermal tumor formation, the epidemiologic data demonstrating protection against colorectal cancer by diets high in calcium and vitamin D is substantial (Garland et al., 1985; Chakrabarty et al., 2005), and as will be discussed later in this article, the role of calcium appears to be synergistic with that of vitamin D.

REGULATION OF KERATINOCYTE PROLIFERATION AND DIFFERENTIATION BY CALCIUM AND 1,25(OH)$_2$D

Calcium and 1,25(OH)$_2$D are critical for keratinocyte differentiation. Calcium concentrations below 0.07 mM promote proliferation, whereas increasing the extracellular calcium concentration (Cao) above 0.1 mM (calcium switch) induces differentiation. Among the changes are the translocation of proteins such as E-cadherin to the membrane to form the E-cadherin/catenin complex (adherens junctions). Proteins associated with this complex include phosphatidyl inositol 3 kinase (PI3K), various catenins including β-catenin, and phosphatidyl inositol 4-phosphate 5-kinase 1α (PIP5K1A). These proteins in this complex mediate much of the ability of calcium and vitamin D to promote differentiation (Tu et al., 2001, 2005; Xie et al., 2005, 2009; Xie and Bikle, 2007; Tu C. et al., 2008). Other important proteins whose translocation to the membrane promotes the differentiation process include the calcium sensing receptor (Casr), phospholipase C-γ1 (Plcg1), and the Src family of tyrosine kinases, the activation of which phosphorylate the catenins facilitating their binding to E-cadherin to form the E-cadherin/catenin complex. These changes then lead to the sequential induction of proteins including keratins Krt1 and Krt10 (Yuspa et al., 1989), profilaggrin (the precursor of filaggrin [Flg]), involucrin (Ivl), and loricrin (Lor). These and other proteins are cross linked into the insoluble cornified envelope by the calcium induced transglutaminase 1 (Tgm1) (Thacher and Rice, 1985; Hohl, 1990), the final step in the differentiation process.

The Casr underlies the ability of the keratinocyte to respond to calcium (Tu et al., 2004, 2011, 2012; Tu C. et al., 2008). The Casr through the scaffold protein filamin activates the RhoA pathway that in turn activates the src kinase family, which phosphorylate the catenins, facilitating their binding to E-cadherin (Tu et al., 2011). We cloned the Casr from keratinocytes (Oda et al., 1998) and subsequently developed a mouse expressing a floxed form of *Casr* (exon 7 encoding the entire transmembrane domain and intracellular portion of the gene; Chang et al., 2008; Tu C. et al., 2008). This enables us to delete the gene in the tissue of our choice, in this case the keratinocyte where we demonstrated the central role of Casr in calcium signaling within the keratinocyte and its impact on differentiation (Tu C. et al., 2008; Tu et al., 2012). Mice lacking the Casr develop a defective permeability barrier due to abnormal production of essential lipids and proteins required for barrier formation as well as a defective innate immune response. Similar abnormalities develop in mice with *VDR* or *Cyp27b1* gene deletions. Moreover, deletion of Casr also results in reduced expression of *Vdr* and *Cyp27b1* (Tu et al., 2012), likely contributing to the failure of the epidermis of Casr deficient mice to differentiate normally. On the other hand 1,25(OH)$_2$D induces the *Casr* (Canaff and Hendy, 2002). Just as 1,25(OH)$_2$D/VDR induces *Casr*, calcium/Casr is required for *Vdr* and *Cyp27b1* expression, demonstrating the strong interaction between calcium and vitamin D signaling in the skin with respect to differentiation. Thus, the actions of 1,25(OH)$_2$D/VDR enhance the keratinocyte response to the prodifferentiating actions of calcium (Ratnam et al., 1999), whereas the effect of Casr in the expression of *Vdr* and *Cyp27b1* (Tu et al., 2012) enhances the prodifferentiating actions of 1,25(OH)$_2$D (Su et al., 1994). With respect to cancer, this synergistic interaction is an important concept to which we will return.

The synergism between Casr and VDR is well-demonstrated by the joint regulation of the expression of a number of

genes by calcium and $1,25(OH)_2D$ including the phospholipase C (*Plc)* family members (Xie and Bikle, 1997) important for differentiation, the processing of the lipids required for permeability barrier formation (Oda et al., 2009), and the enhancement of the innate immune response via induction of toll like receptor 2 (TLR2) and its coreceptor CD14, that initiate the innate immune response in skin leading to the expression of defensins such as cathelicidin (Schauber et al., 2007). Moreover, both calcium and $1,25(OH)_2D$ inhibit genes such as *Myc* (Matsumoto et al., 1990) and *Ccnd1* (Bikle, 2011) while inducing cell cycle inhibitors Cdnk1a (aka $p21^{cip}$) and Cdnk1b (aka $p27^{kip}$), which contribute to the antiproliferative actions of calcium and $1,25(OH)_2D$. The roles of calcium/Casr and $1,25(OH)_2D$/VDR in immune regulation as well as in proliferation and differentiation likely contribute to their roles in protection of the skin against the development of skin cancer.

THE ROLES OF VDR AND Casr IN CANCER PROTECTION

Zinser et al. (2002) made the first clear demonstration of the predisposition to tumor formation in the skin of mice lacking VDR. They administered the carcinogen 7, 12 dimethylbenzanthracene (DMBA) to *Vdr* null mice and wildtype mice and found that nearly all the *Vdr* null mice developed papillomas, whereas few if any of the wildtype mice did. Others have confirmed these results (Indra et al., 2007). Moreover, *Vdr* null mice have also been shown to be predisposed to tumor formation following prolonged UVB exposure first by Ellison et al. (2008) and subsequently by our own group (Teichert A. E. et al., 2011). Following UVB both SCC and BCC develop, not just papillomas. Part of this predisposition to tumor formation is due to a defective DNA damage repair process (review in Bikle, 2012a). The appearance of BCC suggested that the hedgehog (HH) signaling pathway was involved, as mutations in this pathway underlie essentially all BCC (Aszterbaum et al., 1998). However, skin lacking VDR also results in BCC when β–catenin signaling is increased (Pálmer et al., 2008). Thus, we became interested in the interacting roles of HH and β-catenin signaling in tumor suppression by VDR, a subject to which I will return.

When we knocked out both *Vdr* and *Casr* in keratinocytes ($^{epid}Vdr^{-/-}/^{epid}Casr^{-/-}$, DKO), tumors developed spontaneously, something that we had not observed in mice in which either gene was deleted by itself (Bikle et al., 2015). In this case the tumors were SCC. Colorectal cancer provides a good model for the development of skin tumors in that abnormalities in calcium, vitamin D and β-catenin signaling have also been implicated in the development of colorectal cancer with human colorectal cancer cell lines (Chakrabarty et al., 2003, 2005; Bhagavathula et al., 2007; Liu et al., 2010; Wang et al., 2010). A frequently used model involves a mutated *Apc* resulting in increased Wnt/β-catenin signaling (Arimura et al., 2009). Activation of the Wnt/β-catenin pathway increases proliferation and reduces apoptosis, whereas inhibition of this pathway has the reverse effect (Varnat et al., 2009). As it does in keratinocytes calcium via the Casr blocks the translocation of β-catenin to the nucleus in part by increasing its binding to the E-cadherin/catenin complex in the membrane, thus blocking its transcriptional activity (Chakrabarty et al., 2003). Like the situation in keratinocytes, $1,25(OH)_2D$ synergizes with calcium in these actions by inducing the expression of Casr (Chakrabarty et al., 2005), increasing the expression of the cell cycle inhibitors Cdkn1a and Cdkn1b, and inhibiting the expression of Myc, Ccnd1, survivin (Birc5), and thymidylate synthase (Tyms; Bhagavathula et al., 2007; Liu et al., 2010). In addition to its stimulation of E-cadherin/catenin complex formation, thus limiting the access of β-catenin to the nucleus, $1,25(OH)_2D$ induces an inhibitor of Wnt //β-catenin signaling, dickkopf 1 (Dkk1; Pendas-Franco et al., 2008). Our studies in which *Casr* was deleted from the intestinal epithelial cell (Rey et al., 2011) demonstrated hyperproliferation in these cells. This was accompanied by increased localization of β–catenin in the nuclei signifying activation of β-catenin signaling. Again comparable to that shown in the skin, mice lacking *Vdr* in the intestinal epithelium were predisposed to carcinogen induced tumor formation (Byers et al., 2011). Whether deleting both *Vdr* and *Casr* will lead to spontaneous colorectal tumors remains to be tested.

$1,25(OH)_2D$ has also been shown to be protective against UVA induced oxidative induced mutations in DNA (Gordon-Thomson et al., 2012). UVA induces reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide, and hydroxyl radicals as well as nitric oxide (NO) via stimulation of NO synthase. NO combines with O_2^- to form peroxynitrite that causes oxidative and nitrative modifications of DNA bases such as 8 oxo 2 deoxyguanosine (D'Orazio et al., 2013). These UVA fingerprint lesions along with UVB fingerprint lesions have been found in human SCC (Agar et al., 2004). Of interest is that the protection by $1,25(OH)_2D$ of the formation of these UVA fingerprint mutations may not require the VDR (Gordon-Thomson et al., 2012), and the role of calcium in their formation has not been studied.

THE HEDGEHOG (HH) PATHWAY IN EPIDERMAL TUMOR FORMATION

Activation of HH signaling has been shown to result in BCC in both animals and humans (Hahn et al., 1996), and appears also to predispose to UVB induced SCC (Ping et al., 2001). This was first demonstrated when *PTCH1* mutations were found to be the cause of the basal cell nevus syndrome (BCNS) (Gorlin Syndrome), a syndrome in which patients readily develop BCCs (Hahn et al., 1996; Aszterbaum et al., 1998). This syndrome has been reproduced in a mouse model with *Ptch1* mutations (Aszterbaum et al., 1999). Subsequently it was found that essentially all BCCs, sporadic or part of the BCNS, have mutations in *PTCH1* or other elements of the HH signaling pathway (Aszterbaum et al., 1998), and nearly all BCC in humans or mice overexpress Ptch1 or one of the other components of the HH pathway (Tojo et al., 1999; Bonifas et al., 2001). A drug, vismodegib, targeting the HH pathway, has recently been developed as the first effective non-surgical

treatment for advanced BCC (Sekulic et al., 2012; Tang et al., 2012).

In the basal state Ptch1 inhibits the function of smoothened (Smo), like Ptch1 a membrane protein. Smo, in the inhibited state, keeps the Gli family of transcription factors bound to suppressor of fused (Sufu) within the cytoplasm, thus limiting their translocation to the nucleus (Barnfield et al., 2005; Svärd et al., 2006). Sonic hedgehog (Shh) binding to Ptch1 reverses its inhibition of Smo enabling the release of the Gli factors from Sufu and their entry into the nucleus. Gli1 and 2 are the main actors in gene transcription (Mimeault and Batra, 2010). Their transcriptional activity includes the increased expression of other components of the HH pathway, as well as anti apoptotic and cell cycle factors including Bcl2, Ccnd1,2, E2f1, Cdc45, thus promoting proliferation, while suppressing genes associated with keratinocyte differentiation including the VDR (Grachtchouk et al., 2000; Nilsson et al., 2000; Regl et al., 2002, 2004a,b). When Gli1, Gli2, or Shh are overexpressed in basal keratinocytes, BCC develop (Oro et al., 1997; Grachtchouk et al., 2000; Nilsson et al., 2000). Similarly human keratinocytes overexpressing Shh develop BCC like lesions when grafted unto nude mice (Fan et al., 1997).

Vdr null animals overexpress elements of the HH signaling pathway in their epidermis (Teichert A. et al., 2011), although they show reduced expression in the cycling portion of their hair follicles (Teichert et al., 2010). Consensus sequences for vitamin D response elements (VDRE) in the promoters of Shh, Ptch1, Ptch2, Gli1, and Gli2 have been identified (Reddy et al., 2004; Wang et al., 2005; Pálmer et al., 2008; Luderer et al., 2011), and we (Teichert A. et al., 2011) have demonstrated that $1,25(OH)_2D$ inhibits their expression in a VDR dependent fashion. Moreover, vitamin D *per-se* has been shown to suppress Smo (Bijlsma et al., 2006; Tang et al., 2011), presumably by a non-genomic mechanism. These findings in the epidermis stand in stark contrast to the role of VDR during HF cycling where the unliganded VDR appears to promote the ability of HH signaling to initiate early anagen (Lisse et al., 2014).

β-CATENIN SIGNALING IN EPIDERMAL TUMOR FORMATION

Benign hair follicle tumors, pilomatricomas or trichofolliculomas, result from over activation of the wnt/β-catenin pathway (Gat et al., 1998; Chan et al., 1999; Xia et al., 2006). However, this depended on the VDR status of the animal. Pálmer et al. (2008) demonstrated an interaction between VDR and β-catenin in transcriptional regulation. They found putative response elements for VDR and β-catenin /LEF1 in a number of genes including those of the HH signaling pathway. Moreover, an analog of $1,25(OH)_2D$ could block the formation of these hair follicle tumors induced by overactivation of the wnt/β-catenin pathway. On the other hand, mice lacking VDR developed BCC rather than the more benign trichofolliculomas when the wnt/β-catenin pathway was overactivated. In humans trichofolliculomas were found to have high nuclear levels of both β-catenin and VDR, whereas BCC have high levels of β-catenin but low levels of VDR (Pálmer et al., 2008). These data indicate the importance of the interactions between VDR and β-catenin in the hair follicle and in the epidermis in both mice and humans. As in HH signaling, these interactions are complex in that VDR is required for β-catenin activation in the hair follicle (Lisse et al., 2014), such that when β-catenin transcriptional activity is prevented as in the VDRKO, hair follicle formation is blocked (Huelsken et al., 2001). On the other hand suppression of β-catenin transcriptional activity by VDR in the epidermis appears to be protective with respect to tumor formation (Wei et al., 2007).

As noted earlier both the Casr and VDR are required for the formation of the E-cadherin/catenin complex, which is not only required for the differentiation of the keratinocyte, but by keeping β-catenin bound to the membrane prevents its nuclear translocation and activation of genes promoting proliferation. Loss of the E-cadherin/catenin complex is a well-known marker of malignant transformation in a number of epithelial cells (Ghahhari and Babashah, 2015). Moreover, we (Tu et al., 2007; Tu C.L. et al., 2008) have shown that deletion of the Casr from keratinocytes reduces their stores of calcium and blocks their response to extracellular calcium (Cao) including the formation of the E-cadherin/catenin complex.

