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The calcium sensing receptor: from calcium sensing to signaling

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The Ca^{2+}-sensing receptor (the CaSR), a G-protein-coupled receptor, regulates Ca^{2+} homeostasis in the body by monitoring extracellular levels of Ca^{2+} ($[Ca^{2+}]_o$) and responding to a diverse array of stimuli. Mutations in the Ca^{2+}-sensing receptor result in hypercalcemic or hypocalcemic disorders, such as familial hypocalciuric hypercalcemia, neonatal severe primary hyperparathyroidism, and autosomal dominant hypocalcemic hypercalciuria. Compelling evidence suggests that the CaSR plays multiple roles extending well beyond not only regulating the level of extracellular Ca^{2+} in the human body, but also controlling a diverse range of biological processes. In this review, we focus on the structural biology of the CaSR, the ligand interaction sites as well as their relevance to the disease associated mutations. This systematic summary will provide a comprehensive exploration of how the CaSR integrates extracellular Ca^{2+} into intracellular Ca^{2+} signaling.

the Ca^{2+}-sensing receptor (the CaSR), Ca^{2+} signaling, extracellular domain (ECD), Ca^{2+}-binding sites

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After Sydney Ringer serendipitously discovered calcium (Ca^{2+}) to be essential for the contraction of isolated hearts in 1883 [1], Ca^{2+} has received substantial attention and research on Ca^{2+} has grown at an exponential rate as it was recognized that Ca^{2+} is a universal carrier of biological information [2]. Ca^{2+} controls matters of life and death as it modulates the process of fertilization as well as apoptosis.

Before the discovery of the calcium-sensing receptor (the CaSR), Ca^{2+} was mainly considered as a crucial second messenger through rapid yet efficient alterations in the intracellular calcium; it modulates extensive molecular signaling components through calcium channels and exchangers as well as pumps [3]. When Ca^{2+} plays a role as first messenger for the CaSR, it integrates calcium signaling from changes in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) to intracellular signaling networks that are critically important for many (patho)physiological processes, including cardiovascular remodeling, tumor cell migration and cancer metastasis [4–7]. Ca^{2+}-sensing receptors (the CaSRs) along with 14 other members of family C of the G protein-coupled receptors (cGPCRs), i.e., the metabotropic glutamate receptors (mGluR) and γ-aminobutyric acid $(GABA)_B$ receptors, cooperatively respond to small changes in $[Ca^{2+}]_o$ to activate multiple signaling pathways. The cGPCRs are also integrators of diverse extracellular signaling molecules, including metabolites neurotransmitters (i.e., amino acids), pheromones, and sweet substances using their conserved long N-terminal extracellular domains (ECD) with a Venus Fly Trap (VFT) motif that contains a flexible hinge region. The CaSRs distributed in multiple organs play a central role in regulating $[Ca^{2+}]_o$ homeostasis [8–12] as well as monitoring protein/amino acid metabolism by sensing amino acids, especially aromatic amino acids, in blood and related extracellular fluids.

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Although Ca^{2+} is considered as a universal signal for the regulation of cells, it can be toxic to cells if it stays too long at an excessively high concentration. Pulsatile Ca^{2+} signals have evolved to prevent such toxicity. Two major mechanisms are involved in the production of the temporal Ca^{2+} signals that can, in turn, lead to more complex downstream signaling. The first one happens in skeletal and cardiac cells when the intracellular Ca^{2+} signaling pulses are produced on demand as the cells respond to periodic stimulation that leads to membrane depolarization. This kind of signal can be observed in muscle cells when they contract and also in nerve terminals when neurotransmitters are released upon a brief localized pulse of intracellular Ca^{2+} [2]. The second type appears as an oscillation when the cells receive continuous stimulation over a period of time. This rhythmic Ca^{2+} signal has a wide range of frequencies that are tightly related to a variety of cellular responses (i.e., gene expression, fertilization, cell growth, transmitter release, etc.) [13].

In this review, we will review the structural information known about the CaSR and explore its molecular properties as an important therapeutic target in disorders with abnormal $[Ca^{2+}]_o$ homeostasis.

