Calcium-sensing receptor in physiology and in calcitropic and non-calcitropic diseases

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Abstract

The Ca^{2+}-sensing receptor (CaSR) is a dimeric family C G-protein-coupled receptor that is expressed in calcitropic tissues such as the parathyroid glands and kidneys, and signals via G-proteins and beta-arrestin. The CaSR plays a pivotal role in bone and mineral metabolism by regulating parathyroid hormone secretion, urinary Ca^{2+} excretion, skeletal development and lactation. The importance of the CaSR for these calcitropic processes is highlighted by loss- and gain-of-function CaSR mutations, which cause familial hypocalciuric hypercalcaemia and autosomal dominant hypocalcaemia, respectively, and also by alterations in parathyroid CaSR expression, which contribute to the pathogenesis of primary and secondary hyperparathyroidism. Moreover, the CaSR is an established therapeutic target for hyperparathyroid disorders. The CaSR is also expressed in organs not involved in Ca^{2+} homeostasis, where it has non-calcitropic roles that include lung and neuronal development, vascular tone, gastro-intestinal nutrient sensing, secretion of insulin and entero-endocrine hormones, and wound healing. Furthermore, abnormal expression or function of the CaSR is implicated in cardiovascular and neurological diseases, as well as in asthma, and the CaSR is reported to protect against colorectal cancer and neuroblastoma, but increase the malignant potential of prostate and breast cancers. This review will discuss these physiological and pathophysiological roles of the CaSR.

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Introduction

The extracellular calcium (Ca^{2+})-sensing receptor (CaSR) is an ~120-160 kDa G-protein-coupled receptor (GPCR) that is most highly expressed in the parathyroid glands and kidneys[1,2], where it influences systemic Ca^{2+} homeostasis by detecting increases in the prevailing circulating Ca^{2+} concentration, which lead to intracellular signalling events that mediate a decrease in parathyroid hormone (PTH) secretion and reduction in renal tubular Ca^{2+} reabsorption (FIG. 1)[3]. The importance of the CaSR, which is a family C GPCR, for the regulation of circulating Ca^{2+} concentrations, i.e. its calcitropic actions, has been demonstrated by the identification of germline loss- and gain-of-function mutations affecting this GPCR and its intracellular partner proteins that result in inherited hypercalcaemic and hypocalcaemic disorders such as familial hypocalciuric hypercalcaemia (FHH) and autosomal dominant hypocalcaemia (ADH), respectively[4]. Furthermore, the CaSR, which is present as a dimer of ~240-310 kDa[5] has been shown to represent a therapeutic target for such calcitropic disorders, and cinacalcet, a CaSR positive allosteric modulator (PAM), is used in clinical practice to treat hyperparathyroid disorders, and calcilytic drugs that are CaSR negative allosteric modulators (NAMs) are being investigated as a targeted therapy for symptomatic forms of ADH[6]. The CaSR is also expressed in other tissues, such as the intestine, pancreatic islets, lungs, brain, skin and vasculature, where it has been shown to be involved in non-calcitropic actions that include regulation of molecular and cellular processes such as gene expression, proliferation, differentiation and apoptosis, as well as influencing the physiological regulation of entero-endocrine hormone secretion, cardiac function, vascular tone, and also lung and neuronal development (TABLE 1)[7-14]. Furthermore, abnormal expression or function of the CaSR in these non-calcitropic tissues has been reported to contribute to the pathogenesis of cardiovascular diseases, asthma, Alzheimer's disease, and breast and colon cancer[9,14-16]. This review focuses on the evolutionary origins, structure and signalling pathways of the CaSR, together with the roles of the CaSR in calcitropic and non-calcitropic diseases. Many of these aspects were discussed at the Third International Symposium on the Ca^{2+}-Sensing Receptor (Florence, May 2017), which brought together researchers who are studying these basic, translational and clinical aspects of CaSR physiology and pathophysiology.

Structure of the CaSR

The CaSR belongs to the family C GPCRs, which play pivotal roles in neurotransmission, nutrient-sensing and Ca^{2+} homeostasis[17]. These receptors are functionally active as homodimers or heterodimers[18-20], and are characterised by the presence of a large extracellular domain (ECD), which contains a bilobed venus flytrap (VFT) module (FIG. 2) that closes upon ligand binding, and has structural similarity to nutrient-scavenging bacterial periplasmic proteins[21]. The CaSR is considered to have arisen during vertebrate evolution[22], and has been shown to be functionally active in vertebrates ranging from cartilaginous fishes to terrestrial mammals[22]. Recent X-ray crystallography studies have demonstrated the human CaSR to have a glycosylated ECD, which binds Ca^{2+}_o at three distinct sites within the VFT, and also at a site located between the VFT and cysteine-rich domain (CRD) (FIG. 2)[23]. The CaSR also binds amino acids within the cleft of the VFT (FIG. 2)[23,24], and the binding of both Ca^{2+} and amino acids may be required to fully activate the CaSR[23]. The CaSR VFT is predicted to contain additional binding sites for anions such as phosphate and sulphate, and these anions likely stabilise the CaSR in an inactive conformation[23]. The CaSR is maintained as a dimer (FIG. 2) by covalent and non-covalent interactions that involve the N-terminal VFT lobe of each monomer, and ligand-binding has been shown to induce closure of the VFT, as well as extending the dimer interface to include the C-terminal VFT lobe and the CRD[23]. These agonist-induced conformational changes of the CaSR ECD may lead to rearrangements of the helices within the transmembrane domain (TMD) that facilitate G-protein binding and intracellular signal transduction[3,25]. The CaSR TMD consists of seven hydrophobic helical domains connected by three extracellular loops and three intracellular loops (FIG. 2). The TMD anchors the CaSR in the plasma membrane, and also plays a critical role in signal transduction. Indeed, mutational analysis has identified residues within intracellular loops 2 and 3, which are essential for the activation of downstream effector proteins[26]. The CaSR TMD has not yet been crystallised, however, homology modelling studies using the related metabotropic glutamate receptor crystal structure indicates the presence of a cavity located between the mid-portion and extracellular aspect of the CaSR TMD, which is the site of binding of allosteric modulators such as the phenylalkylamine calcimimetic drugs and amino-alcohol calcilytic drugs (FIG. 2)[27]. The intracellular domain (ICD) of the CaSR

regulates downstream signalling events[27], and also undergoes phosphorylation and ubiquitination, which facilitates desensitisation and degradation or recycling of the CaSR[28,29]. Moreover, the ICD is considered to be the site of binding for the intracellular adaptor-related protein complex 2 (AP2) (FIG. 2), which facilitates clathrin-mediated endocytosis of GPCRs such as the CaSR[30].

CaSR signalling

The CaSR activates downstream signalling cascades by coupling to heterotrimeric guanine-nucleotide binding proteins (G-proteins) such as the $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$ family of proteins[31,32]. The $G_{q/11}$ proteins are critical for CaSR signal transduction, and germline mutations of G-protein subunit α_{11} ($G\alpha_{11}$) have been shown to cause hypercalcaemic and hypocalcaemic disorders (TABLE 2)[33]. Through $G_{q/11}$, the CaSR activates phospholipase C (PLC) to convert phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which in turn releases Ca^{2+} from intracellular stores by binding to IP_3 receptors on endoplasmic reticulum and activates Ca^{2+} influx via store-operated channels located in the plasma membrane (FIG. 2)[31]. The lipid derived second messenger, DAG, activates protein kinase C (PKC), which regulates protein phosphorylation cascades involved in cell survival, differentiation and proliferation such as the mitogen activated protein kinases (MAPKs) (FIG. 2)[31,34]. Key components of the MAPK cascade activated by the CaSR include the extracellular-signal regulated kinases 1/2 (ERK1/2), p38 kinase (p38K) and c-Jun N-terminal kinase (JNK)[35,36]. By coupling to the pertussis toxin-sensitive $G_{i/o}$ proteins, the CaSR suppresses adenylyl cyclase activation and cyclic AMP (cAMP) production (FIG. 2), and also stimulates MAPKs such as ERK1/2[31,34,35]. The CaSR may also stimulate the MAPK pathway via a G-protein independent mechanism, which involves the beta-arrestin proteins[37]. The CaSR additionally has been reported to couple to the $G_{12/13}$ proteins to activate RhoA, which is a small GTPase protein that plays a role in the Wnt3a-beta-catenin signalling cascade[38] and also facilitates cadherin-mediated intercellular adhesion[11]. CaSR signal transduction also determines the level of cell-surface expression of this GPCR via a process known as agonist-driven insertional signalling (ADIS), which increases anterograde trafficking of newly synthesised CaSRs to the plasma membrane (FIG. 2), and likely prevents the CaSR from undergoing functional desensitization in response to continual exposure to Ca^{2+}_o[39,40]. CaSR signalling

is additionally mediated by the σ-subunit of the heterotetrameric AP2 complex, and germline loss-of-function AP2σ mutations have been shown to impair intracellular Ca^{2+} and MAPK signalling responses in CaSR-expressing cells, and to cause hypercalcaemia (TABLE 2)[30,41-43]. These CaSR-mediated signalling cascades modulate diverse physiological functions in calcitropic and non-calcitropic tissues, including hormone secretion, growth, survival, migration, adhesion, and differentiation (see sections below). In calcitropic tissues, loss- and gain of CaSR expression and/or signalling lead to hypercalcaemic and hypocalcaemic disorders, respectively. However, in non-calcitropic tissues reduced CaSR activity/signalling has been associated with disorders ranging from impaired wound healing to vascular calcification and colorectal carcinoma[11,16,44], and upregulation of CaSR expression in cells and tissues with a low basal level of CaSR expression and activity, such as neurons and airway smooth muscle cells, has been reported to occur in brain injury or asthma[14,45], with the resulting increase in CaSR activity and signalling further contributing to disease progression.

CaSR ligands and biased signalling

The CaSR binds to an array of physiological ligands such as cations (e.g. H^+, Na^+, Ca^{2+}, Mg^{2+}), L-amino acids and polyamines, and crystal structures indicate that the CaSR may also bind anions such as PO_4^{3-} and SO_4^{2-} [23,31]. Low molecular weight molecules which allosterically enhance or suppress CaSR activity and are referred to as calcimimetic or calcilytic compounds, respectively, have also been identified[6]. Such calcimimetic and calcilytic compounds are either in clinical use or under investigation for the management of calcitropic and non-calcitropic disorders, which are associated with activating or inactivating mutations, aberrant expression of the CaSR, or its downstream signaling molecules[3,14,42,46-48].