The β-catenin and HH pathways interact (Bienz, 2005; Pálmer et al., 2008). Using a constitutively active *Smo* [*Gt(ROSA)26Sor*$^{tm1(Smo/EYFP)Amc}$] in keratinocytes to induce BCC, two groups (Yang et al., 2008; Youssef et al., 2012) found a rapid increase in genes of the Wnt/ β-catenin pathway. *Dkk1* overexpression or deletion of *Ctnnb1* prevented the development of BCC. Both HH and Wnt/ β-catenin pathway constituents were found to be over expressed in a series of human BCC (Youssef et al., 2012). Putative β-catenin /LEF1 response elements as well as VDRE mentioned earlier have been found in a number of HH pathway genes (Pálmer et al., 2008), which unlike the VDRE appear to be stimulated by activated β-catenin with an increase in *Shh* expression (Schneider et al., 2010). The role of VDR in the regulation of these two pathways is shown in **Figure 1**.

LONG NON-CODING RNAS (LncRNA)

Approximately 20,000 protein-coding genes are encoded by the human genome. This represents less than 2% of the total genomic sequence. That said 90% of the genome is actively transcribed without protein coding potential (Mercer et al., 2009). These non-coding transcripts are arbitrarily divided into short and long non-coding RNAs with small non-coding RNAs defined as less than 200 bases, including tRNAs, microRNAs, and small nuclear (snoRNAs), and long non-coding RNAs (lncRNAs) as those with lengths larger than 200 bases, some over 100 kb in length (Gibb et al., 2011). Much of the transcriptome is comprised of lncRNAs (∼80%; Mercer et al., 2009), and over 23,000 have been identified so far. Like mRNAs they are spliced and contain polyadenylation signals, (Mattick, 2011). Many regulatory processes are controlled by lncRNAs including embryonic pluripotency, differentiation, and body axis patterning, promoting developmental transitions (Mattick, 2011; Batista and Chang, 2013). LncRNAs can influence the epigenetic programs of the transcriptome through their regulation of histone modifications (Spitale et al., 2013). Of particular relevance to this review is that lncRNAs

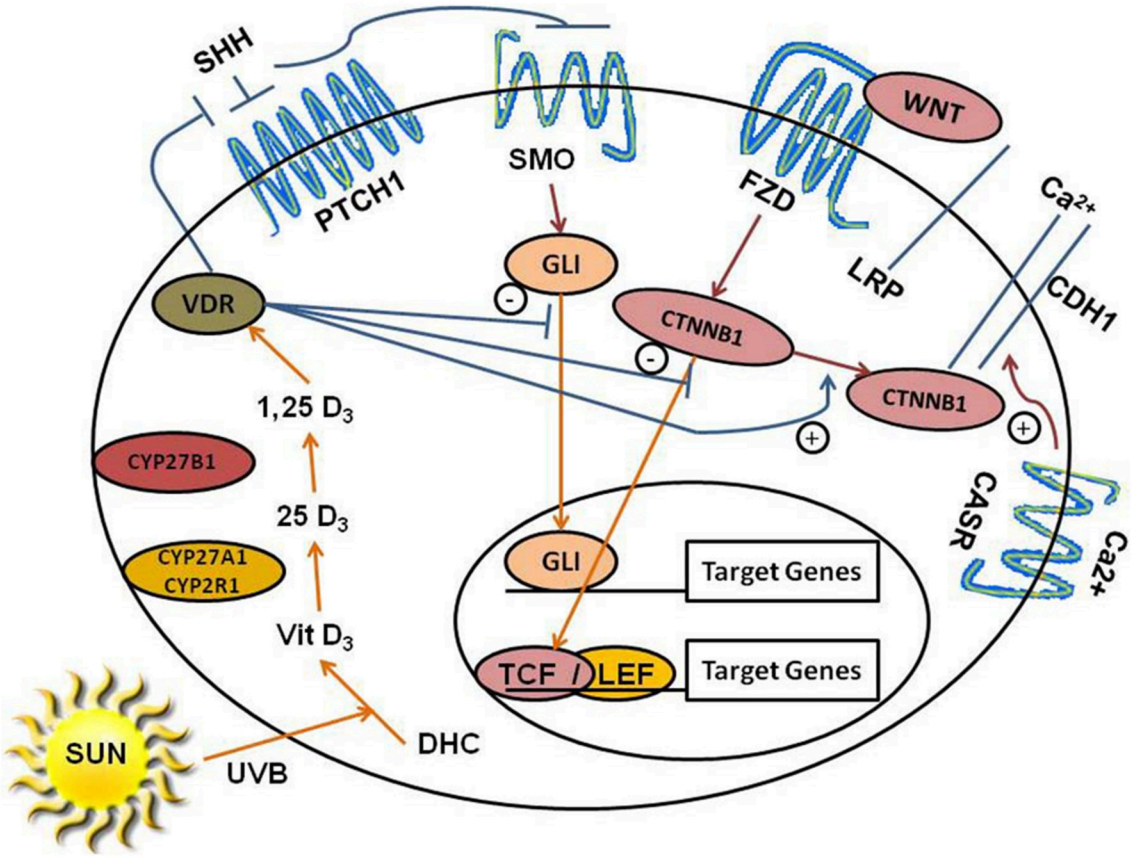

FIGURE 1 | Regulation of HH and Wnt/β-catenin signaling by 1,25(OH)$_2$D/VDR and calcium/Casr. The keratinocyte expresses VDR and is capable of making its own 1,25(OH)$_2$D$_3$ from the vitamin D$_3$ produced from 7-dehydrocholesterol (DHC) under the influence of UVB, as it has both Cyp27a1/Cyp2r1 (which convert vitamin D$_3$ to 25OHD$_3$) and Cyp27b1 [which converts 25OHD$_3$ to 1,25(OH)$_2$D$_3$]. The keratinocyte also expresses the calcium sensing receptor Casr required for calcium induced differentiation. 1,25(OH)$_2$D/VDR suppresses *Shh* and *Gli1* expression, inhibiting the HH pathway in keratinocytes. 1,25(OH)$_2$D/VDR binds CTNNB1(β-catenin) and increases CDH1(E-cadherin) levels in the plasma membrane reducing the amount of β-catenin available for binding to TCF/LEF in the nucleus limiting its transcriptional activity. Calcium acting through its receptor is required for the formation of the E-cadherin/catenin complex in the plasma membrane. In combination these actions reduce the proliferative actions of Shh and Wnt/β-catenin signaling in keratinocytes, limiting their ability to induce tumors in the skin.

regulate cancer development through a number of effects on tumor cell proliferation, blocking growth suppressors, inducing angiogenesis, and promoting invasion and metastasis (Gibb et al., 2011; Gutschner and Diederichs, 2012; Li et al., 2013).

We (Jiang and Bikle, 2014) evaluated the potential role of lncRNAs in VDR protection against skin tumor formation using *in vitro* cultured mouse keratinocytes and an *in vivo* mouse model, comparing cell or mouse epidermis from wildtype or *Vdr* null animals, in an array containing 90 well-annotated mouse lncRNAs. We found increased expression of several well-known oncogenes, including *H19, HOTTIP,* and *Nespas*, and reduced expression of tumor suppressor genes such as *Kcnq1ot1, lincRNA-p21* in VDR deleted keratinocytes whether from cultured cells or epidermis. These results point to an additional mechanism for protection by VDR against skin cancer formation that we are in the early stages of exploring. Whether concomitant *Casr* deletion will amplify these results has not yet been determined.

CONCLUSIONS

Both calcium and vitamin D signaling through their respective receptors Casr and VDR are important for the normal functions of the skin. In this article we have focused on their roles in tumor development when such signaling is disrupted by deletion of the receptors. At this point we have not observed tumor formation in the skin of mice lacking only the Casr, but have observed that mice lacking both Casr and VDR develop tumors of greater malignancy and spontaneity than seen in mice lacking only the VDR. Analyses of gene expression in the epidermis from mice lacking one or both of these receptors clearly demonstrate the synergism between VDR and Casr in gene regulation including genes involved with cancer such as the E-cadherin/catenin pathway that plays such an important role in the epithelial/mesenchymal transition leading to tumor formation. We have focused on two pathways, HH and Wnt/β-catenin, that are regulated by VDR and Casr, and that are plausible participants in epidermal tumor formation when

their regulation is disrupted. In addition we have introduced a new mechanism, alterations in lncRNA expression toward an oncogenic profile, when VDR is deleted. No doubt other pathways will emerge that contribute to a greater or lesser degree to the predisposition of the epidermis lacking VDR and/or Casr to tumor formation.

AUTHOR CONTRIBUTIONS

DB is the senior author of the paper. He wrote the text and ran the laboratory where the work was done. YJ performed the experiments showing the development of SCC in mice lacking both the VDR and CaSR, as well as the LncRNA profiling. TN assisted with many of the technical aspects of the experiments described in recent publications. YO provided much of the data for the publications involving VDRKO mice. CT developed the CaSRKO mouse and did most of the studies with this mouse.

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REVIEW
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The Calcium-Sensing Receptor and the Reproductive System

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Active placental transport of maternal serum calcium (Ca^{2+}) to the offspring is pivotal for proper development of the fetal skeleton as well as various organ systems. Moreover, extracellular Ca^{2+} levels impact on distinct processes in mammalian reproduction. The calcium-sensing receptor (CaSR) translates changes in extracellular Ca^{2+}-concentrations into cellular reactions. This review summarizes current knowledge on the expression of CaSR and its putative functions in reproductive organs. CaSR was detected in placental cells mediating materno-fetal Ca^{2+}-transport such as the murine intraplacental yolk sac (IPYS) and the human syncytiotrophoblast. As shown in *casr* knock-out mice, ablation of CaSR downregulates transplacental Ca^{2+}-transport. Receptor expression was reported in human and rat ovarian surface epithelial (ROSE) cells, where CaSR activation stimulates cell proliferation. In follicles of various species a role of CaSR activation in oocyte maturation was suggested. Based on studies in avian follicles, the activation of CaSR expressed in granulosa cells may support the survival of follicles after their selection. CaSR in rat and equine sperms was functionally linked to sperm motility and sperm capacitation. Implantation involves complex interactions between the blastocyst and the uterine epithelium. During early pregnancy, CaSR expression at the implantation site as well as in decidual cells indicates that CaSR is important for blastocyst implantation and decidualization in the rat uterus. Localization of CaSR in human extravillous cytotrophoblasts suggests a role of CaSR in placentation. Overall, evidence for functional involvement of CaSR in physiologic mammalian reproductive processes exists. Moreover, several studies reported altered expression of CaSR in cells of reproductive tissues under pathologic conditions. However, in many tissues we still lack knowledge on physiological ligands activating CaSR, CaSR-linked G-proteins, activated intracellular signaling pathway, and functional relevance of CaSR activation. Clearly, more work is required in the future to decode the complex physiologic and pathophysiologic relationship of CaSR and the mammalian reproductive system.

Keywords: calcium-sensing receptor, reproduction, testes, ovaries, uterus, placenta

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Abbreviations: AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; EGFR, epidermal growth factor receptor; Gd^{3+}, Gadolinium; GV, germinal vesicle; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; Mg^{2+}, Magnesium; PI-3K, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; CaSR, Calcium-sensing receptor; Ca^{2+}, Calcium; IPYS, Intraplacental yolk sac; ROSE cells, Rat ovarian surface epithelial cells; STB, Syncytiotrophoblast; PCOS, polycystic ovarian syndrome; PTH, Parathyroid hormone; PTHrP, Parathyroid hormone-related protein; PY, tyrosine phosphorylation.

INTRODUCTION

Calcium (Ca^{2+}) is indispensable in the context of mammalian reproduction (Baczyk et al., 2011; Correia et al., 2015; Kornbluth and Fissore, 2015). Firstly, Ca^{2+} contributes to crucial developmental processes such as skeletal formation and mineralization (Riccardi et al., 2013; Kovacs, 2014, 2015), lung (Riccardi et al., 2013) and kidney development (Gilbert et al., 2011) or formation and maturation of neuronal circuits and long-term memory (Leclerc et al., 2011). Therefore, Ca^{2+} must be supplied in sufficient quantities to the growing offspring, which is accomplished by active Ca^{2+}-transport from maternal to fetal or neonate blood circulation across placental and mammary tissue, respectively (Olausson et al., 2012; Kovacs, 2014, 2015, 2016). Secondly, Ca^{2+} is the most universal second messenger. It is modulated downstream of numerous receptors and can activate diverse cytoplasmic signaling proteins (Berridge et al., 2000). Not surprisingly, therefore, Ca^{2+} signaling pathways also play crucial roles in early reproductive events like gamete formation and maturation in the male and female gonads, and fertilization as well as pre- and peri-implantation development in the female reproductive tract (Kashir et al., 2013; Armant, 2015).

Both, extracellular and intracellular Ca^{2+}-linked processes require a tightly regulated extracellular calcium (Ca_o^{2+}) concentration. Mammals have therefore developed a carefully balanced Ca_o^{2+} homeostatic system, which is based on Ca_o^{2+}-sensors. The calcium-sensing receptor (CaSR), is the master regulator of Ca_o^{2+} concentration (Brown, 2013; Tyler Miller, 2013; Alfadda et al., 2014). CaSR controls secretion of a regulatory hormone, parathyroid hormone (PTH), that in turn impacts on Ca_o^{2+} via cells in the target tissues kidney, intestine, and bone (Brown, 2013).

CaSR is additionally expressed in other adult tissues, including the central and peripheral nervous system (Ruat and Traiffort, 2013; Jones and Smith, 2016), the cardio-vascular system (Smajilovic et al., 2011; Schepelmann et al., 2016), the lung (Riccardi et al., 2013), the pancreas (Squires et al., 2014), the epidermis (Tu and Bikle, 2013), or the intestine (Macleod, 2013). There, the function of CaSR is not related to control of Ca_o^{2+} -homeostasis. Instead, CaSR modulates functions such as proliferation and differentiation, apoptosis and chemotaxis, ion channel activity, or hormone secretion, to name a few.

The outstanding role of Ca^{2+} in reproduction together with CaSR expression in reproductive organs implicates a role of CaSR in reproductive processes. This review first introduces CaSR and its functional versatility. It then gives a survey on organs and processes required for reproduction, and summarizes the still sparse information on expression, localization, and function of CaSR in gametes, gonads, uterus, and placenta in health and disease (summarized in **Table 1**). Finally, it indicates research demand in these areas. Expression and function of CaSR in mammary epithelial cells is not addressed in this article as this has been reviewed recently (Kovacs, 2016). Likewise, the role of CaSR in proper development of the skeleton (Riccardi et al., 2013; Kovacs, 2014), the lung (Riccardi et al., 2013; Brennan et al., 2016) and the brain (Liu et al., 2013) is not considered in this article.