1 Biological roles of the CaSR

It has been well known that the serum Ca^{2+} concentration can regulate the secretion of parathyroid hormone (PTH). In 1993, Dr. Edward M. Brown and coworkers [14] cloned the receptor that is primarily responsible for this type of regulation from the bovine parathyroid gland. The receptor was given the name "calcium-sensing receptor" (the CaSR/CaR). The previously observed cytosolic calcium changes in parathyroid cells, as well as in other *in vitro* expression systems, were triggered by changes in the serum calcium concentration and are characteristic "signatures" of the CaSR's activities [15,16]. A unique characteristic of the CaSR is the high degree of cooperativity of its Ca^{2+}-dependent activation, which tightly controls the secretion of parathyroid hormone when the receptor is exposed to changes in serum Ca^{2+} concentration within its responsive range [14]. The CaSRs expressed in various species are highly conserved, indicating the biological importance of this receptor in regulating physiological functions among living organisms.

The major function of the CaSR is to maintain calcium homeostasis through balancing the ingestion and absorption of calcium from the gastrointestinal tract, excretion of calcium by the urinary system and the breakdown and formation of bone [17,18] (Figure 1). The CaSR is mainly expressed in the parathyroid and thyroid glands, as well as in other tissues mentioned above. The CaSR regulates PTH secretion from parathyroid glands and calcitonin secretion from thyroidal C-cells upon detection of variations in the calcium concentration in the extracellular fluid [17].

Moreover, the CaSR has its specific roles in different tissues. As mentioned in the last section, the CaSR was first discovered in parathyroid cells. The main function of the receptor is to mediate the negative feedback of PTH secretion upon stimulation by various agonists [14]. Most of the CaSR expressed in parathyroid cells is located in caveolae, where a significant number of signaling molecules, as well as scaffolding proteins, reside [19]. Similarly, one of the critical roles the CaSR expressed in the kidney is in maintaining calcium homeostasis [20]. However, the CaSR along different parts of the nephron (e.g., the proximal tubule, thick ascending limb, etc.) may have specific functions [21,22].

The CaSR is also found in the gastrointestinal tract, including the esophagus, stomach, small intestine, and colon [23,24]. The activity of the CaSR has been reported to be associated with increased phosphorylation of ERK, intra-

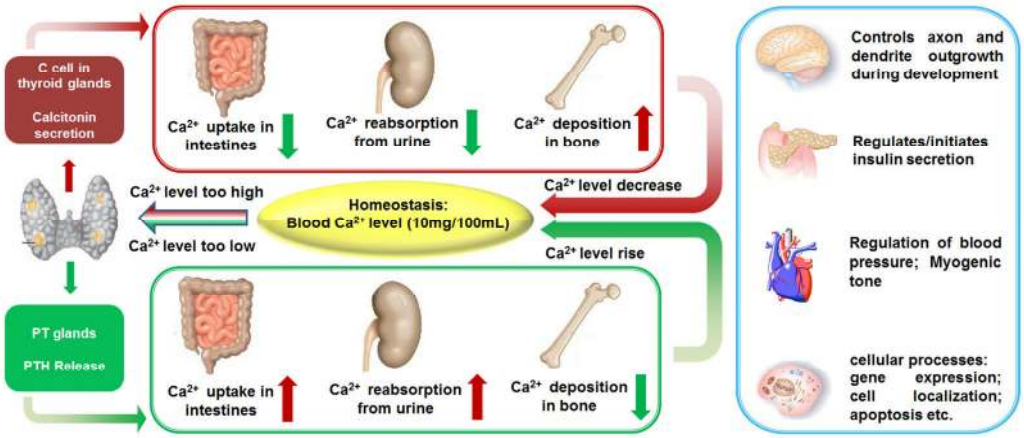

Figure 1 Systematic regulation of Ca^{2+} homeostasis in human body. Two major circulating hormones (PTH and CT) regulate Ca^{2+} homeostasis via several primary organs. If the Ca^{2+} level is too low, PTH is released from parathyroid glands. It then stimulates Ca^{2+} uptake from intestines, increases the rate of Ca^{2+} release from bone and the rate of Ca^{2+} reabsorption in the urinary system. If the Ca^{2+} level is too high, the opposite regulation will occur to control the blood Ca^{2+} level. Thus, the blood Ca^{2+} is strictly maintained between 1.1–1.4 mmol L^{-1}.

cellular calcium mobilization, secretion of IL-8, gastric acid [25–27] and cell proliferation and differentiation [28,29].