Several CaSR ligands, including L-amino acids, polyamines, Mg^{2+}, calcimimetics, and calcilytics, have been shown to induce biased signalling (also referred to as functional selectivity), thereby leading to preferential activation of distinct intracellular signalling responses[37,49]. CaSR-mediated biased signalling may provide an explanation for the CaSR's ability to have differing responsiveness to extracellular Ca^{2+} and mediating diverse physiological functions in different cell systems in response to locally available allosteric factors. This biased signalling may also explain the

differences in the efficacies of calcimimetics or calcilytics in different target tissues. For example, it has been shown that the calcimimetic compound R-568 is 40-fold more potent in suppressing PTH secretion in parathyroid cells than in stimulating calcitonin secretion from thyroid C-cells[50].

The mechanisms underlying CaSR-mediated biased signalling remain to be elucidated, and the recent characterization of a novel ADH-causing mutation, Arg680Gly, has led to the identification of a transmembrane salt-bridge (Arg680-Glu767) (FIG. 3), which regulates signalling via a G-protein independent mechanism[51]. Indeed, mutations disrupting this salt bridge, which is located at the entrance to the TMD allosteric modulator binding pocket, were shown to upregulate beta-arrestin-mediated signalling without altering G-protein-dependent signalling (FIG. 3)[51]. The Arg680Gly CaSR mutation also abrogated the effect of NPS 2143 (FIG. 3)[51], which is an amino-alcohol calcilytic compound[52], most likely by disrupting a hydrogen bond interaction between the wild-type Arg680 residue and NPS 2143[27]. These findings indicate that a structurally distinct class of CaSR NAMs, such as the quinazolinone calcilytic compounds[52], may be required to treat patients with symptomatic hypocalcemia caused by the gain-of-function Arg680Gly CaSR mutation[51].

Role of the CaSR in Ca^{2+} homeostasis

Extracellular Ca^{2+} (Ca^{2+}_o) is critical for a diverse range of biological functions that include mineralisation of bone matrix, neuronal and neuromuscular function, blood coagulation, and provision of a constant supply of Ca^{2+} for intracellular processes that range from signal transduction to the synthesis and secretion of hormones[53]. The concentration of Ca^{2+}_o is tightly regulated by a homeostatic system consisting of the following components: 1) the parathyroid glands, which sense Ca^{2+}_o and secrete PTH; 2) the kidneys and small intestine, which shift Ca^{2+} between the extracellular fluid and external environment; 3) the skeleton, which is the main Ca^{2+} reservoir and buffers acute fluxes in Ca^{2+}_o concentrations; and 4) calcitropic hormones such as PTH and 1,25-dihydroxyvitamin D_3, which mediate interactions between the parathyroid glands and target calcitropic organs such as the bone, kidney and intestine (FIG. 1)[3]. Moreover, during lactation the mammary glands act as a calcitropic endocrine organ, which secretes PTH-related peptide (PTHrP) to mobilise Ca^{2+} from bone. The CaSR is expressed in the

parathyroid glands, kidneys, bone and breast, and its calcitropic actions in these tissues are outlined below.

Parathyroid gland

The CaSR is most highly expressed in the parathyroid glands[2], where it regulates PTH synthesis and secretion in an inverse manner[54]. Thus, increases in Ca^{2+}_o lead to CaSR-mediated suppression of PTH release, whereas a decrease in Ca^{2+}_o releases this suppression to promote tonic PTH release[54]. In keeping with this, mice with a parathyroid-specific CaSR ablation have been shown to develop severe hyperparathyroidism and hypercalcemia[55]. In the parathyroid glands, the CaSR has been shown to interact with Klotho, which is a transmembrane protein, to regulate PTH secretion[56]. Moreover, expression of the parathyroid CaSR is upregulated by 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which acts on vitamin D response elements within the *CASR* gene promoter[57]. CaSR-mediated PTH secretion leads to activation of the PTH1 receptor (PTH1R) in bone and the kidneys to increase: osteoclast-mediated bone resorption; Ca^{2+} reabsorption by the renal thick ascending limb (TAL) of the loop of Henle and distal renal tubule; phosphate excretion by the proximal renal tubule; and synthesis of $1,25(OH)_2D$ by the proximal renal tubule, which increases intestinal Ca^{2+} absorption (FIG. 1)[3,58,59]. These calcitropic effects lead to an increase in Ca^{2+}_o concentrations, which in combination with $1,25(OH)_2D$, causes feedback inhibition of PTH release (FIG. 1)[3].

Kidney

The CaSR is highly expressed in the basolateral membrane of the renal TAL (FIG. 1), where it regulates Ca^{2+} reabsorption independently of the actions of PTH[60,61]. The effect of the TAL CaSR on renal Ca^{2+} excretion is likely mediated by the claudin-14 tight junction protein, which regulates the paracellular reabsorption of divalent cations[59,62,63]. The CaSR is also expressed at a lower level in the proximal renal tubule[64] where it regulates expression of the 1-α-hydroxylase enzyme, thereby influencing $1,25(OH)_2D$ synthesis[62]. The proximal renal tubular CaSR has also been shown to inhibit PTH-mediated phosphate excretion[65]. The kidney CaSR is considered to play a key physiological role in the defense against hypercalcaemia, by promoting renal Ca^2 excretion independently of the actions of PTH[60,66], and also by

potentially inhibiting 1,25$(OH)_2$D synthesis[62,67]. The CaSR is also expressed in the distal convoluted tubule[64], where it may promote Ca^{2+} reabsorption via the transient receptor potential vanilloid member 5 (TRPV5) channel[68]. Furthermore, the CaSR is expressed in the renal collecting ducts[64], where it prevents the development of hypercalciuria-mediated nephrocalcinosis by enhancing urinary acidification and water excretion[69]. Finally, the CaSR has been reported to be expressed in the juxtaglomerular apparatus, where it inhibits renin secretion[70], although the physiological consequence of this finding remains to be established.

Skeleton

The CaSR is expressed within bone and the growth plate, and its roles in these tissues have been characterized by using mice with osteoblast- and chondrocyte-specific ablations of the CaSR[55,71,72]. These studies have demonstrated that the chondrocyte CaSR has a critical role in growth plate chondrogenesis, longitudinal bone growth and skeletal mineralization, by counteracting PTH-related protein (PTHrP)/PTH1R signalling[73]. Indeed, mice with chondrocyte-specific ablation of the CaSR have shortened long bones, and also undermineralisation of the ribs, long bones and calvariae[55]. Mice with an osteoblast-specific CaSR ablation also showed reductions in bone mineralisation and long bone size, as well as developing spontaneous long bone fractures[55,71]. These findings occurred in association with impaired osteoblast differentiation and increased osteoclast activation, and highlight a role for the osteoblast CaSR in skeletal development, mineralisation and remodelling[71].

Breast

The CaSR has been shown to be expressed in human and mouse breast epithelial cells, and its expression is increased during lactation[74,75]. The CaSR promotes lactation by increasing the supply of Ca^{2+} for milk production through effects on the plasma membrane Ca^{2+}-ATPase 2 (PMCA2), which transports Ca^{2+} from the mammary cell into milk[76]. Studies in mice have shown that during lactation, the breast CaSR also influences Ca^{2+}_o homeostasis in order to regulate the mobilisation of skeletal Ca^{2+} for milk production[9,75]. Thus, in low Ca^{2+}_o states, reduced activation of the breast CaSR leads to increased PTHrP production from breast epithelial cells, which in turn stimulates osteoclastic resorption of Ca^{2+} from

bone (FIG. 1)[9]. These systemic effects of the breast CaSR ensure a steady supply of Ca^{2+} for milk production, and also protect against maternal hypocalcaemia during lactation[9].

Role of CaSR signalling pathways in inherited calcitropic diseases

Loss-of-function of the CaSR or the downstream $G\alpha_{11}$ and AP2σ signalling proteins gives rise to hypercalcaemic disorders, such as FHH types 1-3 (FHH1, FHH2 and FHH3), respectively, as well as primary hyperparathyroidism (PHPT) and neonatal severe hyperparathyroidism (NSHPT); whereas gain-of-function mutations of the CaSR or $G\alpha_{11}$ cause ADH types 1 (ADH1) and 2 (ADH2), respectively (TABLE 2).

Hypercalcaemic disorders

FHH is considered to be a rare disorder, with one study involving FHH patients in the west of Scotland reporting an estimated minimum prevalence of 1:78,000[77]. FHH is characterised by mild-to-moderate elevations of serum Ca^{2+} concentrations, mild hypermagnesaemia, and normal or elevated circulating PTH concentrations[4]. FHH has a similar serum biochemical phenotype to PHPT, and these conditions are distinguished in clinical practice by assessment of urinary calcium excretion, as ~80% of FHH patients are hypocalciuric with a calcium-to-creatinine clearance ratio (CCCR) <0.01[78,79]. However, a low CCCR is also observed in ~10% of PHPT patients, and thus mutational analysis of the known FHH-causing genes may be required to differentiate FHH from PHPT[80]. FHH comprises a genetically heterogeneous group of autosomal dominant disorders, designated as FHH types 1-3 (TABLE 2)[3], and these will be briefly discussed.

FHH1. FHH1 (OMIM #145980) occurs most commonly and accounts for ~65% of all FHH cases[4]. This condition is usually benign and non-progressive, however, a higher prevalence of chondrocalcinosis with increasing age has been noted[81], and some patients may develop recurrent pancreatitis[82]. FHH1 is caused by germline heterozygous loss-of-function mutations of the *CASR* gene, which is located on chromosome 3q21.1 (TABLE 2)[83,84], and to-date >150 different *CASR* mutations have been reported in FHH patients (http://www.casrdb.mcgill.ca)[85], and the majority (>85%) of these

are missense substitutions and the remaining (<15%) are nonsense, deletion, insertion and splice-site mutations that result in truncated CaSR proteins[4,83]. The offspring of FHH1 parents may harbour homozygous or compound heterozygous *CASR* mutations that lead to NSHPT (OMIM #239200), which is a potentially life-threatening disorder characterized by severe hypercalcaemia (serum calcium concentrations typically 3.5-5.0 mmol/L (normal range 2.10-2.55 mmol/L)), bone demineralization leading to fractures, and respiratory distress (TABLE 2)[4]. Hypercalcaemia in some patients with NSHPT may respond to treatment with cinacalcet, a calcimimetic (see below), but for long-term treatment parathyroidectomy is usually required[86]. Some patients who have the clinical features of FHH, but do not harbour *CASR* mutations, may have autoimmune hypocalciuric hypercalcaemia (AHH), which is associated with the presence of autoantibodies against the CaSR and also lymphocytic infiltrates within the parathyroid gland[87,88]. The hypercalcaemia caused by AHH may be responsive to glucocorticoid therapy[87,88].