THE CALCIUM SENSING RECEPTOR, CaSR, AND ITS FUNCTIONAL VERSATILITY

CaSR is a member of the class C of the G protein-coupled receptors and is present in all vertebrate classes. The fully glycosylated monomer has a molecular mass of 160 kDa and consists of a large N-terminal glycosylated extracellular domain, a seven transmembrane domain and an intracellular C-terminal domain. The extracellular domain contains a bi-lobed Venus-flytrap-like domain. The functional receptor is a dimer, where the Venus-flytrap-like domains of two monomers are linked via covalent as well as non-covalent interactions. Ca^{2+}-binding in the cleft between the two lobes of each Venus-flytrap-like domain causes a rotation of one monomer relative to the other, which finally allows for G proteins to interact with the cytoplasmic side of the CaSR (Zhang et al., 2015).

CaSR is, however, promiscuous in ligand binding; it can be stimulated by other divalent (Mg^{2+}) and tervalent (Gd^{3+}) inorganic cations, as well as organic polycations (e.g., neomycin, spermine). Moreover, it is modulated by various physiological stimuli including extracellular pH, L-aromatic amino acids, and ionic strength. Different ligand binding sites can stabilize distinct conformational changes, which results in "ligand-biased signaling." As a consequence, CaSR was shown to interact with the heterotrimeric G proteins $G_{q/11}$, $G_{i/o}$, $G_{12/13}$, and G_s and is able to target all major intracellular signaling pathways. CaSR-mediated activation of $G_{q/11}$ results in stimulation of phospholipase C (PLC), Ca_i^{2+} mobilization from intracellular stores, and activation of protein kinase C (PKC) isoforms. CaSR-coupling to $G_{i/o}$ can inhibit adenylyl cyclase (AC). On the other hand, it can activate mitogen-activated protein kinases (MAPK) such as ERK1/2 and JNK. This can lead to transactivation of the epidermal growth factor receptor (EGFR). Activation of $G_{12/13}$ modulates several pathways. This can lead to migration via rho-mediated actin polymerization and membrane ruffling or induce cell differentiation. It can also target tyrosine kinases, protein phosphatases, or activate certain AC isoforms. CaSR-coupling to G_s also activates ACs. Furthermore, CaSR activation can stimulate PLA, phosphatidylinositol 3-kinase (PI-3K) and PI-4K. Overall, major consequences of CaSR activation in cells are Ca_i^{2+} mobilization, regulation of intracellular cAMP levels, activation of various protein kinases as well as activation of gene transcription factors. CaSR-mediated signaling, however, depends on the cell-type-specific expression of important components of the downstream signaling pathways (Conigrave and Ward, 2013).

An example for the cell-type specific function of CaSR is the contradictory role in cancer development, where it acts as either an oncogene (breast, prostate) or as a tumor suppressor gene (colon, parathyroid) (Brennan et al., 2013; Peterlik et al., 2013; Tennakoon et al., 2016). CaSR activation can also have diametrical consequences in normal and tumor cells as shown for mammary epithelial cells by activating different G-proteins (Mamillapalli et al., 2008). The multiple consequences of CaSR activation are illustrated by its variable impact on

TABLE 1 | CaSR expression and putative functions in healthy reproductive tissues.

Organ/Tissue	Species	mRNA	Anti-CaSR antibody	Protein WB	Protein IFM/IHC	Function related to CaSR	References
MALE REPRODUCTIVE SYSTEM							
	Rat	Testis Epididymis	Rabbit anti-CaSR Acris (N-terminal domain)		Spermatogonia Spermatocytes Spermatids Sertoli cells Epididymal cells	Sperm motility ↑	Mendoza et al., 2012
	Horse		Goat anti CaSR (F19) Santa Cruz (N-terminal domain)	Sperm 100 kDa + 77 kDa	Sperms	Sperm capacitation ↓ Sperm motility ↑	Macías-García et al., 2016
	Human		Rabbit anti-CaSR Sigma (N-terminal domain)		Epithelial cells in normal prostate tissue		Feng et al., 2014
OVAR							
	Human	Ovarian surface epithelial cell lines	Polyclonal anti-CaSR Affinity BioReagents		Ovarian surface epithelial cell lines,120 kDa	Pro-liferation ↑ Ca_i^{2+} ↑ IP3 ↑	McNeil et al., 1998
	Human		Rabit anti-CaSR (C0117-15) US Biological (C-terminal)	Denuded oocyte (metaphase II),130 kDa Granulosa cells of cumuli oophori cells, 120 + 130 kDa	Oocyte (GV, MI, MII state)		Dell'Aquila et al., 2006
	Horse		Rabit anti-CaSR (C0117-15) US Biological (C-terminal)	Denuded oocyte (metaphase II), 130 kDa Granulosa cells of cumuli oophori cells, 120 + 130 kDa	Oocyte (metaphase II) Granulosa cells of cumuli oophori cells	Oocyte maturation↑	De Santis et al., 2009
	Pig	Ocytes Granulosa cells of Cumuli oophori	Goat anti-CaSR Santa Cruz	Oocyte, 160 kDa Somatic cell, 160 kDa		Oocyte maturation↑	Liu et al., 2015
	Japanese Quail		Mouse anti-CaSR Abcam or NPS Pharmaceutical (Extracellular domain)	Granulosa explants 115–125 kDa +100–110 kDa	Granulosa cells of preovulatory follicles Granulosa layer after ovulation	Survival↑ (Decreased apoptosis)	Diez-Fraile et al., 2010
UTERUS							
	Rat	Uterine luminal epithelium Uterine stromal cells	Rabbit anti-CaSR Affinity BioReagents		Implanting blastocyst Luminal and glandular epithelium Subluminal uterine stromal cells at implantation site	Blastocyst implantation Decidua-lization	Xiao et al., 2005
	Rat		Anti-CaSR Affinity BioReagents		Uterine luminal Epithelium Longitudinal and circular smooth muscle layers Blood vessels Luminal and glandular epithelium of the endometrium	Relaxation?	Pistilli et al., 2012

(Continued)

TABLE 1 | Continued

Organ/Tissue	Species	mRNA	Anti-CaSR antibody	Protein WB	Protein IFM/IHC	Function related to CaSR	References
	Human		Anti-CaSR (ab19347) Abcam		Uterine Myometrium (low) Human term placenta	Relaxation?	Crankshaw et al., 2013
PLACENTA							
	Mouse	Intra-placental yolk sac cells, parietal side + columnar side	Mouse anti-CaSR ADD		Intraplacental yolk sac cells, parietal side + columnar side Placental trophoblasts		Kovacs et al., 2002
	Human		Anti-CaSR Novus HL-1499 (WB) anti-CaSR Acris (IHC)	Term Placenta 130 kDa	Term villous STB Term villous cytotrophoblast Term extravillous trophoblast		Papadopoulou et al., 2014
	Human	First trimester villous STB	Monoclonal Anti-CaSR NPS Pharmaceuticals, human extracellular CaSR epitopes amino acids 214–235 or amino acids 374–391		Villous STB (first trimester and term) Extravillous trophoblast (first trimester and term)	Ca_i^{2+} ↑	Bradbury et al., 2002
	Human	Extravillous cytotrophoblast					Bradbury et al., 1998

intracellular Ca^{2+} concentration. CaSR can directly increase cytosolic Ca^{2+} through PLC activation to open Ca^{2+} channels. Alternatively, CaSR can trigger ERK/phospho-ERK pathways, thereby increasing transcription factor (e.g., CREB) activity, which stimulates expression of Ca^{2+}-channels with appropriate response elements. And finally, CaSR can increase K^+-channel activity, and as a consequence increase membrane potential and a Ca^{2+}-driving force. Many intracellular signaling pathways, in turn, are sensitive to cytosolic Ca^{2+}-rises, and as a consequence, cellular processes such as proliferation or migration can result from CaSR activation (Tennakoon et al., 2016).

Overall, CaSR has immense functional versatility by activating different signaling pathways in a ligand- and cell-type specific way. In the context of reproduction (see below), mainly activation of MAPK pathways (e.g., ERK1/2, p38, or JNK) has been investigated so far, probably because MAPK activation is of great importance in reproduction (Li et al., 2009; Almog and Naor, 2010; Fan et al., 2012; Nunes et al., 2015). However, CaSR can also induce other pathways such as the PI-3K/AKT pathway (Bilderback et al., 2002; Liao et al., 2006) in the reproductive system. Activation of diverse intracellular signaling routes should be considered in future studies addressing the function of CaSR in reproduction.

CaSR AND THE MALE REPRODUCTIVE SYSTEM

Spermatogenesis

The mass production of the male gamets starts with puberty, when the hypothalamic-pituitary-gonadal axis is established, and is a continuous, life-long process. Spermatogenesis consists of three major phases (1) the proliferation of the stem cells (spermatogonia) resulting in spermatocytes, (2) two meiotic divisions that give rise to haploid spermatids, and (3) spermiogenesis, the differentiation into mature sperms (for details see Kornbluth and Fissore, 2015). Spermatogenesis occurs in the seminiferous tubulus of the testes and is supported by testicular non-germ line cells, such as Sertoli and Leydig cells, which nourish the sperms and/or produce factors required for proper spermatogenesis. Within the epididymis the sperms complete maturation. Secretory products of the male accessory sex glands (e.g., prostate gland) support the functionality of sperms. Once in the female tract, sperms undergo capacitation to increase motility (hyperactivation) and prepare for fertilization of the oocyte. Only capacitated sperms can bind to molecules in the egg's zona pellucida, which triggers the acrosome reaction. Enzymes released from the acrosome, a Golgi-derived sperm organelle, digest a way through the zona pellucida enabling the sperm to follow and to finally fertilize the egg (de Kretser, 2012). Ca^{2+} is a major determinant of proliferation and differentiation of spermatogonia, sperm motility, capacitation, and acrosome reaction (Breitbart, 2002; Yoshida and Yoshida, 2011; Correia et al., 2015; Kornbluth and Fissore, 2015). In contrast to Ca^{2+} channels, which are well studied in sperms (Correia et al., 2015), sparse information is available on the expression and function of CaSR in sperm or male reproductive tissues (see also **Table 1**).

CaSR Expression in Healthy Male Reproductive Tissue

In rats, CaSR mRNA expression appeared higher in testes compared to epididymis (**Figure 1A**). In testis, CaSR protein was found in Sertoli cells, spermatogonia, spermatocytes, and spermatids with highest expression level in spermatids (**Figure 1B**). Leydig cells and fibroblasts, in contrast, presented CaSR negative. Mature sperms in the epididymis also expressed CaSR, predominantly at the head domain. Epithelial epididymal cells exhibited staining at the apical pole. Since a calcimimetic drug (AMG 641) caused a moderate, but significant increase of motility of isolated rat sperms, a role of CaSR in sperm motility was suggested. A similar stimulatory effect on pig sperms mobility indicated expression of CaSR also in male gametes of this species (Mendoza et al., 2012).

In isolated equine sperms, CaSR protein was located predominantly in the head of the sperm, with lower expression seen along the tail. Proteins detected with an anti-CaSR antibody by western blotting had a molecular weight of 100 and 75 kDa (Macías-García et al., 2016), which is smaller than that of fully glycosylated CaSR transiently expressed in HEK cells (160 kDa) (Pidasheva et al., 2006). The authors suggested involvement of CaSR in sperm capacitation as well as sperm mobility. Capacitation occurs when alterations in the extracellular milieu induce cAMP and activate PKA. The result is tyrosine phosphorylation (PY) of various proteins. Among factors impacting on PY is Ca_o^{2+}. In species such as stallion (González-Fernández et al., 2013), human and mice (Baker et al., 2004), Ca_o^{2+} in the capacitation medium inhibits PY. Ca^{2+}-dependent PY inhibition in stallion sperm was reversed in the presence of a CaSR antagonist (NPS 2143), while a CaSR agonist (AC-265347) inhibited PY similarly to Ca_o^{2+}. The result suggested that CaSR reduces capacitation in stallion sperm. A modulatory role of the pH was observed. In addition, NPS 2143 caused a significant decrease in sperm motility indicating the CaSR is also involved in the regulation of equine sperm mobility (Macías-García et al., 2016).

CaSR and Disorders of the Male Reproductive System

Diabetes, being an increasing problem worldwide[1], can cause testicular damage and male infertility. A recent study demonstrated up-regulated CaSR in testicular tissues in streptozotoxin-induced diabetic rats (Kong et al., 2016). The diabetic rats had significantly lower testes weights and serum levels of testosterone compared to healthy controls. A connection between CaSR activation and testicular damage was found. Specific activation of CaSR by Gd^{3+} increased, while specific inhibition of CaSR by the antagonist NPS 2390 reduced testicular damage in the diabetic rats. Increased lipid peroxidation, decreased anti-oxidative capacity, increased apoptosis of germ cells, and activation of the mitochondrial apoptotic pathways were observed in testicular tissue of diabetic rats and the parameters were further aggravated by the administration of Gd^{3+}, and attenuated by NPS 2390. CaSR was found to be an activator of different MAPK pathways (ERK, p38, JNK). It was concluded that CaSR activation had a pro-apoptotic impact on germ cells in diabetic rats and overall participated in diabetes-induced testicular damage.

While CaSR expression in normal rat Leydig cells could not be shown (Mendoza et al., 2012), cultured Rice H-500 rat Leydig cell tumor express the receptor mRNA and protein (Sanders et al., 2000). H-500 cells are a transplantable model for humoral hypercalcemia of malignancy. Humoral hypercalcemia of malignancy is a syndrome seen in various types of cancers including prostate cancer, but also testicle or breast cancer. It arises from tumor-derived humoral factors which destroy normal calcium homeostasis (Chakravarti et al., 2009). Upon implantation into adult male rats, H-500 cells start proliferation and cause hypercalcemia by abundant release of the humoral factor parathyroid hormone-related protein (PTHrP). PTHrP release, proliferation, and protection of cells from apoptosis, are stimulated by Ca_o^{2+} via CaSR. CaSR-induced proliferation depends on AKT and MAPK p38, but not on MAPK ERK1/2 (Tfelt-Hansen et al., 2004). CaSR also stimulates transcription of the PTHrP gene, and release of PTHrP in H-500 cells by activating PKC as well as various MAPK pathways (ERK1/2, p38, JNK) (Tfelt-Hansen et al., 2003a). Partly, these effects are caused by transactivation of EGFR by CaSR (Tfelt-Hansen et al., 2005). In addition to PTHrP, Ca_o^{2+} also up-regulates mRNA of the oncogene pituitary tumor transforming gene as well as vascular endothelial growth factor genes in H-500 cells, which leads to the robust proliferation and angiogenesis at the site of their implantation (Tfelt-Hansen et al., 2003b). Stimulation of PTHrP release initiates a vicious circle of hypercalcemia maintained by CaSR expression in Leydig cell tumors, which suggests CaSR as a potential therapeutic target for CaSR antagonists. Colloton and coworkers investigated the ability of cinacalcet, an allosteric modulator of CaSR, to attenuate hypercalcemia in mice bearing H-500 cells and indeed demonstrated that cinacalcet effectively reduced tumor-mediated hypercalcemia (Colloton et al., 2013).