The CaSR has been identified in bone as demonstrated by various techniques [30]. Both osteoblasts and osteoblastic cell lines, such as MC3T3-E1 cells, as well as osteoclasts are reported to express the CaSR. The receptor is involved in osteoblast proliferation, likely via JNK signaling [31,32], and participates in both differentiation and apoptosis of osteoclasts through the PLC pathway [33]. Activation of the CaSR is also believed to inhibit the bone resorbing activity of osteoclasts [34].

Experiments using immunocytochemistry showed that the CaSR is widely distributed in the central nervous system with quite a diverse pattern [35]. In the nervous system, the CaSR can also form heterodimers with other family C GPCRs [36,37]. It has been proposed that the CaSR may play a crucial role in regulating ion currents that mediate the initiation of action potentials and the associated neuronal depolarization [38]. The CaSR in the hippocampus has been reported to regulate both Ca^{2+}-permeable, nonselective cation channels as well as Ca^{2+}-activated K^+ channels [35,39,40].

The CaSR is also detected in cardiac tissue by various techniques, including RT-PCR and immunohistochemistry [41]. A few pathological processes have been found to be associated with an increased expression level of the CaSR, for instance, angiotensin II-induced cardiac hypertrophy [42] and hypoxic-oxygenation-treated cardiac myocytes [43].

2 The structural biology of the CaSR

The CaSR expressed in pcDNA-CaSR-transfected human embryonic kidney (HEK293) cells exhibits similar expression patterns as the CaSR proteins identified in parathyroid cells [44]. The Western blot results of the CaSR extracted from HEK293 cells showed several immunoreactive bands using antibodies specific for the CaSR. The minor band at 120 kD corresponds to the non-glycosylated monomeric form of the protein, the major band at 140 kD is the high mannose form, while another major band at 160 kD represents the complex carbohydrates form of the monomeric CaSR [44,45]. Additional bands at higher molecular mass (350 kD) correspond to the dimeric forms of the receptor. The CaSR that is expressed on the cell membrane is the mature receptor that only accounts for a small portion of the total cellular protein [46].

The CaSR comprises four major parts in terms of its structure: a large N-terminal extracellular domain (ECD), a cysteine-rich domain linking the ECD to the first transmembrane helix, a seven transmembrane domain and the intracellular C-terminal. The major function of the CaSR *in vivo* is regulating the calcium concentration in the human blood. Upon stimulation with Ca^{2+} or other polycations, a number of intracellular signal transduction pathways are activated (Figure 2). Among them, the phosphoinositide-specific phospholipase C (PI-PLC) and extracellular signal-regulated kinases (ERK1/2) have been widely applied to the investigation of CaSR activity [48,49].