Loss-of-function mutations affect all regions of the CaSR protein, however, mutations commonly occur within the first 350 amino acid residues of the ECD, which contains the four Ca^{2+} binding sites and the VFT cleft amino acid binding site[23,24]. Indeed, this region is a hotspot for recurrently occurring FHH1 mutations, and some amino acid residues are also affected by multiple different loss-of-function mutations[3,83]. Approximately 50% of the FHH1 mutations are predicted to impair the biosynthesis and post-translational processing of CaSR proteins within the endoplasmic reticulum or Golgi apparatus, thus leading to enhanced proteasomal degradation of these nascent receptors, with reduced anterograde trafficking and cell-surface expression[89,90]. Some other FHH1 mutations, which are located in the ECD and TMD, have instead been demonstrated to induce biased signalling responses by switching the wild-type CaSR from preferentially coupling with intracellular Ca^{2+} (Ca^{2+}_i) signalling to a mutant receptor that signals equally via the Ca^{2+}_i and MAPK pathways, or which acts mainly through MAPK[91,92]. Biased signalling has also been observed with an AHH-causing autoantibody, which enhanced CaSR-mediated accumulation of inositol phosphates but impaired ERK1/2 phosphorylation[93].

FHH2. FHH2 (OMIM#145981) is caused by germline heterozygous loss-of-function mutations of the *GNA11* gene on chromosome 19p13.3, which encodes the $G\alpha_{11}$ protein (TABLE 2)[33]. To date, FHH2-causing mutations have been reported in four probands, and these affected individuals exhibit mild hypercalcaemia (serum adjusted-calcium concentrations <2.80 mmol/L (normal range 2.10-2.55 mmol/L)), normal serum PTH concentrations, and normal or low urinary Ca^{2+} excretion[33,47,94]. The *GNA11* mutations identified in FHH2 patients comprise three missense substitutions, Thr54Met, Leu135Gln, Phe220Ser, and an in-frame isoleucine deletion at residue 200 (Ile200del)[33,47,94]. All of these mutations have been demonstrated to impair CaSR-mediated signalling responses, and are predicted to affect key domains of the $G\alpha_{11}$ subunit[33,47,94]. Thus, the Ile200del and Phe220Ser mutations are located within the $G\alpha_{11}$ GTPase domain and predicted to impair the coupling of $G\alpha_{11}$ with the upstream GPCR (i.e. CaSR) or downstream effector protein (i.e. PLC), respectively[33,47]. However, the Leu135Gln mutation is located within the portion of the $G\alpha_{11}$ helical domain that interacts with downstream effectors, and the Thr54Met mutation is situated at the interface between GTPase and helical domains, and predicted to impair guanine-nucleotide binding at this interface[33,94]. These studies of FHH2 mutations have highlighted residues critical for $G\alpha_{11}$ function.

FHH3. FHH3 (OMIM#600740) represents a more severe form of FHH, and is characterized by significantly higher serum calcium and magnesium concentrations and a significantly reduced FECa, when compared to FHH1[41,95]. Moreover, symptomatic hypercalcaemia, reduced bone mineral density, recurrent pancreatitis and cognitive dysfunction have been reported in some FHH3 patients[41,96]. This disorder is caused by germline heterozygous loss-of-function mutations of the *AP2S1* gene on chromosome 19q13.3, which encodes the AP2σ protein (TABLE 2)[30]. *AP2S1* mutations have been reported in >60 FHH probands to date, and >99% of affected individuals harbour a mutation affecting the AP2σ Arg15 residue, which may give rise to an Arg15Cys, Arg15His or Arg15Leu missense substitution[30,41,42,95,97-99]. To date, only one FHH patient has a mutation not involving the Arg15 residue, but the Met117 residue[100]. The prevalence of FHH3 has been reported to be ~7.8 per 100:000[100]. Patients harbouring the Arg15Leu AP2σ mutation have been shown to be more hypercalcaemic with an earlier age of presentation compared to probands with Arg15Cys or Arg15His AP2σ mutations[41,99]. Crystal

structure analyses have demonstrated the AP2σ Arg15 residue to bind to cell-surface cargo proteins[101], and it is predicted that these FHH3-causing Arg15 mutations disrupt an interaction between the AP2 complex and the CaSR ICD, thereby diminishing endocytosis of this GPCR[30]. Indeed, *in vitro* studies have shown FHH3-causing AP2σ mutations to alter CaSR cell-surface expression and impair signal transduction in a dominant-negative manner[30,41]. Thus, these studies highlight a role for the AP2 complex in Ca^{2+}_o homeostasis.

Hypocalcaemic disorders including ADH

ADH is caused by increased sensitivity of the CaSR signalling pathway to Ca^{2+}_o concentrations [33,102] and has the opposite biochemical phenotype to FHH. Thus, ADH patients have low serum calcium concentrations, mild hypomagnesaemia, normal or low PTH concentrations, and a relative or absolute hypercalciuria[3]. Approximately 50% of ADH patients have symptomatic hypocalcaemia and >30% of patients have renal and/or intracerebral calcifications (TABLE 2)[3]. Some ADH patients with marked CaSR activation may additionally have Bartter syndrome type V, which is characterised by hypokalaemic alkalosis, renal salt wasting and hyperreninaemic hyperaldosteronism (TABLE 2)[103,104]. Active vitamin D metabolites, combined with adequate dietary calcium intake and/or use of calcium supplements, are currently used to treat symptomatic ADH patients, although their use may predispose patients to the development of marked hypercalciuria, nephrocalcinosis, nephrolithiasis and renal impairment[102,105].

ADH is a genetically heterogeneous disorder with two types (ADH type 1 (ADH1; OMIM #601198) and ADH type 2 (ADH2; OMIM #615361) described[3]. ADH1 and ADH2 are caused by germline heterozygous gain-of-function *CASR* and *GNA11* mutations, respectively (TABLE 2)[83,102 33,106-108]. To date, >70 different ADH1-causing CaSR mutations have been identified (http://www.casrdb.mcgill.ca)[85], and ~95% of these are missense substitutions, whereas ~5% are inframe or frameshift insertion/deletion mutations[83]. Structure-function analyses have shown ADH1 mutations to cluster within the second peptide loop of the CaSR ECD (residues 116-136)[109], which contributes to the CaSR dimer interface[23,24,110], and also within the distal portion of the TMD (residues 819-837)[111], which is involved in G-protein binding[112]. Moreover, the Leu173 and Pro221 ECD residues

have been shown to be the site of both ADH and FHH mutations, and structure-function analysis indicates that these residues may act as intra-molecular switches to regulate ligand binding or potentially influence the receptor conformational changes that occur upon agonist binding[83,113]. Some patients with hypoparathyroidism, which may be isolated or due to autoimmune polyglandular syndrome type 1 (APS1) have been found to harbour activating autoantibodies to the CaSR[114-116]. These patients may have mild and asymptomatic hypocalcemia, or develop hypocalcaemic seizures that require treatment with calcium and vitamin D preparations[115].

Six different gain-of-function missense *GNA11* mutations (Arg60Cys, Arg60Leu, Arg181Gln, Ser211Trp, Val340Met and Phe341Leu) have been reported to cause ADH2[33,106-108], and affected individuals have a similar phenotype to ADH1 patients. However, ADH2 patients may have milder alterations in urinary Ca^{2+} excretion compared to ADH1[106], and short stature has also been reported in two ADH2 kindreds[106,117]. Structural modelling studies have shown ADH2 mutations to cluster at the interface between the helical and GTPase domains of the $G\alpha_{11}$ protein, which mediates GDP/GTP exchange; and also to be located at the $G\alpha_{11}$ C-terminus, which is involved in GPCR-G-protein coupling[3,33,108].

Role of the CaSR in common calcitropic diseases

Altered CaSR expression is implicated in the pathogenesis of common parathyroid disorders such as primary and secondary hyperparathyroidism. Moreover, *CASR* SNPs have been reported to be associated with hypercalciuria and nephrolithiasis.

Primary hyperparathyroidism

PHPT is associated with an increased set-point for Ca^{2+}_o-mediated PTH release, which indicates a role for altered CaSR function in the pathogenesis of this disorder. In support of this, germline heterozygous or homozygous loss-of-function *CASR* mutations have occasionally been reported in patients with adult-onset PHPT[118]. However, somatic *CASR* mutations have not been identified in parathyroid tumours from patients with sporadic PHPT[119-121]. Instead, the parathyroid set-point abnormalities associated with PHPT may be partly attributed to alterations in CaSR expression, which is reduced in

the majority of hyperplastic and adenomatous parathyroid tumours[122,123]. In keeping with this, *ex-vivo* studies have shown the set-point for Ca^{2+}_o-mediated PTH release to be inversely related to the amount of CaSR expression in parathyroid adenomas[124]. This reported decreased expression of CaSR in some parathyroid tumors may be associated with downregulation of filamin A[125], which is scaffolding protein that interacts with the CaSR in parathyroid cells[126], and it is reported that there are no alterations in *CASR* gene promoter methylation[127]. The mechanisms causing this reduced CaSR expression in parathyroid tumours remain to be fully elucidated. Common *CASR* polymorphisms have been reported to influence the clinical severity of PHPT. In particular, the Arg990Gly SNP, which is located in the CaSR cytoplasmic domain, has been reported to be associated with lower serum PTH concentrations, increased urinary excretion, and also increased occurrence of nephrocalcinosis in PHPT patients[128,129].

Chronic kidney disease and secondary hyperparathyroidism

Secondary hyperparathyroidism (SHPT) is a common complication of chronic kidney disease (CKD), and may arise early in the pathogenesis of this disorder[130]. Altered CaSR function is involved in the progression of SHPT in CKD patients[130,131]. Thus, hypocalcaemia, which occurs as a consequence of hyperphosphataemia and reduced $1,25(OH)_2D$ synthesis in CKD, inactivates the parathyroid CaSR, thereby increasing PTH secretion[130,131]. Prolonged PTH secretion in CKD is associated with increased parathyroid cell proliferation and gland hyperplasia, and studies in uraemic rats have shown that this is accompanied by reduced CaSR expression, which can be upregulated following calcimimetic treatment[132,133]. The reduction in CaSR expression is most marked in nodular parathyroid gland hyperplasia caused by advanced SHPT[130,134]. Furthermore, *ex-vivo* studies involving freshly excised parathyroid glands from haemodialysis patients with SHPT, have shown CaSR expression to inversely correlate with parathyroid gland weight and to be associated with an increase in the set-point for Ca^{2+}_o-mediated PTH release[135]. Studies in rats have shown that renal CaSR expression is also reduced in CKD and may contribute to the hypocalciuria associated with this disorder[136].