In an immunohistochemical study, comparing human prostate cancer tissue sections in microarrays, CaSR expression was confirmed not only in primary prostate cancer tissue, but also in normal prostate tissue (**Figure 1A**). No significant difference in the CaSR expression level between these tissues was observed. However, a significant higher expression level of CaSR was found in the metastatic prostate cancer tissues obtained from bone (Feng et al., 2014). For breast cancer cells it was demonstrated that the elevated Ca^{2+}-release during bone remodeling represents a chemoattractant, which promotes migration of cancer cells to bone via activated CaSR (Saidak et al., 2009). CaSR mRNA and protein is also detected in the highly bone metastatic prostate cancer cell lines PC-3 and C4-2B, while comparatively lower expression of CaSR is found in non-skeletal metastatic, epithelial-derived prostate cell line LNCaP cells (Liao et al., 2006). Prostate cancer most commonly metastasizes to bone, preferring areas of high bone turnover. As a result of bone remodeling, Ca^{2+} and growth factors are released. In PC-3 and C4-2B, but not LNCaP cells elevated Ca_o^{2+} activated CaSR. Activation resulted in stabilization of cyclin D, promoted PI-3K/AKT/ mTOR signaling, and increased

[1]http://www.who.int/mediacentre/factsheets/fs312/en/ (Accessed June 04, 2016).

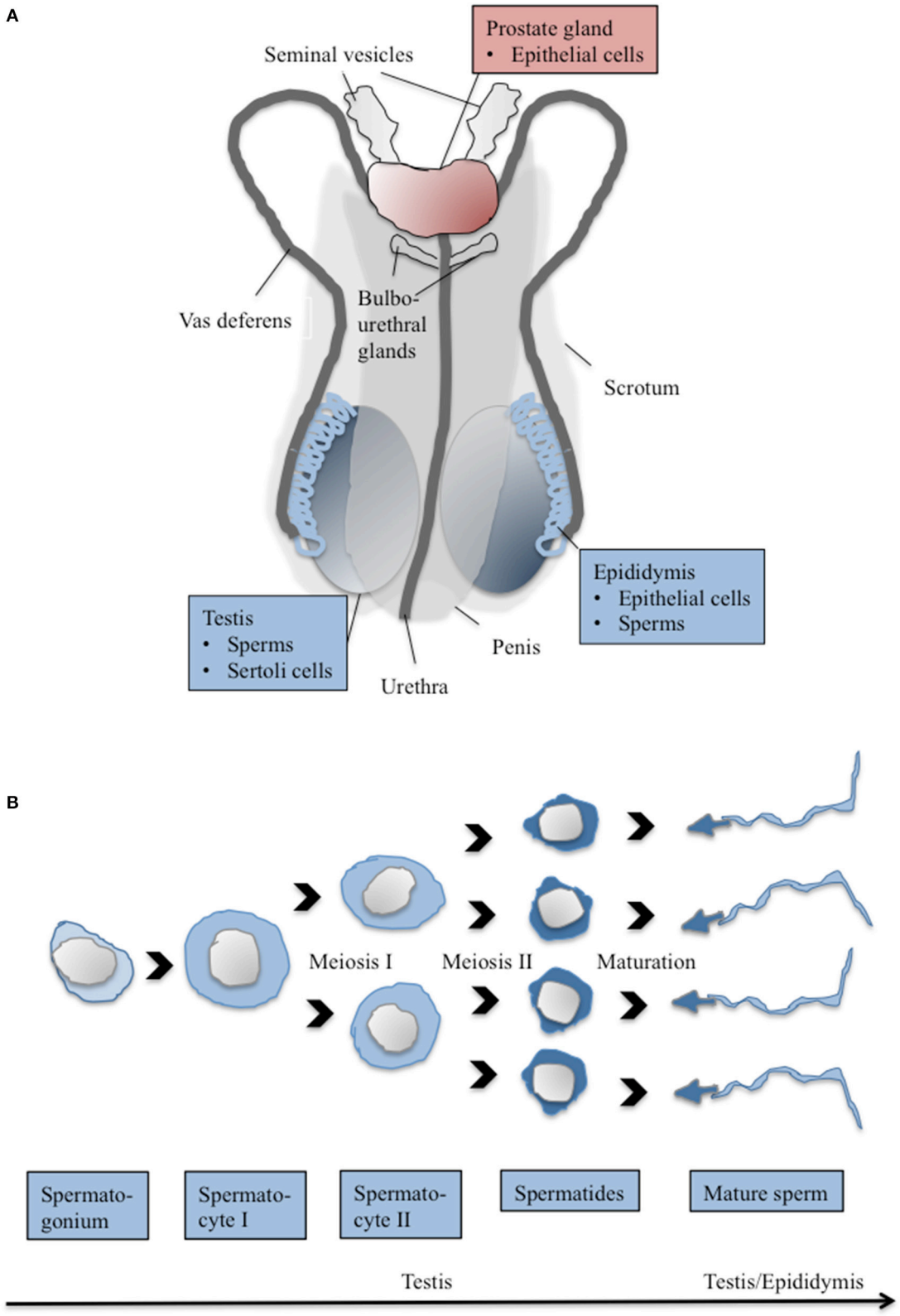

FIGURE 1 | The cartoon depicts (A) major components of the human male reproductive system and (B) major steps in spermatogenesis. Organs/tissues/cells, which were shown to express CaSR are depicted in color. Red color, CaSR expression shown in human ± other mammalian species; blue color, CaSR expression shown in mammalian species other than human (for details on species and assumed function of CaSR see Text and **Table 1**).

metastatic potential (Liao et al., 2006). A few clinical studies supported the notion that CaSR promotes lethal prostate cancer. First, the CaSR Q1011E minor allele, which is common in populations with African ancestry, appeared to be associated with a less aggressive form of prostate cancer among African-American men (Schwartz et al., 2010). A second study assessed genetic variations across CaSR and lethal prostate cancer risk in Caucasian men. Common genetic variations in CaSR were found associated with both higher and lower risk for lethal prostate cancer. The association was stronger in patients with lower plasma levels of vitamin D (Shui et al., 2013). Third, in a study that correlated primary tumor CaSR expression with the risk for lethal prostate cancer, a higher CaSR tumor expression was associated with an approximately two-fold higher risk for lethal progression. This risk was independent of Gleason grade and pathological stage. Higher CaSR expression was significantly associated with lethal progression among cases with lower tumor vitamin D receptor expression but not among cases with high tumor vitamin D receptor expression (Ahearn et al., 2016).

Research Demands

Reports on CaSR expression in human male reproductive system are currently mainly related to cancer. For more detailed information on the role of CaSR in tumors, the reader is referred to other reviews (Singh et al., 2013; Mateo-Lozano et al., 2016; Tennakoon et al., 2016). The high CaSR expression in Leydig and prostate cell tumors asks for further evaluation of the prognostic usability of CaSR. As CaSR promotes bony metastasis of cancer, this raises the possibility of reducing the risk of such metastases with CaSR-based therapeutics.

Infertility is among the most serious social problems affecting advanced nations today. Infertility affects both men and women. A variety of known factors is associated with male infertility, but in 30–45%, the cause of the abnormal semen parameters is not identified (Jungwirth et al., 2012). In this context, knowledge of all molecules that impact on proper sperm development is of relevance. If CaSR would be demonstrated to significantly improve or reduce sperm quantity or quality, agonists or antagonists of the receptor, respectively, could serve for treatment. But currently, many open questions remain. The intracellular signaling pathways associated with the observed effect of CaSR on (rat, porcine, and equine) sperm motility and capacitation remain to be investigated. The predominant physiological ligands responsible for these effects are unknown. Of interest, Ca^{2+} concentrations in the uterine fluid vary during estrous cycle in various species (Casslén and Nilsson, 1984; Alavi-Shoushtari et al., 2012; Alavi Shoushtari et al., 2014). Polyamines (spermine, spermidine, and putrescine), which are type I calcimimetics (Brown, 2010) are secreted by the prostate gland into semen. Whether polyamines, or other CaSR ligands in the semen could be sensed by CaSR expressed on sperms and have effects, is not known. It is unknown, whether CaSR under physiological conditions impacts on sperm cell apoptosis. Likewise, CaSR may modulate acrosome reaction or spermatic hyperactivation. The function of CaSR expressed in (rat) Sertoli cells and epididymal cells remains to be investigated. CaSR expression was not observed in healthy rat Leydig cells. However, there is functional evidence for a divalent cation (Ca^{2+}) receptor present on the surface of murine cells inducing Ca^{2+} release from ryanodine receptor-gated intracellular Ca^{2+} stores (Adebanjo et al., 1998). Since a raise of extracellular Ca^{2+} can induce testosterone secretion of Leydig cells (Meikle et al., 1991), re-evaluation of CaSR expression in Leydig cells in mammalian species might be of interest when looking for new therapies to increase or reduce hormone secretion. The molecular size of CaSR protein detected in equine sperm is small compared to the mature glycosylated CaSR. The reason for this difference is currently unknown. It may relate to different glycosylation of CaSR or alternatively spliced CaSR in the tissue. In addition, a recent study indicated that the western blotting profiles in tissues depend on the anti-CaSR antibody used (Graca et al., 2016). Various anti-CaSR antibodies were used in the studies summarized in this review. They are listed in **Table 1**.

CaSR IN OVARY AND OOCYTES

Ovaries and Oogenesis

Only a small number of mature oocytes is released during the reproductive years of a woman. Ovulation occurs in cycles consisting of follicular phase, ovulation and luteal phase. The follicular phase is characterized by estrogen dominance, while in the luteal phase, progesterone is the dominant sex hormone. Due to the cyclic release of the steroids, the female body exhibits cyclicity, which is accompanied by structural and functional changes in the ovaries, oviducts, and the uterus. In most animals, this is called estrous cycle, while higher primates including human experience menstrual cycles. The reason of the changes occurring during female cyclicity is the dual function of the female reproductive tract. Under estrogen dominance, the body is prepared to receive the male gametes and enable fertilization, while under progesterone dominance the body is prepared for implantation and nourishment of the conceptus (for details see Findlay, 2012).

The ovarian surface is covered with a single layer of epithelial cells. Ovarian surface epithelial cells are responsible for the most malignant forms of ovarian carcinoma. In the ovarian cortex, ovarian follicles of various sizes and at different stages of development are located. The follicles are the functional units of the ovary. They contain a single oocyte and are surrounded by epithelial granulosa cells. Theca cells are closely associated with the follicles. Theca cells differentiate from the interfollicular stroma due to signals from growing follicles and produce the androgen substrate, which is required for estrogen biosynthesis in the granulosa cells. After ovulation, theca cells are transformed from androgen-producing to progesterone-producing cells, thereby becoming the pregnancy-maintaining cells of the corpus luteum. The polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism, oligo- and/or anovulation, and polycystic ovarian morphology. PCOS is associated with insulin resistance, hyperinsulinemia and central obesity. Excessive proliferation of theca cells is often associated with PCOS; this is a major cause of infertility due to ovarian hyperandrogenism (Magoffin, 2005).

Oogenesis starts with the formation of primary oocytes during the fetal period *in utero*. Stem cells (oogonia) first proliferate and then enter the first meiotic division, which is halted in a prophase state of meiosis I until sexual maturity. This first meiotic arrest is characterized by a large nucleus called the germinal vesicle (GV see **Figure 2B**). The oocyte and the surrounding granulosa cells, together constituing the primordial follicles, have complex paracrine interactions during follicle growth and development. Oocyte maturation depends on secretory products of the surrounding cells (Findlay, 2012).

Oocyte maturation—defined as the period of progression from the first to the second meiotic arrest—starts with puberty, when during the follicular phase of each menstrual/estrous cycle, a pool of follicles is recruited. Hormonal signals stimulate the oocytes as well as their surrounding granulosa cells to grow and theca cells attach to the follicle. One dominant follicle continues growing. Increasing estrogen levels produced by the follicle's granulosa cells cause a pulsatile release of luteinizing hormone (LH) from the pituitary gland, which stimulates the release of the oocyte from the ovary (ovulation). Upon that stimulus, meiosis I continues and is terminated shortly before ovulation. Oocytes at metaphase I (MI) stage are defined as oocytes with no GV (intact nucleus) and no polar body (see **Figure 2B**). Granulosa cells and the oocyte cooperate and transmit signals involved in maintaining or releasing the meiotic arrest in the oocyte. The arrest in prophase I is mediated by elevated levels of cyclic adenosine monophosphate (cAMP). Activation of MAPKs triggers degradation of cAMP, allowing oocyte maturation to proceed. Ca^{2+} plays a role in controlling LH-induced as well as spontaneous oocyte maturation, possibly by modulating intracytoplasmic cAMP concentrations via Ca^{2+}-sensitive AC (Silvestre et al., 2011, 2012; Chen et al., 2013). By termination of meiosis I, the diploid primary oocyte generates two haploid daughter cells. One cell becomes the secondary oocyte (MII stage of oocyte), while the other cell forms the first polar body (see **Figure 2B**). The released oocyte is surrounded by the zona pellucida, a thick specialized extracellular matrix as well as additional layers of granulosa cells (corona radiata).

The ovulated oocyte, which enters the fallopian tube, is now arrested at metaphase of meiosis II to prevent parthenogenesis (self-fertilization). For transition of the unfertilized egg to a developing embryo, egg activation is initiated by alterations in Ca_i^{2+}, which is triggered by fusion of the sperm with the egg involving a sperm-derived factor. The type of Ca^{2+}-signal is species dependent, a prolonged series of Ca_i^{2+} oscillations in the egg cytoplasm is characteristic in e.g., mammals, while other species (e.g., fish) exhibit a single increase in Ca_i^{2+} concentration (Kornbluth and Fissore, 2015). Egg activation comprises a series of events including cortical granule exocytosis, modifications of the zona pellucida and plasma membrane to prevent polyspermy, completion of meiosis II in the egg, recruitment of maternal mRNAs into polysomes for translation, and formation of male and female pronuclei (Schultz and Kopf, 1995; Wang and Machaty, 2013; Swann and Lai, 2016).