Idiopathic hypercalciuria and nephrolithiasis

Idiopathic hypercalciuria (IH) is associated with nephrolithiasis, and may be familial[137], although germline *CASR* mutations have not been identified in families with autosomal dominant inheritance of IH[138]. However, common SNPs located within the coding and regulatory regions of the *CASR* gene have been implicated in nephrolithiasis in patient-based studies. Thus, the common Arg990Gly *CASR* SNP has been reported in association with an increased risk of hypercalciuric nephrolithiasis[139], and a common *CASR* promoter-region SNP (rs6776158 (G>A)), which has been shown to impair *CASR* transcriptional activity *in vitro* and decrease CaSR expression within the renal medulla, is reported to be associated with an increased risk of Ca^{2+}-containing renal calculi[140]. The mechanisms underlying the development of nephrolithiasis in patients harbouring the rs6776158 *CASR* promoter-region SNP remain to be established as these patients did not have alterations in the urinary concentrations of calcium or phosphate[140].

Drugs for calcitropic diseases

CaSR allosteric modulators, which comprise calcimimetic and calcilytic compounds, are being used as a targeted therapy for parathyroid disorders and also for symptomatic hypercalcaemia and hypocalcaemia caused by germline loss- and gain-of-function CaSR mutations, respectively[52,141].

Calcimimetics

Calcimimetics are ligands that mimic the effects of Ca^{2+}_o at the CaSR, and are divided into two types: type I calcimimetics are agonists, and include naturally occurring ligands; and type II calcimimetics are PAMs that increase the sensitivity of the CaSR to Ca^{2+}_o[3,142]. Calcimimetic compounds, which decrease PTH secretion and parathyroid gland proliferation, are used for the treatment of hyperparathyroid disorders[143,144]. Cinacalcet, which is a phenylalkylamine compound that binds to the CaSR TMD, was the first calcimimetic to be licensed as a therapy for hyperparathyroid conditions such as secondary hyperparathyroidism due to end-stage renal failure; inoperable forms of primary hyperparathyroidism; and parathyroid carcinoma[6]. More recently, etelcalcetide, which is an intravenously administered synthetic polycationic peptide that acts as a type II calcimimetic and agonist of the CaSR[145], has been

approved for the management of secondary hyperparathyroidism in adult patients on haemodialysis[146]. Etelecalcetide has been shown to bind to the Cys482 residue within the CaSR VFT[147].

Calcimimetics also represent a targeted therapy for symptomatic forms of FHH[52], and have been shown to improve the signalling responses of cells expressing loss-of-function CaSR, $G\alpha_{11}$ or AP2σ mutant proteins *in vitro*[42,46,47,92,148], and to significantly lower serum calcium concentrations in patients with symptomatic hypercalcaemia caused by FHH types 1-3[42,48,149,150]. Cinacalcet has also been successfully used to manage life-threatening hypercalcaemia in NSHPT patients harbouring a heterozygous CaSR mutation, Arg185Gln[151], but is ineffective for NSHPT caused by biallelic deletional CaSR mutations[152].

Calcilytics

Calcilytics are synthetic NAMs that bind to the CaSR TMD[27], and comprise two main classes of compounds, which are the amino alcohols (e.g. NPS-2143, ronacaleret, NPSP795 and JTT-305/MK-5442) and quinazolinones (e.g. ATF936 and AXT914)[6]. These compounds were originally investigated as potential therapies for osteoporosis, as they induced a transient rise in PTH secretion, which had the potential to induce anabolic effects on bone mass[153]. However, clinical trials have shown that calcilytics such as ronacaleret and JTT-305/MK-5442 are not effective for treating postmenopausal osteoporosis[154,155], and these compounds have instead been investigated as a potential targeted therapy for ADH[52]. *In vitro* studies have shown the NPS-2143 calcilytic to rectify the increased signalling responses associated with gain-of-function CaSR and $G\alpha_{11}$ mutations, which cause ADH1 and ADH2, respectively[46,92,156,157]. However, NPS-2143 has limited efficacy for severe gain-of-function mutations, which cause Bartter syndrome type V[157]; whereas the quinazolinone-derived calcilytic drugs have successfully rectified constitutive activation caused by Bartter syndrome-associated CaSR mutations[158]. Calcilytics have also been assessed *in vivo*, and single dose studies have shown NPS-2143 to significantly increase plasma calcium and PTH concentrations in mouse models for ADH1 and ADH2[48,156,159]. Moreover, longer-term studies involving ADH1 mouse models have shown the JTT-305/MK-5442 calcilytic to prevent the development of nephrocalcinosis, which was observed in mice treated with the drug vehicle or recombinant PTH[159]. Intravenous administration of the NPSP795

calcilytic compound in a phase IIa clinical trial involving five ADH1 patients, significantly increased plasma PTH concentrations and reduced urinary calcium excretion[160].

Non-calcitropic roles of the CaSR

The CaSR is widely expressed in tissues not involved in Ca^{2+}_o homeostasis, thereby indicating that it likely has non-calcitropic roles. These non-calcitropic roles of the CaSR involve regulation of physiological and pathophysiological processes in a tissue-dependent manner, and these include: proliferation, differentiation and apoptosis; vascular tone; renal and intestinal water transport; inflammation; maintenance of the integrity of epithelial layers in the intestine and skin; neuronal development and function; and entero-endocrine hormone secretion (TABLE 1)[7,9-11,13,14,161,162]. Thus, abnormal CaSR function or expression in non-calcitropic organs may be associated with cancer, cardiovascular diseases, asthma, gastro-intestinal disorders, Alzheimer's disease, pancreatic insulin secretion and poor wound healing (FIG. 4)[163,164]. Some of these physiological and pathophysiological non-calcitropic roles of the CaSR are reviewed briefly, below.

Tumourigenesis

The CaSR regulates cell fate and its expression has been reported to be increased or decreased in different cancers, thereby suggesting that the CaSR may have possible roles as an oncogene or tumour suppressor in different organs. For example, increased CaSR expression is found in metastatic breast and prostate cancers[16,165,166], whereas loss of CaSR expression is found in colorectal cancer and some neuroblastic tumours[16,167]. In breast cancer cells, the CaSR has been reported to act as an oncogene and increase proliferation and inhibit apoptosis[168] by likely switching G-protein coupling from $G\alpha_i$, to $G\alpha_s$, which leads to an upregulation of PTHrP expression[169]. This increase in PTHrP results in the inhibition of the apoptosis inducing factor (AIF) and cell cycle inhibitor $p27^{kip1}$, thereby reducing apoptosis and promoting cell proliferation, respectively[168]. Moreover, *in vitro* studies involving the stimulation of malignant breast carcinoma cells with Ca^{2+}_o or the R-568 calcimimetic have shown the CaSR to increase the migratory potential of these tumour cells, and also promote the secretion of pro-angiogenic and chemotactic factors that are implicated in breast cancer metastases[170,171]. Furthermore, metastatic breast

cancer cells in bone that express the CaSR may exacerbate PTHrP-mediated osteolysis by sensing high concentrations of Ca^{2+} released during bone remodelling[172], and thereby promoting further PTHrP secretion. Thus, the CaSR has been hypothesised to drive a vicious cycle of skeletal metastasis[173]. In support of this, PTHrP secretion from breast cancer cells has been shown to be stimulated by high (7.5-10 mM) Ca^{2+}_o concentrations[173], which are present in the bone microenvironment during remodelling[172], and breast cancer cells overexpressing the CaSR have been demonstrated to cause an increase in osteolytic lesions when injected intratibially into Balb/c-Nude mice[174]. Thus, these findings indicate that the CaSR plays a role in the pathogenesis of breast cancer skeletal metastases.

The CaSR is involved in the differentiation of neural progenitor cells and has a tumour suppressor role in malignancies affecting the developing nervous system. Thus, the CaSR is expressed in neuroblastic tumours, which comprise neuroblastomas, ganlioneuroblastomas and ganglioneuromas, and its expression in these tumours is associated with favourable prognostic outcomes such as low clinical stage and age at diagnosis <1 year[175]. In contrast, in highly malignant and undifferentiated neuroblastomas, the CaSR has been shown to be epigenetically silenced, and overexpression of the CaSR or treatment with cinacalcet in neuroblastoma cell lines reduces tumourigenicity by decreasing proliferation and increasing apoptosis[167,176].

The CaSR may also mediate the effects of dietary Ca^{2+} on the development of prostate and colorectal cancer. Thus, a high dietary intake of Ca^{2+} is reported to enhance prostate tumourigenesis by potentially acting on the CaSR and the TRPC6 channel, which is a Ca^{2+}-permeable channel[177]. In contrast, dietary calcium is reported to be protective against colorectal cancer, and this effect is likely mediated by the CaSR as it was observed only in patients with CaSR-expressing tumours[178]. In the colon, the CaSR has tumour suppressor roles and been shown to: inhibit proliferation, induce apoptosis and differentiation; prevent epithelial-to-mesenchymal transition[179-182]; and suppress canonical Wnt signalling whilst activating the non-canonical Wnt pathway[183]. Furthermore, CaSR expression has been demonstrated to be lost during colorectal tumourigenesis, mainly through epigenetic mechanisms[184-186].

Nervous system

The CaSR is widely expressed in the central and peripheral nervous system including in: nerve terminals and fibre tracts; myelin-producing oligodendrocytes; astrocytes and microglial cells[187,188]. Moreover, the CaSR is considered to play a role in the development of the hippocampus, granule cell layer of the cerebellum, sympathetic nervous system, and also in GnRH neuronal migration[188-191]. The CaSR has been shown to modulate the growth of sympathetic axons as well as promoting dendritic growth within the hippocampus[191]. The CaSR may also regulate neuronal excitability within the hippocampus by regulating neuronal potassium (K^+) channels and the sodium (Na^+) leak channel non-selective protein (NALCN)[12]. Furthermore, the CaSR may facilitate neuronal migration by sensing alterations in Ca^{2+}_o or polycations such as spermine, which are present within the brain, and also by enhancing the production of chemokines such as monocyte chemoattractant protein-1[189,190]. The CaSR is also expressed in the sub-fornical organ (SFO), which is involved in Na^+ sensing and water intake[192], and the SFO-expressed CaSR may potentially influence body fluid composition[12].