CaSR in Ovarian Surface Epithelial Cells

Expression of CaSR in the ovary was first described in human surface epithelial cells (**Figure 2A** and **Table 1**). Proteins detected by western blotting show a molecular weight of 120–140 kDa (McNeil et al., 1998). Increasing Ca_o^{2+} concentrations induced a significant proliferative response in these cells, indicating that the cells can sense Ca^{2+} concentrations in the surroundings. Induced proliferation of these cells due to high Ca^{2+} concentrations may be of physiological relevance during the healing of the ruptured surface following each ovulation, when neighboring cells are locally exposed to ovarian follicular fluid. At least in pig ovarian follicular fluid, Ca^{2+} concentrations of around 2.34 mmol/L have been measured (Schuetz and Anisowicz, 1974); CaSR is active above threshold Ca_o^{2+} levels between 0.5 and 2 mmol/L (Conigrave and Ward, 2013). Upon treatment of human surface epithelial cells with the CaSR agonists Gd^{3+}, Ca^{2+} as well as neomycin, intracellular Ca^{2+} release occurred. The same observation was made in rat ovarian surface epithelial (ROSE) cells. There, inositol triphosphate production increased after stimulation with Gd^{3+} and Ca^{2+}. Expression of an interfering mutant CaSR inhibited the proliferative response to elevated extracellular Ca^{2+}. CaSR agonists induced tyrosine phosphorylation, ERK activation and proliferation. Expression of interfering mutants for Ras, Raf, and MKK1 indicated that proliferation of ROSE cells in response to increased Ca_o^{2+} involves cross-talk between CaSR and a tyrosine kinase-dependent Ras-Raf-MKK1-ERK signaling pathway. Interestingly, agonists of CaSR also increased the kinase activity of Src, which is a proto-oncogene (Hobson et al., 2000). This work was later extended by Bilderback et al. (2002), who demonstrated an additional ERK-independent, but PI-3K/AKT-dependent component in the proliferative response of ROSE cells. Activation of these pathways has also been observed upon stimulation of CaSR in prostate and Leydig cell lines (see Section CaSR and Disorders of the Male Reproductive System)

Ovarian surface epithelial cells are the cell type primarily responsible for malignant ovarian carcinoma, which is the most lethal female genital malignancy. McNeil et al. (1998) observed increased expression of CaSR mRNA in two ovarian tumor cell lines (BG-1 and CAOV-3). More evidence for a link between CaSR and ovarian cancer is given by the observation that the G allele of the CaSR rs17251221 polymorphism seems to protect against ovarian cancer (Yan et al., 2015). CaSR is considered as a molecule that can either promote or prevent tumor growth depending on the type of cancer (Tennakoon et al., 2016). The exact role of CaSR the development of ovarian cancer remains to be determined.

CaSR in Follicular Cells

CaSR is present at the surface of human oocytes and granulosa cells within the corona radiata (**Figure 2B** and **Table 1**). Western-blot analysis revealed a single 130 kDa protein in denuded oocytes and a protein doublet of 130/120 kDa in cumulus cells (Dell'Aquila et al., 2006). The expression and localization of CaSR protein in human oocytes at different maturation stages (GV, MI, and MII) was studied by immunofluorescence microscopy. Increased CaSR protein expression in the MI stage as compared

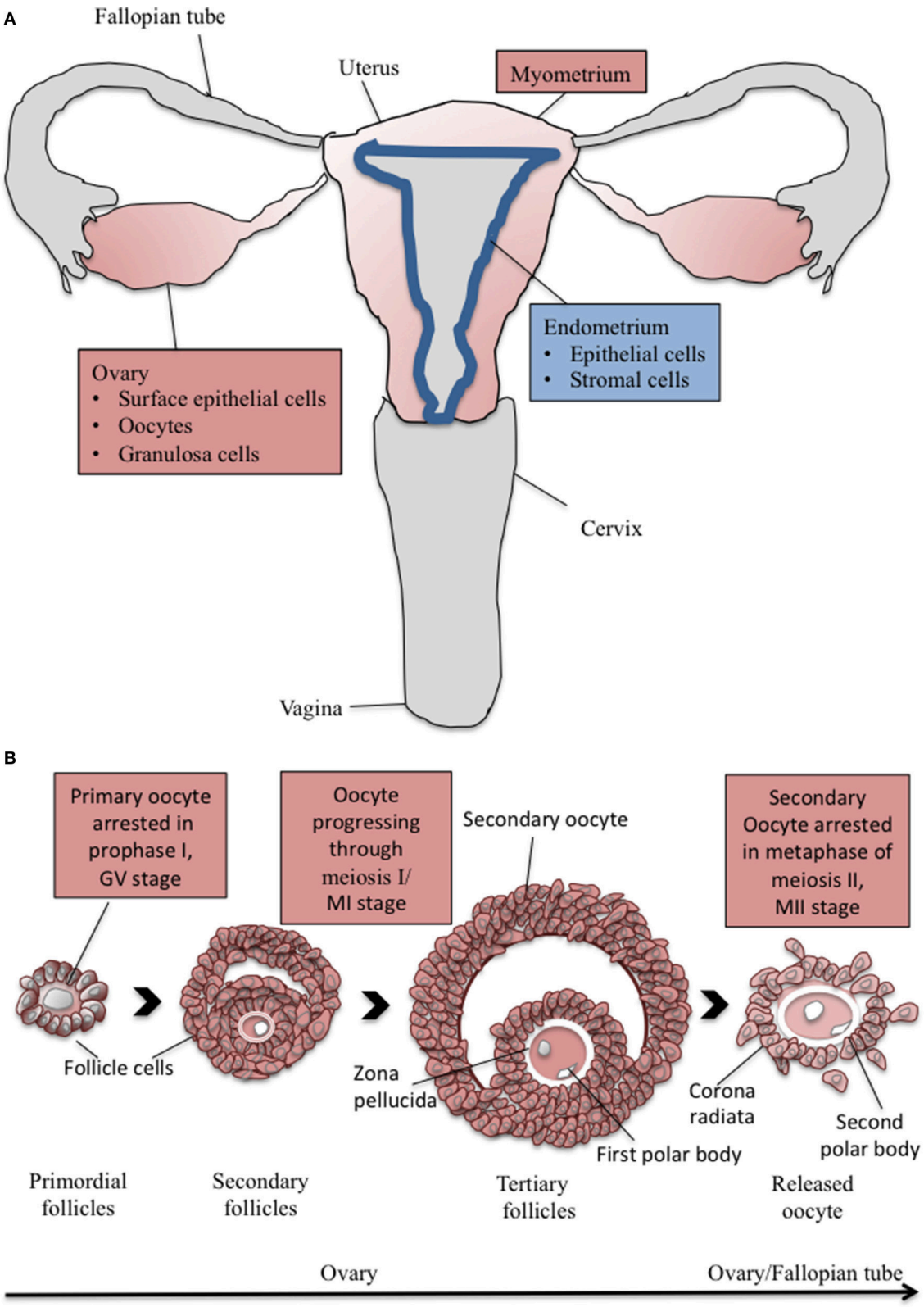

FIGURE 2 | The cartoon depicts (A) major components of the human female reproductive system and (B) major steps in oogenesis. Organs/tissues/cells, which were shown to express CaSR are depicted in color. Red color, CaSR expression shown in human ± other mammalian species; blue color, CaSR expression shown in mammalian species other than human (for details on species and assumed function of CaSR see Text and **Table 1**).

to GV and MII oocytes (**Figure 2B**) suggested a role of CaSR in the process of meiotic maturation, which would correlate with the observed role of external Ca^{2+} in mobilization of intracellular Ca^{2+} during oocyte maturation, activation, and fertilization (review by Tosti, 2006).

CaSR mRNA and protein expression was also shown in equine follicles (**Table 1**). Again, western blot analysis revealed a single 130 kDa protein in denuded oocytes and a protein doublet of 130/120 kDa in cumulus cells (De Santis et al., 2009). When studied by confocal microscopy, CaSR was demonstrated at the plasma membrane and, more pronounced, within the cytoplasm of oocytes at all examined stages of meiosis (GV, MI, MII). Corona radiata cells exhibited strong plasma membrane labeling. CaSR was also detected in the transzonal cytoplasmic processes of corona radiata cells, which penetrate through the zona pellucida and contact the oocyte membrane. A role of CaSR in oocyte maturation was investigated. The CaSR agonist NPS R-467, which is an allosteric modulator of CaSR sensitizing the CaSR to Ca^{2+} without activating it, in the absence of Ca^{2+}, had a stimulatory effect on oocyte maturation. Pre-incubation with the CaSR antagonist NPS 2390 attenuated the effect. Stimulation of maturation by CaSR agonist depended on the presence of external Ca^{2+}(2.92 mM) and was not observed at suboptimal external Ca^{2+}(0.5 mM). However, variations of Ca^{2+} between 0.5 and 4 mM Ca^{2+} in the absence of the agonist did not stimulate *in vitro* oocyte maturation. In oocytes treated with NPS R-467, CaSR immunostaining increased at the plasma membrane, while it was reduced in the cytosol. Finally, treatment of oocytes as well as cumulus cells with CaSR agonist resulted in an increased activity (phosphorylation) of MAPK (ERK) in these cells (De Santis et al., 2009). Activation of MAPK is necessary for gonadotropin-induced meiotic resumption of oocytes (Fan and Sun, 2004).

CaSR mRNA and protein is also present in porcine oocytes and cumulus cells (**Table 1**). In contrast to human and equine oocytes, a 160 kD protein was detected by western blotting. The effects of gonadotropins and EGF, two key factors involved in oocytes maturation (Conti et al., 2006; Uhm et al., 2010), on CaSR expression and localization in porcine oocytes were tested. CaSR expression was up-regulated in oocytes matured in gonadotropin-containing, but not EGF-containing medium. Cortical distribution of CaSR was enhanced with gonadotropins but not EGF. Porcine cumulus-oocyte-complexes exposed to CaSR agonist NPS R-568 in gonadotropin-containing medium showed increased maturation rate, while CaSR antagonist NPS 2390 to medium supplemented with gonadotropins significantly decreased oocyte maturation. MAPK (ERK) phosphorylation was increased during *in vitro* maturation as well as after NPS R-568 treatment. Treatment with NPS 2390 resulted in reduced levels of phosphorylated MAPK during oocyte maturation. It was concluded that CaSR participates in gonadotropin-induced oocyte nuclear maturation through MAPK signal transduction (Liu et al., 2015).

Apoptosis of granulosa cells is an important process in follicular atresia. The causal relationship between Ca^{2+} and induction of apoptosis was investigated in cultured avian granulosa cell explants of Japanese quail (*Coturnix japonica*). Increasing extracellular Ca^{2+} resulted in a biphasic response of the cells; an initial inhibitory effect on apoptosis was followed by a delayed phase of increased apoptosis. As the initial inhibitory effect of the Ca^{2+} on apoptosis was mimicked by applying the CaSR agonists Mg^{2+} and Gd^{3+}, an involvement of CaSR in inhibition of apoptosis was suggested (Mussche et al., 2000). In a follow up paper, the same group by immunocytochemistry confirmed expression of CaSR in granulosa cells of quail pre-ovulatory follicles as well as in the remnants of the granulosa layer after ovulation (**Table 1**). CaSR was not detected in the granulosa cells of smaller undifferentiated follicles. The presence of CaSR in follicles destined to ovulate was confirmed by western blotting showing a protein of 115-125 kDa. The rate of apoptosis of F1 granulosa explants (F1 being the largest preovulatory follicle) stimulated by either gonadotropin withdrawal alone or in combination with C_8-ceramide was significantly decreased with a CaSR agonist (NPS R-568) as well as the ions Ca^{2+} and Mg^{2+}. The authors suggested that activated CaSR may play a role in securing the survival of avian follicles after their selection (Diez-Fraile et al., 2010).

Research Demands

Disturbance in oocyte meiotic events can lead to subfertility or premature aging by reducing the functional ovarian reserve. Any molecular target that would allow for promoting or reducing oocyte maturation would therefore be of interest. Expression of CaSR in human, equine, and porcine oocyte has been shown. In analogy to sperms, the molecular weight of the detected CaSR protein in oocytes and granulosa cells differs between the species. Reasons for that are given in Section Research Demands (page 7). Current data support a role of CaSR in maturation (completion of first meiosis) of oocytes and a contribution of the MAPK ERK signaling pathway. However, the interplay of CaSR with other molecules that maintain oocytes in the meiotic arrest and those that initiate meiotic resumption (such as LH) (Celik et al., 2015) is currently not well explored.

The oocyte–somatic cells interaction is very important. Low quality of somatic cells and difficulties in the interaction between oocyte and granulosa cells can reduce the fertilization and implantation capacity of the oocyte/zygote, which again results in poor pregnancy outcome. The expression of CaSR in granulosa cells of various species was confirmed, but current data addressing the function of CaSR in these cells are still limited and point toward an anti-apoptotic function. Expression of CaSR in theca cells remains unexplored. PCOS is a common multifaceted metabolic disease in women of fertile age, which has a strong genetic component. Recent results provided evidence that CaSR Hin1I gene polymorphism represents a candidate for the genetic contribution to the development or the severity of insulin secretion in women with PCOS; So far, the exact molecular mechanism underlying this association is largely undetermined (Ranjzad et al., 2011). It remains to be demonstrated whether theca cells are also affected.

Finally, CaSR expression was observed in epithelial surface cells of ovaries, where it promotes a proliferative response. Up-regulation of the response in case of tumors need to be further explored before therapeutic use of antagonist can be considered.

CaSR IN THE UTERUS

Uterus and Implantation

During mammalian development, parts of the Müllerian ducts develop into the female uterus. The extent of duct-fusion is species-dependent, resulting in different shapes such as simplex uterus in humans and primates, or duplex uterus in rodents such as rats and mice. Irrespective of the shape, the function of the uterus is to enable implantation of the fertilized egg (zygote), to house the growing conceptus and to expulse the fetus during delivery (Spencer et al., 2012).

The uterus consists of a tripled-layered wall, composed of perimetrium, myometrium and endometrium. The endometrium is a mucosal layer which undergoes marked changes in thickness and structure during the estrous cycle in animals such as rats (Westwood, 2008), but most pronounced changes are seen during the menstrual cycle of humans/primates (Fazleabas and Strakova, 2002). The early embryo enters the uterus in the morula state (12–16 cells). Then, transformation into the blastocyst occurs, which is comprised of a layer of trophectoderm cells that contacts the uterine epithelium and starts to form the placenta, and the inner cell mass that gives rise to the embryo. The blastocyst can implant after shedding the zona pellucida; in human, implantation occurs 9 days post coitum (p.c.), while in rats and mice it occurs 4–5 days p.c. The principle purpose of implantation is to enable a contact between maternal blood supply and the developing embryonic blood vessels. The mechanism of implantation of the conceptus is, however, species dependent. Based on the interaction between blastocyst and uterine cells it is classified as being interstitial (e.g., human, guinea pig), centric (e.g., rabbits, domestic animals), and eccentric (e.g., rats, mice) (Lee and DeMayo, 2004). During interstitial implantation the conceptus breaks through the surface epithel of the maternal uterus and invades the underlying stroma. Centric implantation means fusion of blastocyst with uterine epithelium without penetration and in eccentric implantation, the luminal epithelium invaginates and surrounds the blastocyst. Due to implantation, endometrial stromal cells undergo the decidual reaction and become decidual cells (Gellersen et al., 2007).

Successful implantation requires a delicate interplay of many molecules in a limited period of time (the window of implantation). If communication between the embryo and the endometrium fails, then implantation fails. This is an important cause of infertility. There are estimations that only 50–60% of all conceptions advance beyond 20 weeks of gestation and from the pregnancies that are lost, 75% represent a failure of implantation. Failed implantation is also a major limiting factor in assisted reproduction. To treat disorders such as infertility, which increases worldwide due to an increasing toxic environment, but also diseases such as obesity, it is important to understand the molecular mechanisms of implantation and placentation (Norwitz et al., 2001). Many factors including steroid hormones, cytokines, and growth factors, but also Ca_i^{2+} play a role in the course of implantation (Ruan et al., 2014). Ca_i^{2+} seems to promote the blastocyste-endometrium interaction (Thie and Denker, 2002). Of interest, the Ca_o^{2+} concentration in rat uterine secretion changes at the time of implantation (Nilsson and Ljung, 1985).

Expression of CaSR in the Uterus

Xiao et al. (2005) found CaSR mRNA expression in rat uterus (**Figure 2A** and **Table 1**). CaSR mRNA and protein appeared in the luminal epithelium on day 1 of pregnancy. Expression of CaSR was switched from the luminal epithelium to stromal cells on days 1–3 of pregnancy. Expression diminished on day 4, but was again induced by the implanting blastocyst. The results suggested that CaSR expression in the stromal cells in the receptive status of the uterus was induced by the implanting blastocysts, while in epithelial cells during day 1 through day 5, the expression of CaSR was regulated by some non-embryonic factors. An embryo transplantation model confirmed that CaSR expression in the uterus was induced by the implanting blastocysts. Artificial decidualization caused upregulation of CaSR expression in the decidualized cells. Furthermore, estrogen as well as progesterone induced the expression of CaSR protein in the uterus. The strong CaSR expression at the implantation site and decidual cells in rat uterus suggests that CaSR is of relevance for blastocyst implantation and decidualization (Xiao et al., 2005).

In addition to the endometrium, also the myometrium, the thick muscular coat of the uterus, has important functions during reproduction as it contributes to sperm and embryo transport, but also to implantation. Ca^{2+} plays an important role for the uterine contractility (Aguilar and Mitchell, 2010). Based on the observation that uterine contractions can be inhibited by the CaSR ligands spermine and high concentrations of Mg^{2+}, it was speculated that CaSR was involved in the regulation of myometrial contractility (Pistilli et al., 2012). CaSR expression was confirmed in the estrogen-dominated rat uterus. Highest expression of CaSR was seen in the luminal epithelium, but it was also detected in longitudinal and circular muscle layers of the myometrium (**Figure 2A** and **Table 1**). Oxytocin-induced contraction of the rat uterus was not only inhibited by various CaSR agonists such as polyvalent cations and polyamines, but also by two synthetic positive allosteric CaSR modulators, (R)-calindol and (R)-cinacalcet. However, the positive allosteric modulation of CaSR did not show the appropriate stereoselectivity. (S)-cinacalcet, which is usually less potent than the (R)-enantiomer, inhibited contraction to the same extent. Furthermore, the negative allosteric modulator calhex 231 also caused concentration-dependent relaxation. Thus, the pharmacological profile of inhibition of contractility by CaSR ligands was not consistent with their effects being mediated through CaSR, but was rather consistent with promiscuous actions of the ligands. Later, the same authors investigated CaSR expression and function in the pregnant human myometrium. Despite bright staining in the human placenta, only sparse expression of CaSR receptors in pregnant human myometrium was observed (**Figure 2A** and **Table 1**). Exposure of human myometrial strips to CaSR receptor ligands showed that calindol and

cinacalcet, CaSR agonists, were ineffective as inhibitors of contractions, while the CaSR antagonist calhex 231 produced partial inhibition of contractility (Crankshaw et al., 2013). In summary, the role of CaSR in the rat myometrium remains unclear.

Research Demand

In assisted reproduction, implantation failure is the pregnancy rate-limiting step. The mechanisms underlying human embryo-endometrium signaling are not fully understood, but this is required to improve assisted reproduction outcomes. A detailed understanding of the implantation process is also crucial to develop effective interventions to prevent early pregnancy loss. The small number of studies currently addressing CaSR expression in the uterus demonstrated expression in rat endometrium, but more functional studies confirming the suggested role in implantation and decidualization are required, not only for the rat system, but also for humans and all other species, where assisted reproduction could be of interest. It is known that studies done in animal models do not always translate well to humans. Human *in-vitro* models of implantation (Weimar et al., 2013) may provide an alternative possibility to study human CaSR function during implantation and decidualization. Expression of CaSR in the myometrium was so far only investigated in rat and human. The function of CaSR in the myometrium remains unclear.

PLACENTA

Placenta Formation and Function

Placentas are unique and transient organs, which develop in female mammals during pregnancy at the interface of maternal and fetal circulation. The organ exhibits an amazing range of morphological variations across species (Benirschke et al., 2012).

Formation of a placenta is of great importance for the development of the embryo and starts very early in pregnancy. The first differentiation process gives rise to trophoblast cells. Trophoblasts initiate implantation of the embryo into the maternal endometrium by interaction with decidual cells of the maternal uterus. By proliferation, invasion and differentiation, trophoblast cells are the most important builders of the placenta. With progression of (human) placenta formation, two distinct populations of trophoblast cells, the villous and the extravillous trophoblast, which exhibit distinct functions, become evident. The expanding chorionic villi of the placenta, which mediate materno-fetal transport of nutrients are covered by a multinucleated layer of syncytiotrophoblast (STB). This cell layer is maintained by proliferation and fusion of the underlying villous cytotrophoblasts. Extravillous trophoblasts, in contrast, invade the uterus and interact with maternal cells. These cells are important for proper placentation.

The process of implantation of the conceptus is species dependent and can be either **invasive** as seen in humans, most primates, dog, cat mouse, rat, or rabbit meaning that the conceptus will break through the surface epithel of the maternal uterus and invades the underlying stroma or can be **non-invasive** (pig, sheep, cow, horse), integrating the uterine epithelium in the placenta. The depths of invasion as well as the degree of proximity between maternal and fetal circulation can vary largely among species. In humans and guinea pigs, the conceptus invades the stroma so deeply that the uterine surface epithelium is restored over it (interstitial implantation). Other species (dog, cat, rat), may invade the stroma only partially and project into the uterine lumen (eccentric implantation). This can result in a contact to other sites of the uterine lumen and additional placental development (e.g., bidiscoid placenta in rhesus monkey, zonary placenta in dog and cat). While in some species the maternal tissue remains relatively intact (dog, cat), meaning that trophoblast cells contact maternal capillary endothelium, in other species, trophoblast cells also invade the maternal endothelium and ultimately bath in maternal blood (human, rabbit, rat, mouse). These types of placentas are classified as haemochorial, which means that all maternal tissue layers are removed and the chorion (i.e., the trophoblast) bathes directly in maternal blood. But even structures of murine and human placentas differ to some extent. Whereas, the term human placenta is monochorial, which means that one continuous trophoblast cell layer, the STB, separates maternal and fetal blood circulation, the murine placenta is trichorial. Moreover, in rodent placenta, but not in human, other primate, or ruminant placentas the primitive yolk sac, which in all species participates in nutrient exchange between the fetal and maternal circulations before the formation of the placenta, is incorporated into the placenta and turns into the intraplacental yolk sac (IPYS). The IPYS is positioned between fetal vessels and maternal blood spaces, well situated for exchange of substances between mother and fetus. It is a bilayered membrane, where smaller parietal or cuboidal cells on a thick basement membrane (Reichert's membrane) overlie maternal blood spaces and vessels, while tall columnar cells on the visceral or endothelial side overly the fetal vessels. Between these layers is the sinus of Duval, which communicates with the yolk sac cavity and the uterine lumen (Metz et al., 1976).

To ensure optimal fetal development, the placenta fulfills a plethora of functions including gas exchange, nutrient transfer, hormone secretion, and immunological functions. Materno-fetal transfer of the ion Ca^{2+} is indispensable for proper development including mineralization of fetal skeleton. About 30 g Ca^{2+} are actively transported across the human placenta, predominantly during the last trimester of pregnancy. As fetal serum Ca^{2+} concentrations are set at significant higher concentrations than maternal serum concentrations, trans-placental Ca^{2+} transport occurs against an extracellular Ca^{2+}-concentration gradient. Placental transfer of Ca^{2+} involves various proteins, including Ca^{2+} channels, Ca^{2+}-ATPases and intracellular Ca^{2+}-binding proteins (Calbindin-D9k). Current knowledge on placental Ca^{2+} transfer as well as regulation of maternal and fetal Ca^{2+} homeostasis have been reviewed in several recent publications (Baczyk et al., 2011; Olausson et al., 2012; Kovacs, 2014, 2015, 2016).

Despite this active Ca^{2+} transfer, the placental trophoblasts still uses Ca^{2+} as a second messenger to activate diverse cellular functions such as differentiation or proliferation, or—in the case of extravillous trophoblasts—also invasion (Baczyk et al., 2011).

CaSR Expression and Function in Trophoblast and Yolk Sac Cells

An involvement of CaSR in regulation of murine placental Ca^{2+} transfer was confirmed by heterozygous or homozygous ablation of the CaSR gene in mice. *Casr* knock down reduced placental Ca^{2+} transfer. CaSR disruption may, directly or indirectly, downregulate PTHrP or influence the PTHrP effect on the placenta (Kovacs et al., 1998). PTHrP reaches the maternal circulation during pregnancy most likely from the placenta and breasts, as well as possibly from the uterus and fetal tissues. PTHrP significantly stimulates the placental Ca^{2+} transport via proteins involved in transport (Kovacs et al., 1996; Strid et al., 2002; Bond et al., 2008). A Nuf mice with an activating mutation of CaSR exists (Hough et al., 2004), but, placental Ca^{2+} transport has not been analyzed in this strain. In murine placentas, CaSR mRNA was detected by *in situ* hybridization in both types of IPYS cells, but not in the trophoblasts. By immunohistochemistry, using a monoclonal antibody directed against a region of the CaSR that has been deleted in the *Casr*-null mice, the CaSR was found to be expressed in both layers of the IPYS (**Table 1**). CaSR was also present in the surrounding labyrinth trophoblasts of wt placenta, but was absent in placentas obtained from *Casr*-null mice. Other molecules important for materno-fetal Ca^{2+}-transfer, including PTHrP, PTH/PTHrP receptor, calbindin-D9k, or Ca^{2+}-ATPase, were also higher expressed in the IPYS of the murine placenta compared to the trophoblasts. Overall, this suggests that IPYS is the important route of Ca^{2+}exchange between mother and fetus in the mouse (Kovacs et al., 2002).

Some evidence for involvement of human CaSR in transplacental Ca^{2+}-transfer has been obtained recently. Reduced expression of CaSR in placentas derived from women suffering from gestational diabetes mellitus compared with healthy placentas was found. This was associated with lower Ca^{2+} levels measured in cord blood of infants from women suffering from gestational diabetes mellitus supporting its role in placental Ca^{2+}-transfer (Papadopoulou et al., 2014). In humans, expression of CaSR was found in both villous and extravillous tissue of first trimester and term placentas (**Table 1**). In chorionic villi, CaSR was mainly detected at the apical membrane of the STB, which contacts maternal blood and at lower levels in cytotrophoblast cells (**Figure 3**). This location would be in line with the control of placental Ca^{2+}movement. The CaSR was also localized in extravillous trophoblast cells in close proximity to maternal blood vessels (**Figure 3**). It was speculated that CaSR senses maternal extracellular Ca^{2+} levels during the process of placentation and participates in regulation of placental development (Bradbury et al., 2002). Earlier, Bradbury and coworker had demonstrated that isolated human extravillous cytotrophoblasts expressed the full-length transcript of CaSR. A splice variant lacking exon 3 was also found, which encoded a truncated protein of 153 amino acids (compared with 1078 amino acids for the full length protein). Upon translation, this CaSR splice variant, however, would not be incorporated into the plasma membrane. The extravillous cytotrophoblasts responded to elevation of extracellular Ca^{2+}, and also to extracellular Mg^{2+} with a bi-phasic elevation of intracellular Ca^{2+} (Bradbury et al., 1998).

Research Demand

CaSR modulates transplacental Ca^{2+} transfer, at least in the mouse. Some evidence also exists for a role in human transplacental Ca^{2+} transfer. In breast cells, CaSR is expressed in the basolateral membrane of the lactating alveolus and regulates PTHrP secretion. CaSR senses the availability of Ca^{2+} for milk production, and stimulates production of PTHrP when the Ca^{2+}supply is insufficient. CaSR also impacts on a Ca^{2+}-ATPase (PMCA2) to stimulate Ca^{2+} secretion into milk when the Ca^{2+} supply is adequate (Kovacs, 2016). In the murine placenta, CaSR impacts on PTHrP, but detailed information of CaSR function in the murine or human hemochorial placentas is lacking. Information on expression of CaSR in the epitheliochorial placentas of other species is lacking.

CaSR can regulate proliferation, differentiation or apoptosis, but neither in extravillous nor villous human trophoblasts this issue has been addressed so far. Expression of CaSR in extravillous trophoblasts suggests an important role in placentation. In this context, not only Ca^{2+}, but also other CaSR ligands such as L-amino acids (Conigrave et al., 2000) may be modulators of placental tissue development in response to e.g., maternal Ca^{2+} concentration or maternal diet. Signaling pathways targeted by CaSR in the placenta are also unknown. A detailed understanding of the modulators of placentation is necessary before effective interventions can be considered.

Finally, it remains to be demonstrated whether CaSR is the only relevant Ca^{2+}-sensing protein of the placenta. In 1989, Juhlin and colaborators generated several monoclonal anti-parathyroid antibodies (E11, G11, B6) by immunization of mice with intact parathyroid cells. Two of these antibodies (G11 and B6) interfered with the Ca^{2+}-sensing mechanism of parathyroid cells (Juhlin et al., 1987). When monoclonal antibody E11 was applied in immunohistochemistry on placenta and uterus of the pregnant rat, positively stained rat placental cells were found at the end of pregnancy. Staining was confined exclusively to the columnar epithelial cells lining the sinuses of Duval, i.e., in the IPYS. In the uterus, positive staining of the epithelium lining the uterine lumen was obtained prior to and during implantation (days 5–6) (Bernadotte et al., 1989). When G11 and E11 were applied on sections of human placenta, both antibodies labeled the cytotrophoblast cells of anchoring and floating villi as well as cytotrophoblasts in the chorionic plate. The STB, however, was not stained. Isolated cytotrophoblast were found to react with G11 and E11. In these cells, a temporary increase of Ca_i^{2+} upon elevation of external Mg^{2+} was observed, which was blocked by G11 antibodies. Raised extracellular Ca^{2+} inhibited release of PTHrp from the cells, and this inhibition was blocked by the G11 antibody (Hellman et al., 1992). E11 was also used in immunoelectron microscopical studies showing positive staining of cytotrophoblast cells of the human placenta, and trophoblast cells lining fetal blood vessels in the rat placenta (Bjerneroth et al., 1992). Both antibodies, however, detected a 500 kDa protein, which is much larger than CaSR (Juhlin et al., 1990). When the protein was then cloned from human placenta, it was found to belong to the LDL-receptor superfamily of glycoprotein and it was identified as gp330 (megalin, LRP2) (Lundgren et al., 1994; Hjälm et al., 1996).