Abnormal CaSR function has been implicated in central nervous system disorders such as Alzheimer's disease (AD), ischaemic brain injury and epilepsy. Indeed, a patient-based study showed the presence of a polymorphic dinucleotide repeat within intron 4 of the CASR to be significantly associated with the occurrence of AD[193]. Moreover, *in-vitro* studies have shown that: CaSR function can be upregulated by proteins such as the amyloid-β 1-42 peptide fragment (Aβ(1-42)) and apolipoprotein E, which are involved in the pathogenesis of AD[193]; and that the calcilytic drug NPS 2143 inhibits the secretion of Aβ(1-42) from human astrocytes and cortical neurons *in vitro*[194]. The neuronal CaSR forms heteromeric complexes with the $GABA_B$ receptor 1 (GBR1), and mouse model studies have demonstrated in ischaemic brain injury that the stoichiometry of these complexes is altered with upregulation of CaSR expression and down regulation of GBR1, which potentiates ischaemic neuronal death[195]. These studies also revealed that calcilytic treatment in mice with brain injury was neuroprotective and improved learning and memory retention[45]. CaSR mutations have been reported in patients with epilepsy[196], and *in vitro* studies of a CaSR Arg898Gln mutation, which co-segregated with

epilepsy in one family, in which the proband was normocalcaemic[196], revealed that this is a gain-of-function mutation that increased CaSR expression[197].

Cardiovascular

The CaSR is expressed in arterial vessels and influences cardiovascular function through direct effects within the vasculature. Thus, the CaSR is expressed in human vascular smooth muscle cells (VSMCs) and endothelial cells[198,199], and it has been reported to likely play a role in VSMC proliferation[200], and also in the regulation of blood pressure and blood vessel tone[13]. Indeed, ablation of the VSMC CaSR in mice has been shown to cause endothelium-independent decreases in the contractility of the aorta and mesenteric arteries, and also to decreases in diastolic and mean arterial blood pressures[13]. Furthermore, in rabbit mesenteric arteries, CaSR stimulation induced endothelium-dependent vasorelaxation through two different pathways; one involving the production of nitric oxide (NO), and the other involving activation of the intermediate Ca^{2+}-activated potassium (IKCa) channels[201]. The VSMC CaSR also protects against the development of vascular calcification, and histological studies of lower-limb arteries from CKD patients have demonstrated reduced expression of the CaSR within the calcified medial arterial layer[44]. These findings are supported by studies of primary VSMCs, in which loss of CaSR expression or overexpression of a loss-of-function mutant CaSR in these cells leads to increased mineralisation[44]. Furthermore, the CaSR represents a potential therapeutic target in patients with vascular calcification, and a randomised controlled trial involving CKD patients on haemodialysis showed that cinacalcet treatment resulted in beneficial effects on arterial calcification, and particularly for calcification involving the cardiac valves[202].

The CaSR is also expressed in the heart and has been shown to be functionally active in rodent ventricular cardiomyocytes, where it promotes apoptosis[203] and may also protect against myocardial ischaemic injury[204]. Moreover, patient-based studies have demonstrated that a common CaSR SNP, Ala986Ser, is associated with an increased risk of cardiovascular diseases such as myocardial infarction[15,205], and it remains to be established whether this is a direct consequence of abnormal CaSR function within the heart or vasculature, or due to an indirect effect of the polymorphic CaSR on Ca^{2+}_o homeostasis[15].

Pulmonary development and function

The CaSR has been reported to be to be expressed in human fetal lungs, where it promotes fluid secretion into the pulmonary lumen, which mediates lung growth and development[206], and studies in mice have also indicated a role for the CaSR in regulating lung branching morphogenesis[207]. The CaSR is also expressed in adult human and mouse airway smooth muscle and bronchial epithelium[14], and CaSR expression has been demonstrated to be increased in the airways of asthmatic patients and mice[14]. Moreover, over-expression of CaSR is considered to be activated by polycations in the asthmatic airway mucosa, thereby leading to bronchoconstriction[14]. Calcilytic treatment was shown to diminish signalling responses that caused airway contractility, and nebulised calcilytic administration significantly suppressed airway hyper-reactivity and inflammation in a mouse model of allergic asthma[14]. Thus, these observations highlight the potential of inhaled calcilytics as a treatment for asthma[14]. Pathological upregulation of CaSR expression has also been implicated in idiopathic pulmonary artery hypertension, and calcilytic treatment in rodents has been demonstrated to prevent the development of this disorder and ameliorate the secondary occurrence of right ventricular hypertrophy[208].

Gastro-intestinal tract

The CaSR, which is expressed in the gastro-intestinal (GI) tract of amphibia, birds, fish and mammals[209], has been reported to act as a nutrient sensor that influences gastric acid and entero-endocrine hormone secretion, as well as regulating intestinal fluid homeostasis, and intestinal barrier function and inflammation (Table 1)[10,209-211].

Nutrient sensing and entero-endocrine hormone secretion: The CaSR senses nutrients such as Ca^{2+} and aromatic amino acids within the GI lumen and responds to alterations in these nutrients by regulating hormone secretion from entero-endocrine cells, which are located throughout the GI tract and include: gastrin-secreting G-cells; ghrelin-secreting gastric cells; cholecystokinin (CCK)-secreting I-cells; and glucagon-like peptide-1 (GLP-1) and peptide YY (PYY)-secreting L-cells[212-214]. The CaSR is expressed in gastric G-cells, and studies involving CaSR null mice have shown the CaSR to mediate Ca^{2+}- and

aromatic amino acid-induced gastrin secretion[215]. Gastrin promotes histamine release from gastric body enterochromaffin-like (ECL) cells, which in turn enhances acid secretion from gastric parietal cells[215]. The CaSR is also expressed in gastric parietal cells and human studies have shown this GPCR to increase gastric acid secretion by influencing H^+K^+ATPase activity[216,217].

Activation of the CaSR in the small intestine has also been found to improve post-prandial glucose tolerance in wild-type rats[218]. This effect of the GI-expressed CaSR on glucose tolerance may be mediated by a reduction in gastric emptying rate[218], as well as through effects on GLP-1, which plays a key role in enhancing glucose-dependent insulin release[219]. Indeed, *ex-vivo* studies involving mouse intestine have shown that the CaSR is expressed in GLP-1-secreting L-cells and also that oligopeptides enhance GLP-1 secretion by activation of the CaSR[219].

The GI-expressed CaSR also reduces food intake by modulating the secretion of entero-endocrine hormones[210]. Thus, the CaSR has been shown to inhibit the release of ghrelin and increase the secretion of PYY, which are neuropeptides involved in the hypothalamic regulation of appetite[210]. The CaSR may also exert these anorectic effects by increasing the secretion of GLP-1[210], which in turn suppresses appetite following food ingestion, as well as by increasing CCK secretion from I-cells[220], that leads to a delay in gastric emptying. CaSR activation may also cause emesis and studies involving mice have demonstrated that deoxynivalenol, which is an emesis-inducing toxin, acts by increasing CaSR-mediated secretion of CCK and PYY[221]. These findings may potentially explain the GI adverse effects such as nausea and vomiting, which are experienced by >30% of patients being treated with cinacalcet, which is used for pharmacological activation of the CaSR [146]. Moreover, cinacalcet has been shown to impair gastric emptying in rats[222], whereas, evocalcet, which is a recently reported orally active pyrrolidine-derived calcimimetic compound, does not alter gastric emptying in rats, and has also been shown to cause reduced emesis compared to cinacalcet in studies involving marmosets[222].

Intestinal fluid secretion: Activation of the CaSR within colon epithelial cells has been shown in rodent studies to inhibit fluid secretion induced by secretagogues such as cholera toxin[223]. Cholera toxin causes secretory diarrhoea by increasing cyclic nucleotide generation within the colonic epithelium and also by stimulating the enteric nervous system (ENS) to release secretagogues such as vasoactive peptide[224].

The colonic CaSR has been shown to counteract these effects by activating phosphodiesterases, which degrade the cyclic nucleotides generated by cholera toxin, and also by inhibiting the ENS[223,224]. These findings reveal the CaSR to be a potential therapeutic target for secretory diarrhoea caused by bacterial toxins[223].

Intestinal barrier function and inflammation: Alterations in intestinal epithelial barrier function are implicated in the pathogenesis of inflammatory bowel disorders such as Crohn's disease[225]. The CaSR is expressed in colonic epithelial cells as well as in colonic myofibroblasts, which are located at the basal surface of the epithelium and regulate epithelial barrier function[226,227]. Cellular studies involving colonic myofibroblasts have shown the CaSR to increase secretion of bone morphogenetic protein-2 (BMP-2), which promotes colonic epithelial barrier maturation[227]. The CaSR also inhibits tumour necrosis factor alpha (TNFα) secretion from colonic myofibroblasts[228], and thus this GPCR may protect against intestinal inflammation. The epithelial CaSR also plays a key role in intestinal barrier function, and studies of intestinal epithelium-specific CaSR null mice have shown reduced transepithelial resistance in association with reduced colonic expression of tight junction proteins such as claudin-2[229]. This defect in epithelial barrier function was associated with an altered composition of the intestinal microbiome[229] that comprised a decrease in the amount of beneficial lactobacilli, but an increase in Deferribacteraceae bacteria, which are linked to colitis[229]. This dysbiosis of the intestinal microbiota has been associated with pro-inflammatory responses in intestinal epithelium-specific CaSR null mice[230]. Indeed, in response to chemically induced colitis, these mice had more severe colitis with delayed recovery, when compared with the CaSR-expressing littermate controls[229].

Pancreatic islets and glucose homeostasis

The CaSR is highly expressed in pancreatic islet α- and β-cells [2,231], and studies of isolated human islets and insulin-secreting cell lines have shown that CaSR activation leads to the upregulation of PLC and MAPK-mediated signalling responses in association with a transient rise in insulin and glucagon secretion[231-233]. Furthermore, studies involving an ADH1 mouse model, known as *Nuclear flecks* (*Nuf*), have shown that CaSR activation in heterozygous ($Casr^{+/Nuf}$) and homozygous ($Casr^{Nuf/Nuf}$) mice is

associated with impaired glucose tolerance, when compared to wild-type (*Casr*$^{+/+}$) mice. This impaired glucose tolerance was ameliorated by calcilytic treatment[7]. *Casr*$^{+/Nuf}$ and *Casr*$^{Nuf/Nuf}$ mice also had hypoinsulinaemia and reduced pancreatic islet mass, which was associated with reduced β-cell proliferation[7]. In addition, *Casr*$^{Nuf/Nuf}$ mice had a lack of glucose-mediated suppression of glucagon secretion, which was associated with altered α-cell membrane depolarization[7]. These *in vivo* and *ex vivo* studies have highlighted roles for the CaSR in the regulation of pancreatic islet mass, and in α- and β-cell function.