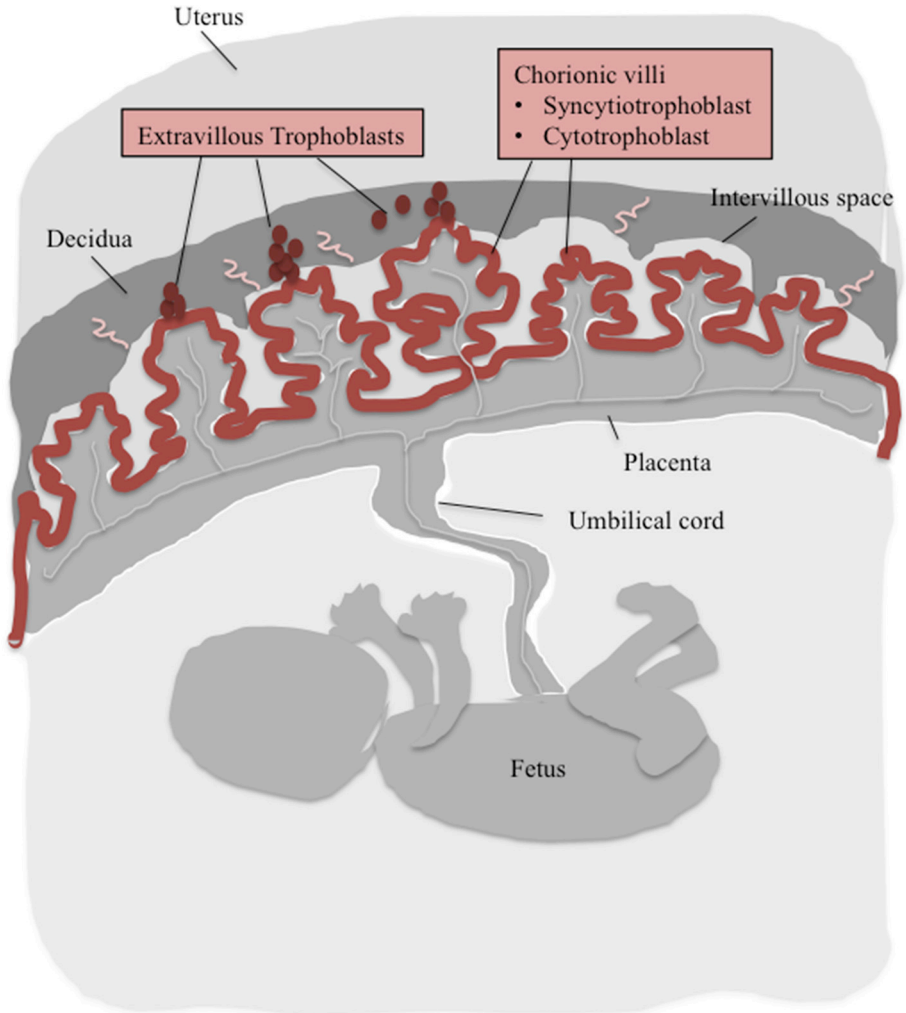

FIGURE 3 | The cartoon depicts major components of the human placenta. Cells, which were shown to express CaSR are depicted in red (for details on assumed function of CaSR see **Table 1**).

In addition to expression in human parathyroid cells, kidney proximal tubule cells and placental cytotrophoblasts, the protein was also detected in other reproductive tissues such as epididymal epithelial cells and mammary epithelium. It was suggested to have Ca^{2+} sensing functions (Lundgren et al., 1997). Unfortunately, further investigations to clarify the role of this protein in Ca^{2+} sensing were not performed.

SUMMARY AND OUTLOOK

CaSR is expressed in many cells of male and female reproductive organs. Due to its functional diversity, CaSR could be involved in a variety of reproductive processes ranging from proliferation or maturation of germ cells to implantation of the zygote, and from placentation to transplacental transport processes. Apart from physiologic actions, current data also suggest a role of CaSR in diseases of reproductive organs or pregnancy. Currently, however, we know very little about CaSRs physiologic and pathophysiologic functions in reproduction. Exploration of CaSR function in the context of diseases of reproduction such as male and female infertility and early pregnancy loss, PCOS, or gestational diabetes mellitus as well as tumors of reproductive organs may add novel possibilities for diagnosis and treatment.

AUTHOR CONTRIBUTIONS

IE designed and wrote the article and meets all criteria for authorship.

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MINI REVIEW
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Calcium Sensing Receptor as a Novel Mediator of Adipose Tissue Dysfunction: Mechanisms and Potential Clinical Implications

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Obesity is currently a serious worldwide public health problem, reaching pandemic levels. For decades, dietary and behavioral approaches have failed to prevent this disease from expanding, and health authorities are challenged by the elevated prevalence of co-morbid conditions. Understanding how obesity-associated diseases develop from a basic science approach is recognized as an urgent task to face this growing problem. White adipose tissue (WAT) is an active endocrine organ, with a crucial influence on whole-body homeostasis. WAT dysfunction plays a key role linking obesity with its associated diseases such as type 2 diabetes mellitus, cardiovascular disease, and some cancers. Among the regulators of WAT physiology, the calcium-sensing receptor (CaSR) has arisen as a potential mediator of WAT dysfunction. Expression of the receptor has been described in human preadipocytes, adipocytes, and the human adipose cell lines LS14 and SW872. The evidence suggests that CaSR activation in the visceral (i.e., unhealthy) WAT is associated with an increased proliferation of adipose progenitor cells and elevated adipocyte differentiation. In addition, exposure of adipose cells to CaSR activators *in vitro* elevates proinflammatory cytokine expression and secretion. An increased proinflammatory environment in WAT plays a key role in the development of WAT dysfunction that leads to peripheral organ fat deposition and insulin resistance, among other consequences. We propose that CaSR may be one relevant therapeutic target in the struggle to confront the health consequences of the current worldwide obesity pandemic.

Keywords: CaSR, inflammation, adipose tissue dysfunction, obesity, adipocyte, preadipocyte

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INTRODUCTION

Obesity has turned into a pandemic disease, with a worldwide prevalence that has more than doubled in the last three decades, even after multiple attempts to stop its expansion[1]. In 2014, more than 1.900 billion adults were overweight, a number that comprises more than 600 million obese individuals[1]. Besides being associated with disorders such as type 2 diabetes mellitus, hypertension,

[1] http://www.who.int/mediacentre/factsheets/fs311/en/.

cardiovascular disease, and cancer (Guh et al., 2009), obesity in itself is a death risk factor (Flegal et al., 2013). There is no doubt that obesity reduces the quality of life and affects the world economic development and productivity (Williams et al., 2005; Slagter et al., 2015).

For decades, many investigations have focused on identifying primary causes, preventive measures, and treatments for halting obesity. Despite these efforts, the long-term impact has been very small (Hafekost et al., 2013), and clinical trials testing different lifestyle-oriented approaches have consistently yielded disappointing results (Langeveld and DeVries, 2015; Ross et al., 2015; Mason et al., 2016). Moreover, the epidemiology reveals that governmental public health interventions focused on diet and physical activity have not been able to decrease the prevalence of obesity or even slow down its increase (Popkin et al., 2012; Cabrera Escobar et al., 2013; Hawkes et al., 2015). Pharmacological approaches have also failed to provide safe and efficacious therapies with long-term relevant results (Yanovski and Yanovski, 2014; Balaji et al., 2016). Considering this scenario, it is clear that there is an urgent need for a deeper understanding of the development of the obesity-associated diseases, and the study of adipose tissue plays a pivotal role in this sense. As written by Elmquist and Scherer (2012), "The solution for the obesity epidemic might lie in better understanding adipocyte biology." It is now known that white adipose tissue (WAT) dysfunction is key in the pathophysiology of obesity-related diseases, and the study of novel regulators of this process is crucial to uncover new therapeutic targets. In this context, our group showed the presence of the Calcium-sensing receptor (CaSR) in human preadipocytes and adipocytes (Cifuentes et al., 2005), and studies in the last decade suggest that its activation is involved in WAT dysfunction (Villarroel et al., 2014). The present review describes the evidence and perspectives of the role of CaSR in WAT and obesity, as a new player in this complex and multifactorial disorder.

WHITE ADIPOSE TISSUE: A KEY HOMEOSTATIC ORGAN

WAT is currently regarded as a dynamic organ with an extraordinary capacity to expand or decrease, according to the energy status of the organism (Pellegrinelli et al., 2016). There is considerable interest in studying WAT due to its relevance as an endocrine organ and as a whole-body metabolic regulator, particularly in light of the current obesity epidemic. The main functional component of WAT is the adipocyte, which specializes in storing energy as triglycerides and releasing it as fatty acids. The tissue is also composed of the so-called stroma-vascular fraction, which contains adipocyte progenitor cells (preadipocytes), and fibroblasts, as well as endothelial, smooth muscle, and immune cells. Besides its storage function, WAT also regulates whole-body energy homeostasis through the production of regulatory paracrine/endocrine molecules, termed adipokines (Rosen and Spiegelman, 2014). These secretory products control a wide variety of biological functions (**Figure 1A**), such as appetite, energy expenditure, body temperature, glucose homeostasis, insulin sensitivity, inflammation, blood clotting, reproduction, and ageing (Berry et al., 2013; Hyvönen and Spalding, 2014).

It is well-accepted that body fat distribution largely determines whether metabolic and cardiovascular comorbidities develop in an obese individual, favoring their occurrence when fat accumulation occurs at the abdominal level ("central obesity"; Jensen, 2008). The subcutaneous and the visceral compartments (**Figure 1B**) have very different clinical implications. Proliferation of the subcutaneous adipose tissue is considered positive, as it leads to increased "healthy" storage capacity (Gustafson et al., 2015). Inflammatory cues associated with obesity lead to impaired expansibility of the subcutaneous depots (Tchernof and Després, 2013). As a consequence, enlargement of visceral preadipocytes drives more inflammation and the increase of ectopic fat depots (Smith, 2015), associated with pathologic effects (Gustafson et al., 2009; Ye and Gimble, 2011). Ectopic fat accumulation leads to lipotoxicity where fatty acids are accumulated in peripheral tissues, impairing cellular signaling, and functions (Lee et al., 2013), constituting what has been termed "adiposopathy"(Bays, 2014).

Adiposopathy takes place when obesity-triggered changes, such as adipocyte hypertrophy and lipid overload, prevent WAT from properly performing its storage and endocrine functions (Gustafson et al., 2009). Dysfunctional adipocytes develop an overall inflammatory state, secreting cytokines that result in the infiltration of macrophages, which in turn produce higher amounts of proinflammatory cytokines. Such environment further compromises normal adipocyte function, particularly triglyceride deposition (Guilherme et al., 2008). This results in increased circulating free fatty acids and ectopic fat accumulation, ultimately leading to insulin resistance, and functional impairment in other organs, especially skeletal muscle.

CaSR: A ROLE IN ADIPOSE TISSUE DYSFUNCTION

CaSR is an extracellular Ca^{2+} sensor, originally described in the parathyroid gland as a regulator of parathyroid hormone secretion and circulating Ca^{2+} levels (Brown et al., 1993; Garrett et al., 1995). CaSR is a G-protein coupled receptor with 7 transmembrane helices and a very complex signaling network (**Figure 1C**) comprising orthosteric and allosteric modulators (Cavanaugh et al., 2012). Among the many non-Ca^{2+}-homeostatic roles that have been described for the CaSR, its involvement in WAT physiology emerged since its presence in human WAT was reported in 2005 (Cifuentes et al., 2005). CaSR activation in WAT is associated, by different mechanisms, with alterations consistent with a dysfunctional phenotype (**Figure 2A**). Activation of CaSR in human adipose cells, as well as WAT explants, elevates the expression of the proinflammatory cytokines interleukin 6 (IL6), chemokine C-C motif ligand 2 (CCL2), interleukin 1β (IL1β), and tumor necrosis factor alpha (TNFα; Cifuentes et al., 2012), which have been linked with adipose dysfunction and the cardiovascular and metabolic consequences of obesity. In addition, CaSR activation stimulates

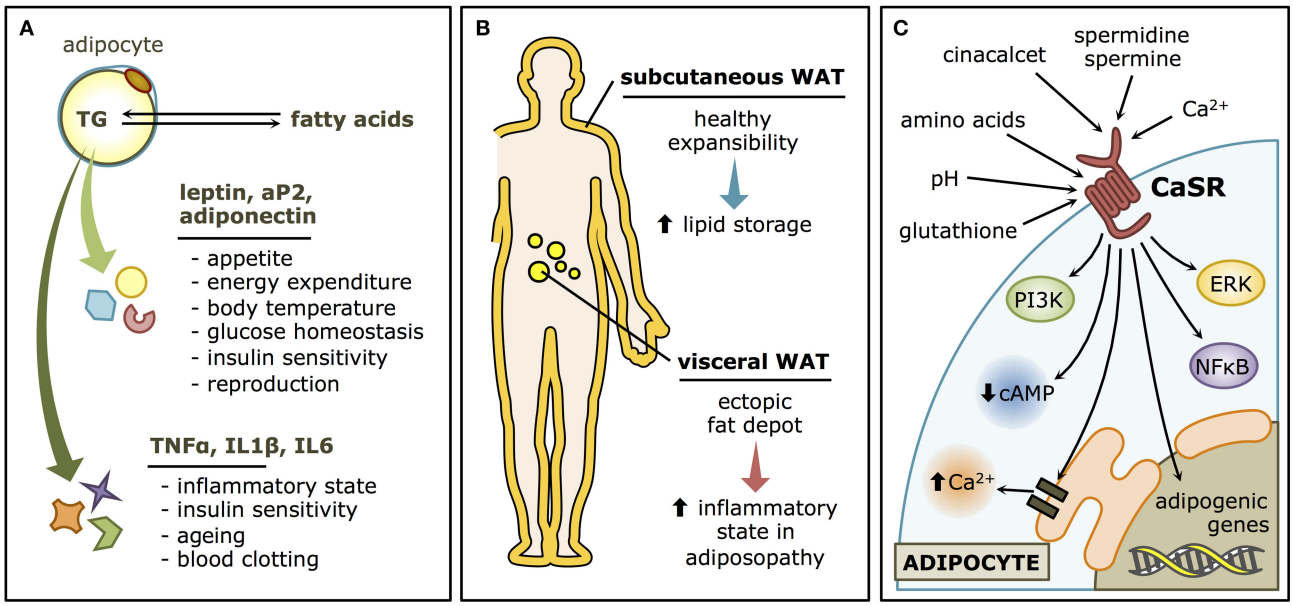

FIGURE 1 | White adipose tissue (WAT) and the Calcium-sensing receptor (CaSR). (A) Functions of the WAT. Adipocytes store lipids in the form of triglycerides and release them as fatty acids. The WAT also has an endocrine role, through the secretion of adipokines such as leptin, adiponectin and aP2 that regulate whole-body metabolism, and cytokines such as TNFα, IL1β, and IL6, with local and distal modulatory functions, that may determine the "low grade inflammatory status" that characterize most obese patients. **(B)** Types of WAT. Subcutaneous WAT is considered rather innocuous (or even beneficial) and has an important expansibility potential in healthy individuals. Visceral WAT, on the other hand, is a major player in adiposopathy, as it contributes to the inflammatory state that characterizes WAT dysfunction. **(C)** CaSR signaling pathways in WAT. CaSR has both physiological activators, such as Ca^{2+} and polyamines, and pharmacological modulators, such as cinacalcet, as well as a variety of allosteric regulators associated with metabolism, such as pH, amino acids, and glutathione. In the adipocyte, the canonical CaSR-associated pathways are thought to be activated, like ERK, PI3K, NFκB, and Ca^{2+} elevations and cAMP decreases through Gα proteins. The adipogenic program is also known to be stimulated by CaSR stimulation.