Skin

Extracellular Ca^{2+} is required for the maintenance of an intact epidermal barrier, and *in vitro* studies have shown that Ca^{2+}_o plays a key role in keratinocyte differentiation[234]. Furthermore, Ca^{2+}_o mediates wound healing and skin re-epithelialisation following injury[235]. This effect is triggered by the epidermal CaSR, which upon activation promotes keratinocyte differentiation, survival, and adhesion[236]. In keeping with this, the barrier function of the skin in epidermis-specific CaSR null mice has been shown to be defective with impaired keratinocyte differentiation[237]. Moreover, mice with combined ablation of the CaSR and vitamin D receptor in keratinocytes were demonstrated to have delayed wound re-epithelialisation as a consequence of impaired keratinocyte adhesion and migration[11]. These findings reveal the importance of Ca^{2+} and vitamin D signalling for epidermal regeneration after injury[11], analogous to the interactions between Ca^{2+} and vitamin D that have been reported for intestine function and colorectal tumourigenesis[238].

Conclusions and future directions

The CaSR is a dimeric family C GPCR that signals via the G-proteins and beta-arrestin, and plays a pivotal role in bone and mineral metabolism by influencing parathyroid hormone secretion, urinary Ca^{2+} excretion, skeletal development, and lactation. Germline loss- and gain-of-function mutations of the CaSR or its intracellular partner proteins lead to inherited calcitropic disorders such as FHH types 1-3 and ADH types 1-2. Loss of parathyroid CaSR expression contributes to the development of primary

and secondary hyperparathyroidism. The CaSR is also widely expressed in non-calcitropic tissues where it influence physiological processes such as nutrient sensing and the secretion of insulin and entero-endocrine hormones, neuronal and pulmonary development, vascular tone and wound healing. Moreover, pathophysiological alterations in CaSR expression or function are associated with cancers of the breast, prostate and colon, as well as with ischaemic brain injury, cardiovascular disease and asthma. The CaSR represents a pharmacological target for calcitropic disorders, and calcimimetic drugs are established as a medical therapy for hyperparathyroid disorders, and shown to be effective for symptomatic forms of FHH. Calcilytic drugs may represent a targeted therapy for ADH and have potential for other hypoparathyroid disorders. CaSR-targeted drugs are also being evaluated for non-calcitropic disorders, and novel strategies such as the use of inhaled calcilytics for asthma to minimize off-target effects, are being evaluated in pre-clinical models. However, a key challenge for the pharmaceutical industry is to develop compounds that can selectively influence biased signalling responses by the CaSR, thereby providing an approach for modulating CaSR function in a tissue- and disease-specific manner. The emergence of such therapies will aid the clinical management of non-calcitropic disorders without leading to off-target effects in calcitropic tissues.

Figure legends

Figure 1. Role of the CaSR in Ca^{2+}_o homeostasis.

A. The CaSR is highly expressed in the parathyroid glands (grey), which are located adjacent and posterior to the thyroid gland (pink). The parathyroid CaSR detects reductions in Ca^{2+}_o, which leads to the release of PTH. PTH acts on the PTH1 receptor (PTH1R) to increase resorption of Ca^{2+} from bone, promote urinary Ca^{2+} reabsorption, and enhance expression of the renal 1-α-hydroxylase (1αOHase) enzyme, which converts the 25-hydroxyvitamin D (25D) precursor metabolite to biologically active 1,25-dihydroxyvitamin D (1,25D). The elevated 1,25D increases absorption of dietary calcium by acting on the intestinal vitamin D receptor (VDR)[3]. The kidney CaSR acts independently of PTH to regulate urinary Ca^{2+} reabsorption[60,61]. Increases in Ca^{2+}_o and 1,25D concentrations lead to negative feedback on the parathyroid glands, thereby inhibiting further PTH release. B. Nephron segment-

specific roles of the CaSR. The CaSR is expressed in the: apical membrane of the proximal tubule (PT), where it regulates 1,25D synthesis and phosphate (Pi) excretion; basolateral membrane of the cortical thick ascending limb (TAL) of the Loop of Henle, and apical and basolateral membranes of the distal convoluted tubule (DCT), where it regulates Ca^{2+} reabsorption; apical and basolateral membranes of the collecting duct (CD), where it regulates H^+ and water excretion; and juxtaglomerular apparatus (JGA), where it regulates renin secretion[58,64]. (+), stimulatory action of CaSR; (-), inhibitory action of CaSR. C. During lactation, the mammary gland CaSR detects reductions in Ca^{2+}_o, which leads to increased PTHrP secretion from mammary epithelial cells into the circulation[9]. PTHrP acts on the PTH1R to increase bone resorption, which in turn releases Ca^{2+}_o for milk production[9]. Stimulatory and inhibitory actions are indicated by solid lines and dashed lines, respectively.

Figure 2. CaSR signalling and trafficking.

The CaSR is functionally active as a constitutive homodimer, and may also form heterodimers with other family C GPCRs such as the metabotropic glutamate receptors and gamma-amino butryric acid (GABA) type B receptors in growth plate chondrocytes and the central nervous system[18-20]. The CaSR ECD binds calcium (Ca^{2+}) at multiple sites within the lobes of the venus flytrap (VFT) and cysteine-rich domain (CRD). The CaSR also binds amino acids within the VFT cleft. Synthetic positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) bind to the CaSR transmembrane domain (TMD). The binding of Ca^{2+} leads to $G_{q/11}$-dependent activation of phospholipase C (PLC) and the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) from membrane bound phosphatidylinositol 4,5-bisphosphate (PIP_2). The increase in intracellular IP_3 levels facilitates the release of Ca^{2+} from intracellular stores such as the endoplasmic reticulum (ER). DAG activates protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway. The CaSR also activates the $G_{i/o}$ protein, which leads to inhibition of adenylate cyclase (AC)-mediated production of cAMP. These signalling events cause a decrease in parathyroid hormone (PTH) secretion and reduction in renal tubular Ca^{2+} reabsorption. CaSR cell-surface expression is regulated by agonist-driven insertional signalling (ADIS), which mediates anterograde receptor trafficking[39]; and also by an endocytic complex comprising clathrin, β-arrestin and the heterotetrameric adaptor-related protein complex 2 (AP2)

complex, which mediates retrograde receptor trafficking[40]. Loss- and gain-of function mutations of the CaSR lead to FHH1 and ADH1, respectively; loss- and gain-of function mutations of the $G\alpha_{11}$ subunit are associated with FHH2 and ADH2, respectively; and loss-of-function mutations of the AP2σ subunit are associated with FHH3.

Figure 3. Disruption of a salt-bridge within the CaSR TMD causes biased signalling.

The salt-bridge is formed by the Arg680 residue located in the proximal portion of transmembrane helix 3 (TM3, shaded blue) and the Glu767 residue located in extracellular loop 2 (ECL2). The Arg680-Glu767 salt-bridge is situated at the entrance of the allosteric modulator binding pocket, which is formed by residues from TM3 and TM5-TM7[27]. The Arg680 residue mediates the binding of the NPS 2143 calcilytic compound[27]. The Arg680-Glu767 salt-bridge is associated with G-protein-mediated signalling, whereas disruption of the salt-bridge by the ADH-causing CaSR mutation, Arg680Gly, selectively increases β-arrestin-mediated signalling, as well as abrogating the inhibitory effect of the NPS 2143 compound[51].

Figure 4. Physiological roles and disease associations of the CaSR.

The CaSR has key physiological roles in calcitropic tissues (e.g. parathyroid glands, kidneys and bone) and in non-calcitropic tissues (e.g. brain, cardiovascular system, lungs, breast, intestine and pancreas, and skin). Altered CaSR activity in calcitropic tissues causes inherited and sporadic diseases of Ca^{2+} homeostasis[3], whereas alterations in CaSR function or expression in non-calcitropic tissues is associated with cardiovascular disease, asthma and malignancy. ADH, autosomal dominant hypocalcaemia; FHH, familial hypocalciuric hypercalcaemia; PHPT, primary hyperparathyroidism; SHPT, secondary hyperparathyroidism.

Key points

- The CaSR is a family C G-protein coupled receptor that is expressed on the cell surface as a dimer, and signals via G-proteins and beta-arrestin
- The CaSR regulates bone and mineral metabolism by influencing parathyroid hormone secretion, urinary Ca^{2+} excretion, skeletal development, and lactation
- Germline *CASR, GNA11* and *AP2S1* mutations cause calcitropic disorders such as familial hypocalciuric hypercalcaemia (FHH) and/or autosomal dominant hypocalcaemia (ADH)
- In non-calcitropic tissues, the CaSR influences biological processes that include: gastro-intestinal nutrient sensing; secretion of insulin and entero-endocrine hormones; vascular tone; and wound healing
- Abnormal expression or function of the CaSR is associated with: primary and secondary hyperparathyroidism; ischaemic brain injury; cardiovascular disease; asthma; and cancers of the breast, prostate and colon
- CaSR-targeted calcimimetic and calcilytic drugs have therapeutic potential for calcitropic and non-calcitropic diseases

Table 1. Major calcitropic and non-calcitropic cellular roles of the CaSR.

Process	Cell type	Role	Reference
Calcitropic			
	Parathyroid	PTH secretion (-)	1,56
		Proliferation (-)	1,133
	Kidney		
	Proximal tubule	Phosphate excretion (-)	65
		$1,25(OH)_2D$ synthesis (-)	62
	TAL	Ca^{2+} reabsorption (-)	60,61
	Distal convoluted tubule	Ca^{2+} reabsorption (+)	68
	Collecting duct	H+ excretion (+)	69
		Water excretion (+)	69
	Juxtaglomerular apparatus	Renin secretion (-)	70
	Bone		
	Osteoblast	Differentiation and mineralization (+)	55,71
	Growth plate chondrocyte	Differentiation (+)	55
	Breast epithelium	Ca^{2+} transport into milk (+)	9,76
		PTHrP production (-)	9,75
Non-calcitropic			
	Cardiovascular		
	Vascular smooth muscle cell	Proliferation (+)	200
		Arterial contractility (+)	13
	Endothelial cell	Nitric oxide production (+)	201
	Ventricular cardiomyocyte	Apoptosis (+)	203
	Foetal lung epithelium	Fluid secretion (+)	206,207
		Proliferation and lung branching morphogenesis (-)	207
	Gastrointestinal		
	Gastric parietal cell	H^+ excretion (+)	216,217
	Gastric mucosal cell	Ghrelin secretion (-)	210,212
	Gastric G cell	Gastrin secretion (+)	215
	Duodenal I cell	Cholecystokinin secretion (+)	220
	Intestinal L cell	Glucagon-like peptide-1 (+)	210
		Peptide YY (+)	210,221
	Colon	Barrier function (+)	229
		Enteric nervous system (-)	224
		Fluid secretion (-)	223,224
		Proliferation (-)	10
	Pancreatic islet		
	α-cell	Glucagon secretion (+)	7,232
	β-cell	Insulin secretion (+)	232,233
	Adipocyte	Proliferation (+)	162
		Differentiation (+)	162
		Inflammatory cytokine expression (+)	162
	Skin keratinocyte	Proliferation (-)	236
		Differentiation (+)	236
		Apoptosis (-)	236
	Central nervous system		
	Cerebellar granule cell	Differentiation and migration (+)	190
	Oligodendrocyte	Differentiation and myelinogenesis (+)	8
	GNRH neuron	Chemotaxis (+)	12
	Sub-fornical organ	Neuronal excitability and blood pressure regulation (+)	12

GNRH, gonadotrophin-releasing hormone; PTH, parathyroid hormone: PTHrP, parathyroid hormone related protein; TAL, thick ascending limb of Loop of Henle; (+), stimulatory action of CaSR; (-), inhibitory action of CaSR.

Table 2. Calcitropic disorders associated with CaSR, $G\alpha_{11}$ and AP2σ mutations or autoantibodies.

Disorder	OMIM[a]	Inheritance	Gene[b]	Chromosomal localisation	Clinical features
Hypercalcaemic disorders					
Genetic causes					
Familial hypocalciuric hypercalcaemia type 1 (FHH1)	145980	Autosomal dominant	*CASR*	3q21.1	Asymptomatic in majority of patients
Familial hypocalciuric hypercalcaemia type 2 (FHH2)	145981	Autosomal dominant	*GNA11*	19p13.3	Asymptomatic in majority of patients
Familial hypocalciuric hypercalcaemia type 3 (FHH3)	600740	Autosomal dominant	*AP2S1*	19q13.3	Hypercalcaemic symptoms in >20% of patients Low bone mineral density in >50% of patients Childhood cognitive deficits in >75% of patients
Neonatal severe hyperparathyroidism (NSHPT)	239200	Autosomal recessive or dominant	*CASR*	3q21.1	Severe hypercalcaemia Hyperparathyroid bone disease
Adult-onset primary hyperparathyroidism (PHPT)	-	Autosomal recessive or dominant	*CASR*	3q21.1	Nephrolithiasis in >40% of patients Low bone mineral density in >25% of patients
Acquired causes due to CaSR autoantibodies					
Autoimmune hypocalciuric hypercalcaemia (AHH)	-	-	-	-	Asymptomatic or symptomatic hypercalcaemia, which may be responsive to glucocorticoids
Hypocalcaemic disorders					
Genetic causes					
Autosomal dominant hypocalcaemia type 1 (ADH1)	601198	Autosomal dominant	*CASR*	3q21.1	Hypocalcaemic symptoms in ~50% of patients Ectopic calcifications in ~35% of patients
Autosomal dominant hypocalcaemia type 2 (ADH2)	615361	Autosomal dominant	*GNA11*	19p13.3	Hypocalcaemic symptoms in >75% of patients Short stature reported in two kindreds
Bartter syndrome type V	601198	Autosomal dominant	*CASR*	3q21.1	Renal salt wasting and hypokalaemia Hypocalcaemic symptoms in >75% of patients
Acquired causes due to CaSR autoantibodies					
Autoimmune hypoparathyroidism	-	-	-	-	Asymptomatic or symptomatic hypocalcaemia

[a]Online Mendelian Inheritance in Man. [b]*CASR* encodes the CaSR; *GNA11* encodes $G\alpha_{11}$; and *AP2S1* encodes AP2σ.
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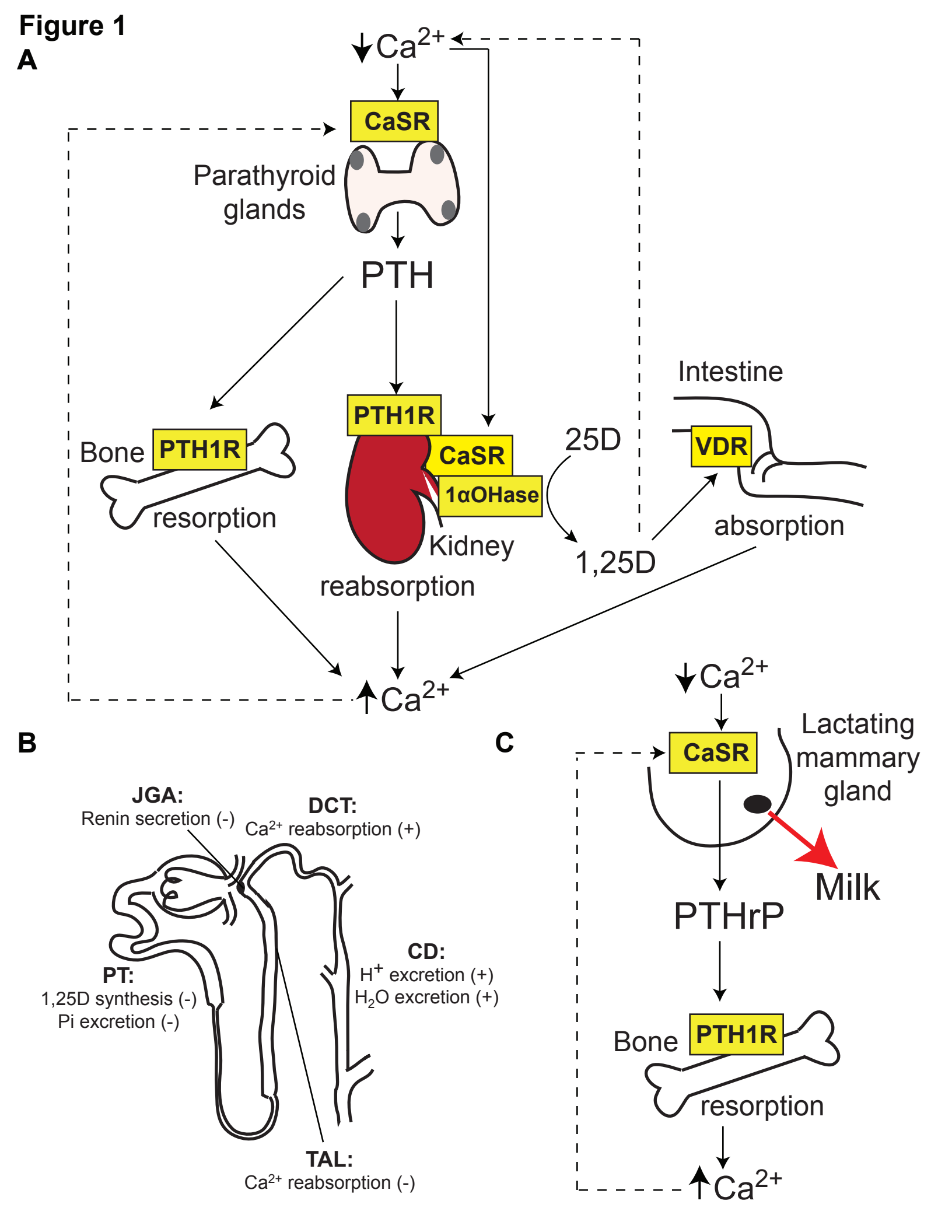
Figure 1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Ca2+
CaSR
Parathyroid glands
PTH
PTH1R
Bone
PTH1R
resorption
CaSR
1αOHase
Kidney
reabsorption
25D
1,25D
Intestine
VDR
absorption
Ca2+
B
JGA:
Renin secretion (-)
DCT:
Ca2+ reabsorption (+)
CD:
H+ excretion (+)
H2O excretion (+)
PT:
1,25D synthesis (-)
Pi excretion (-)
TAL:
Ca2+ reabsorption (-)
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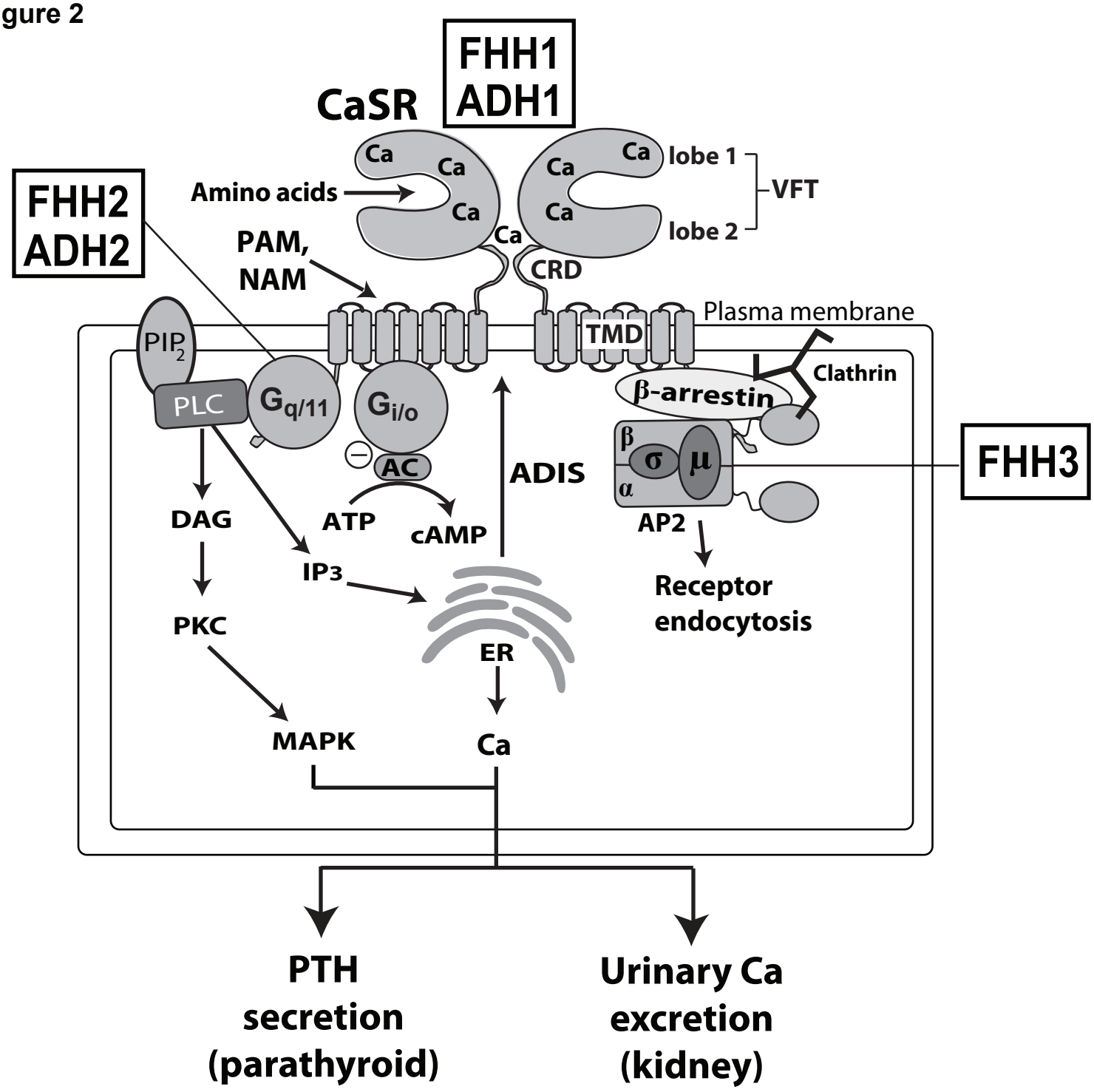
CaSR
Lactating mammary gland
Milk
PTHrP
Bone
PTH1R
resorption
Ca2+