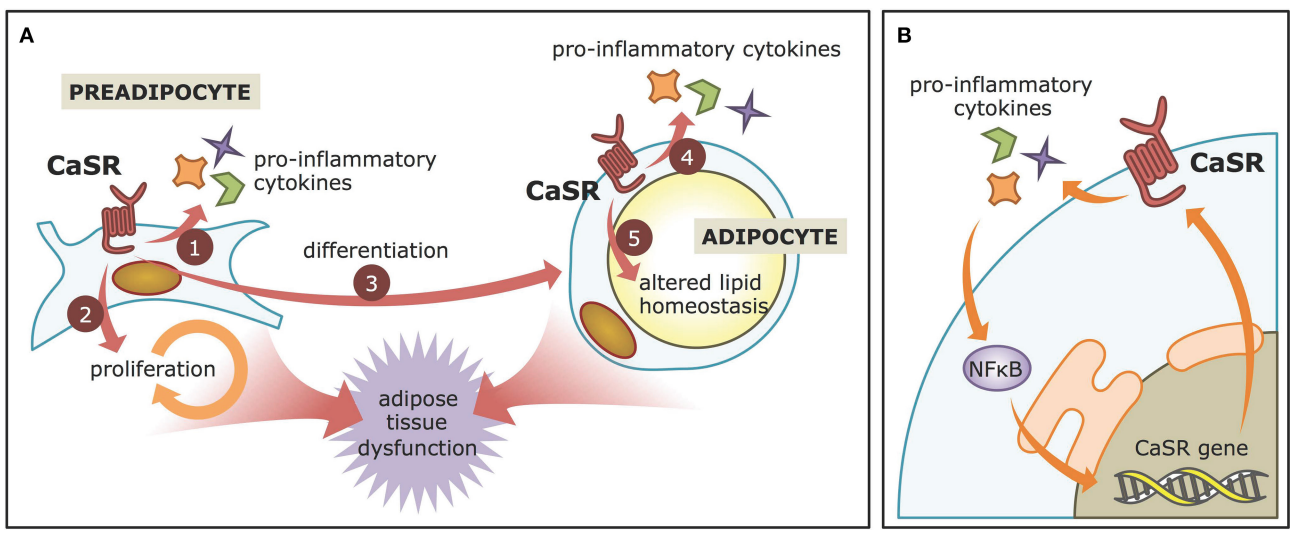

FIGURE 2 | CaSR contributions to adiposopathy. (A) CaSR activation in visceral WAT preadipocytes leads to increased production of pro-inflammatory cytokines, proliferation, and differentiation. In adipose cell models, it also enhances pro-inflammatory cytokine production and may decrease lipid accumulation, thereby contributing to adipose tissue dysfunction. **(B)** The NFκB-CaSR positive feed-back. CaSR activation leads to increased pro-inflammatory cytokines secretion, which are known to activate the NFκB pathway. NFκB, in turn, stimulates CaSR gene expression via specialized sequences in the CaSR promoter.

the proliferation, and proinflammatory cytokine expression in preadipocytes (Rocha et al., 2015). Moreover, CaSR activation elevates adipogenesis in visceral preadipose cells (Villarroel et al., 2013).

Pro-inflammatory Cytokine Expression

Several reports have described how CaSR activation is linked to inflammation in adipose cells, which is the cardinal feature of WAT dysfunction. The human adipose cell line LS14, as well as primary preadipocytes, and adipocytes, not only express CaSR, but also upregulate its protein levels in response to pro-inflammatory cytokines, such as IL1β, IL6, and TNFα (Cifuentes et al., 2010). Furthermore, adipocytes treated with conditioned medium of WAT explants obtained from obese individuals also increase their CaSR protein levels. These elevations at least in part depend on NFκB signaling pathway, as its inhibitor, SN50, reduces the effect. These findings are consistent with previous work showing the presence of NFκB response elements in the CaSR promoter (Canaff and Hendy, 2005). The CaSR, in turn, increases the secretion of proinflammatory cytokines in adipose cells (Cifuentes et al., 2012), possibly resulting in a positive feedback loop. CaSR activation with the calcimimetic cinacalcet increases the expression of IL1β, IL6, TNFα, and CCL2 in LS14 preadipocytes and adipocytes as well as human visceral WAT explants. Again, these changes were dependent on NFκB signaling in LS14 cells (Cifuentes et al., 2012), thus highlighting the intimate, tripartite relationship between CaSR-NFκB-cytokines during inflammation in adipose cells (**Figure 2B**).

Higher Visceral Adipogenesis

Studies in both SW872 and LS14 human adipose cell lines have revealed an adipogenic effect of CaSR (He et al., 2012; Villarroel et al., 2013). Exposure to CaSR activators increases the mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ), a master regulator of the adipogenic genetic program, thereby upregulating its downstream genes, such as adipose fatty acid-binding protein (aP2), lipoprotein lipase (LPL), CCAAT element binding protein α (C/EBPα), glycerol-3-phosphate dehydrogenase (GPD), and adiponectin. In response to body fat overload, healthy subcutaneous adipogenesis is considered a positive measure to increase storage capacity. However, as aforementioned, not all fat depots are equal, and increased visceral WAT is associated with inflammation and obesity-induced cardiovascular and metabolic impairment. In the case of LS14 cells, they are considered a model of visceral adipocytes (Hugo et al., 2006; LaPensee et al., 2008). Given their proinflammatory profile elicited by CaSR activation, this kind of adipogenesis rather contributes to adiposopathy, instead of its prevention or relief.

Preadipocyte Proliferation

In both human LS14 and murine 3T3L1 preadipocytes, different CaSR agonists increase proliferation through the ERK signaling pathway (Hou et al., 2013; Rocha et al., 2015). This mitogenic effect of CaSR activation has also been shown in other cell types, such as rat bone marrow mesenchymal stem cells (Xu et al., 2012), and rat osteoblasts (Chattopadhyay et al., 2004). Moreover, the involvement of the CaSR-ERK signaling axis is consistent with findings in breast cancer cells (El Hiani et al., 2009). As is the case for interpreting the adipogenesis observations, being considered a model of visceral WAT, LS14 proliferation may be linked to the pathologic effects of obesity, instead of a healthy subcutaneous WAT remodeling.

Altered Lipid Handling

Numerous epidemiological, clinical, and cell-based studies have suggested that a diet deficient in calcium is associated with greater fat accumulation (Villarroel et al., 2014). The proposed mechanism involves paradoxically greater cytosolic calcium levels in adipocytes with low calcium diets that trigger changes in triglyceride metabolism (Zemel and Miller, 2004). Interestingly, one of the main downstream signaling events triggered by CaSR activation is elevated cytosolic Ca^{2+}, and rats fed low calcium diets showed an elevated expression of CaSR in WAT (He Y. H. et al., 2011). In addition, the low calcium diet was associated with a decrease in triglyceride breakdown (lipolysis) in rat WAT, which was dependent on CaSR (He Y. H. et al., 2011). The low calcium diet also decreased protein levels of the lipolytic enzymes hormone-sensitive lipase and adipose triglyceride lipase. Using the SW872 human adipose cell line, this CaSR-mediated antilipolytic effect was shown to rely on intracellular Ca^{2+} augmentation, as well as a decrease in cyclic AMP (cAMP) and cAMP-dependent protein kinase A (PKA) signaling (He Y. et al., 2011). Similar results were obtained in primary human adipocytes, where CaSR activation decreased lipolysis via Giα protein and phosphoinositide 3 kinase (PI3K) signaling (Cifuentes and Rojas, 2008). Taken together, the described findings suggest that even though CaSR stimulates WAT proliferation and differentiation, it induces alterations in lipid handling that might contribute to the deleterious effects of obesity. In this context, it may be of great interest to perform controlled trials to evaluate possible changes in visceral adiposity and serum lipid profiles in patients undergoing treatment with cinacalcet.

Integrative Model

Among the many regulators of the CaSR (reviewed in Cavanaugh et al., 2012), the polyamines spermine and spermidine are two orthosteric CaSR agonists with promising roles regarding WAT metabolism. Obese Zucker rats have four times more spermine and spermidine in the adipose tissue in comparison with lean animals, which correlates with increased activity of various triacylglycerol synthetic enzymes (Jamdar et al., 1996). Aside from polyamines, other metabolic indicators potentiate CaSR signaling, such as pH elevations (Doroszewicz et al., 2005), glutathione (Wang et al., 2006), and amino acids (Lee et al., 2007), thus supporting the possibility that outside of the parathyroid gland, CaSR acts more like a metabolic status sensor instead of a Ca^{2+} rheostat. Due to CaSR's unique ability to sense, and thus potentially integrate a variety of signals through distinct allosteric sites, it may be considered a sensor of the local metabolic environment (Breitwieser et al., 2004), which is of great interest in complex tissues, or pathogenic contexts such as dysfunctional

WAT and obesity. Additionally, CaSR folding and traffic have been indicated as key regulators of its activity (Grant et al., 2011). This is interesting given that in obesity, adipocytes are subjected to endoplasmic reticulum (ER) stress (Kawasaki et al., 2012), which specifically compromises protein folding and traffic. Moreover, CaSR synthesis in the ER includes a folding checkpoint and retention step (Cavanaugh et al., 2010), which can be assisted with agonists such as Ca^{2+} and glutathione (Breitwieser, 2014). Considering that adiposopathy leads to oxidative stress and altered glutathione metabolism in adipocytes (Kobayashi et al., 2009), we propose CaSR as a novel potential integrator of environmental cues, which ultimately contribute to adipose tissue dysfunction and pathology.

FUTURE STUDIES

Ongoing studies of CaSR in WAT pathophysiology are contributing to elucidate an important role of the receptor, mainly in the visceral depot or in models of visceral adipose cells. Given the fundamental differences between WAT depots, it is of great relevance that future studies begin to address the role of the receptor in the subcutaneous fraction. Moreover, studying the expression and function of the CaSR in obese and lean individuals, as well as healthy versus unhealthy obese, and exploring whether there exists a sexual dimorphism in WAT CaSR expression and function, are relevant pending issues.

For a better insight on the role of CaSR in the pathogenesis of obesity, studies should also test the effect of CaSR modulation in different obesity models, such as diet-induced and genetically modified mice. Given the function of CaSR in multiple tissues, as well as its preponderance in regulating circulating calcium, it should be considered to modulate its function locally at the WAT. Additionally, its role during the onset and progression of fat accumulation should be tested. Moreover, the WAT of patients with activating or downregulating mutations in the CaSR should be analyzed, in order to detect disturbances in their metabolic or inflammatory state.

In recent years, not only WAT, but also brown adipose tissue (BAT) has received increasing attention. BAT is a type of adipose tissue characterized for being thermogenic and rather metabolically active instead of storage-oriented (Pellegrinelli et al., 2016). Different reports have shown its presence and activity in healthy adult humans, where up to recent years it was thought to be irrelevant (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). BAT is a promising target in the treatment of metabolic diseases, as it inversely correlates with body weight and fasting circulating glucose (Lee et al., 2010). Cold exposure has been shown to stimulate BAT presence in humans (Hanssen et al., 2016) probably through transdifferentiation of WAT (Frontini et al., 2013). White-to-brown transdifferentiation, commonly termed "browning" or "beigeing," has been shown to occur only in subcutaneous (Fisher et al., 2012) but not in visceral WAT, further underscoring the differences between both types of adipose tissue and the need to explore the role of CaSR in the subcutaneous depot. Studies are required to assess the role of CaSR in BAT metabolic and regulatory functions, and also to test whether CaSR pro-inflammatory effect negatively affects WAT browning.

CONCLUSION

Obesity is a complex and multifactorial disease, and even though it is known that energy imbalance is at the core of the problem, it has been extremely difficult to decrease or control the worldwide obesity epidemic. Current efforts aim to reduce obesity-related diseases by better understanding WAT physiology and maintaining its adequate function. The CaSR in WAT is emerging as an important molecule whose activation has been shown to increase the inflammatory state. Moreover, CaSR activation also promotes the proliferation of WAT under inflammatory conditions, which is known to yield less functional fat depots that rather contribute to pathogenesis than to lipid storage. In this sense, inhibition of CaSR activity in the WAT of obese patients may represent a novel therapeutic approach in this obesity epidemic era. The effects here described are in accordance with recent findings indicating that CaSR activation participates in many inflammatory processes, such as recognition of necrotic cells (Rossol et al., 2012) and pathologies like asthma (Yarova et al., 2015), myocardial infarction (Liu et al., 2015), and sepsis (Wu et al., 2015). On the other hand, CaSR-induced inflammation in the gastrointestinal tract has been proposed to be a conditioning agent for the appropriate function of the intestinal epithelial barrier (Owen et al., 2016). Thus, further research should evaluate the potential impact of CaSR activation as a pharmacological target in the pathogenesis of WAT dysfunction.

AUTHOR CONTRIBUTIONS

MC and RB designed and outlined the structure and contents of the review. RB, PM, XD, SL, and MC contributed to the literature review, discussion, and writing of the manuscript. All authors contributed equally to the draft revisions and final approval of the version to be published.

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