Figure 2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Ca
Ca
Ca
lobe 1
lobe 2
VFT
FHH2
ADH2
Amino acids
PAM,
NAM
CRD
Plasma membrane
TMD
PIP2
PLC
Gq/11
Gi/o
AC
ADIS
β-arrestin
Clathrin
β
σ
μ
α
FHH3
AP2
DAG
ATP
cAMP
IP3
Receptor
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PKC
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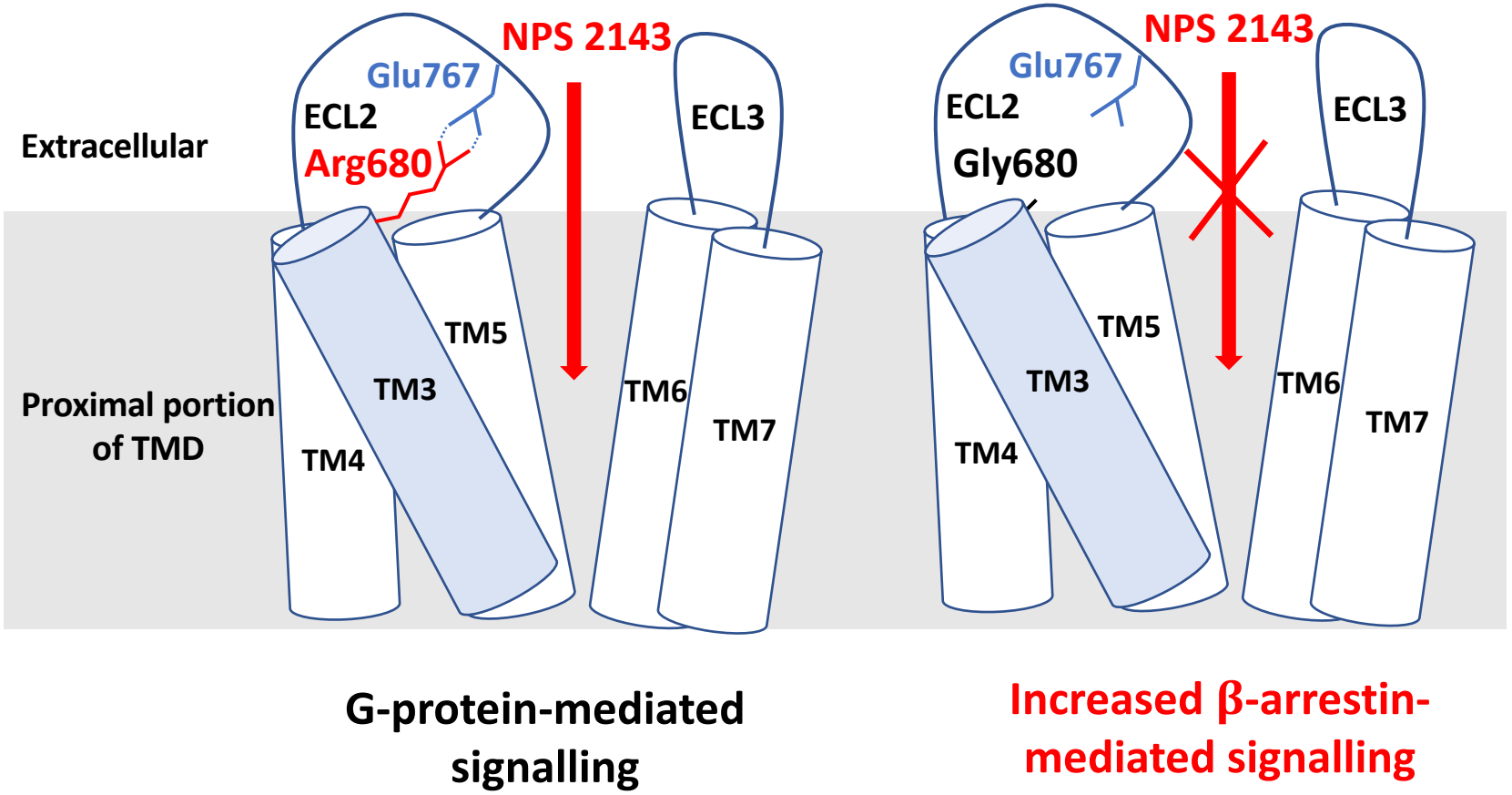
MAPK
Ca
PTH
secretion
(parathyroid)
Urinary Ca
excretion
(kidney)

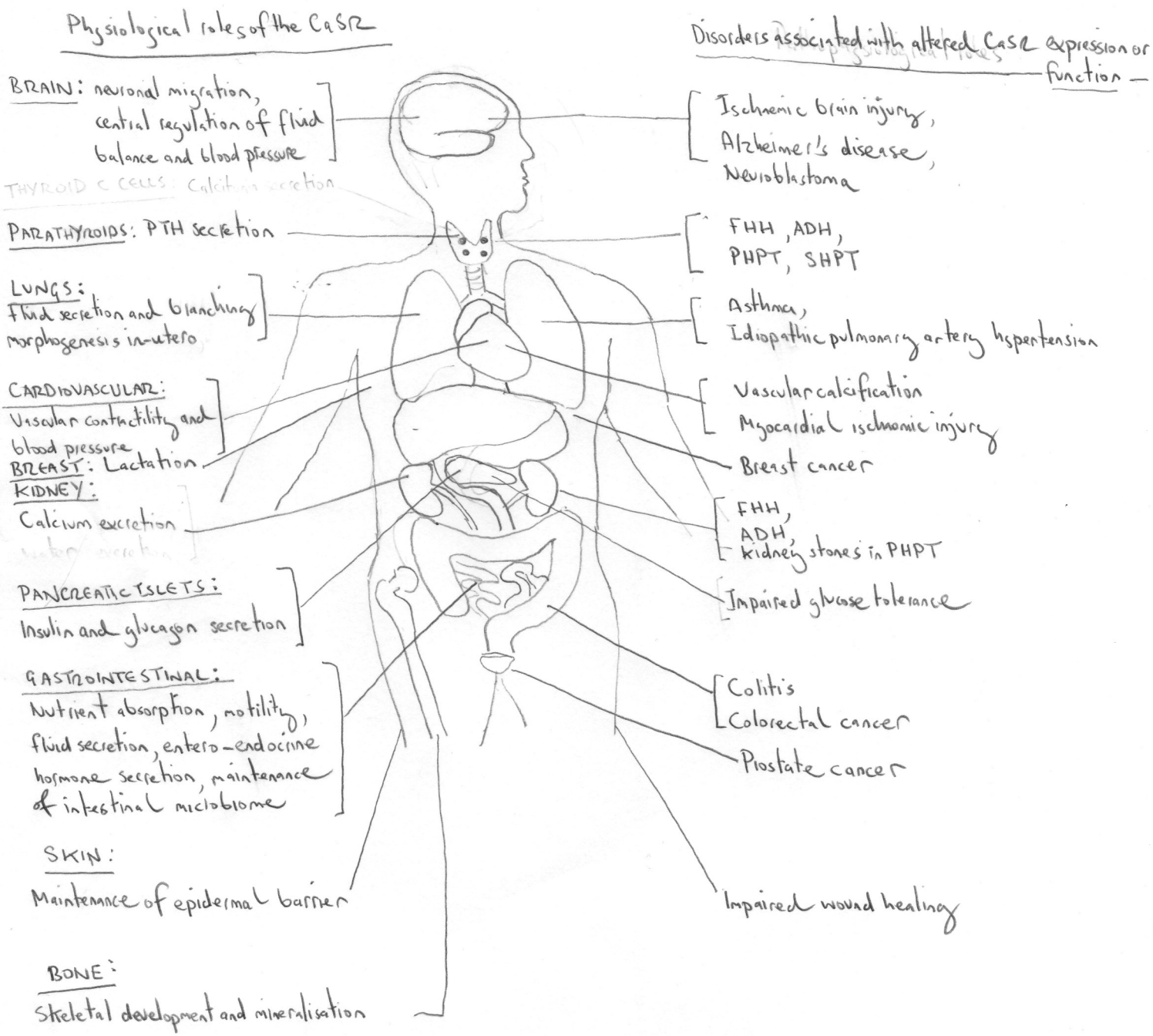
Figure 3



G-protein-mediated signalling

Increased β-arrestin-mediated signalling

Figure 4


Physiological roles of the CaSR
BRAIN: neuronal migration, central regulation of fluid balance and blood pressure
PARATHYROIDS: PTH secretion
LUNGS: fluid secretion and branching morphogenesis in-utero
CARDIOVASCULAR: Vascular contractility and blood pressure
BREAST: Lactation
KIDNEY: Calcium excretion
PANCREATIC ISLETS: Insulin and glucagon secretion
GASTROINTESTINAL: Nutrient absorption, motility, fluid secretion, entero-endocrine hormone secretion, maintenance of intestinal microbiome
SKIN: Maintenance of epidermal barrier
BONE: Skeletal development and mineralisation
Disorders associated with altered CaSR expression or function
Ischaemic brain injury, Alzheimer's disease, Neuroblastoma
FHH, ADH, PHPT, SHPT
Asthma, Idiopathic pulmonary artery hypertension
Vascular calcification
Myocardial ischaemic injury
Breast cancer
FHH, ADH, kidney stones in PHPT
Impaired glucose tolerance
Colitis
Colorectal cancer
Prostate cancer
Impaired wound healing