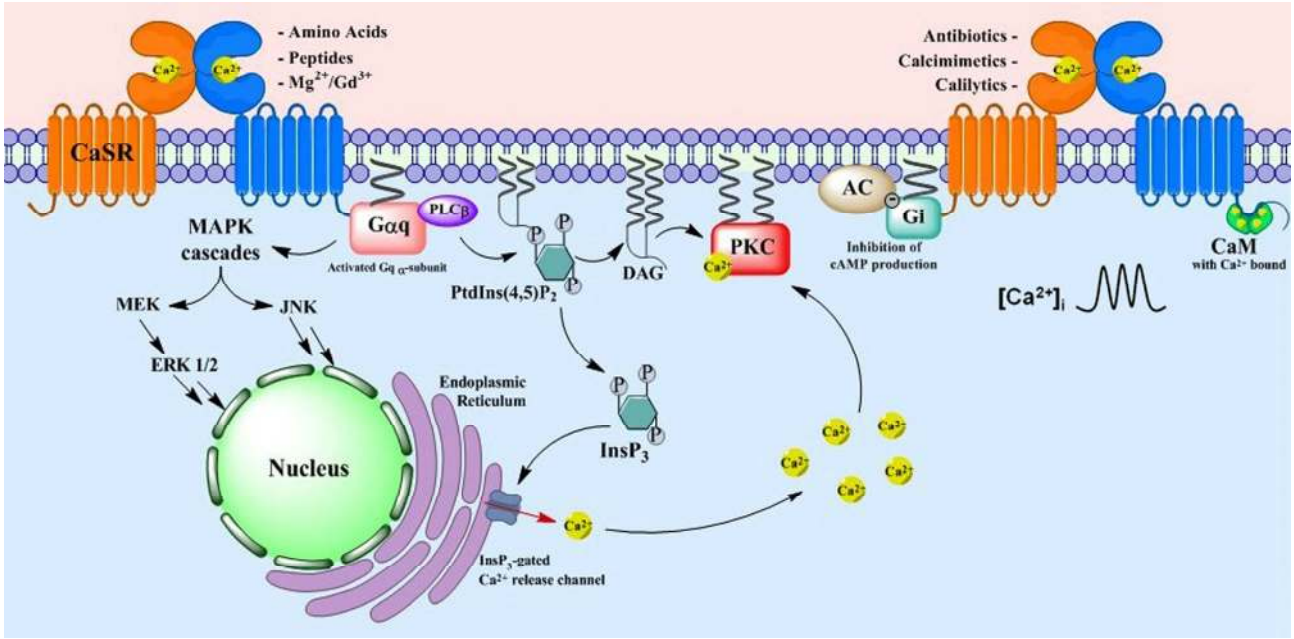

Figure 2 Calcium-sensing receptor-regulated intracellular signaling pathways. The interaction between Ca^{2+} and CaSR results in activation of phosphatidylinositol-specific phospholipase C_β (PLC_β) through recruitment of the $G_{\alpha q/11}$ proteins. PLC_β hydrolyzes phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] to inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ triggers the release of calcium from the endoplasmic reticulum (ER) calcium store via the $InsP_3$ receptor (IP_3R) embedded in the ER membrane. Calmodulin (CaM) binds to the C-terminus of CaSR to maintain proper responsiveness of intracellular Ca^{2+} responses to changes in extracellular Ca^{2+} and regulate cell surface expression of the receptor [47].

Like other members in the family C GPCR proteins, the CaSR possesses the signature large N-terminal ECD. The low but significant amino acid sequence identity between the family C GPCRs and a group of bacterial periplasmic amino acid-binding proteins suggest evolutionary relevance. Indeed, the crystal structure of the ECD of rat metabotropic glutamate receptor (mGluR1) showed a bi-lobed Venus-flytrap-like structure (VFT), which is fully consistent with the structure of the bacterial periplasmic amino acid-binding proteins, switching between "open" and "closed" conformational states upon releasing or binding their endogenous agonists (Figure 3A) [50]. The alignment of the amino acid sequence of the CaSR ECD (AA36-AA513) with mGluRs and the bacterial periplasmic amino acid-binding proteins results in a proposed VFT model of the CaSR ECD with the N-terminal lobe I connecting to the C-terminal lobe II [51]. However, the alignment also shows four segments in lobe I that do not align with the bacterial periplasmic amino acid-binding protein resulting in four unstructured loops (I–IV) [52]. Only a large part of loop III (from residues 365–385) could be deleted without reducing the activity of the CaSR while truncation in the other four loops resulted in a low surface expression of the receptor [52].

The VFT structure of mGluR1 forms a homodimer as verified by the 3D structure of rat mGluR1 VFT. The intermolecular disulfide requires a conserved cysteine in loop 2, and the equivalent residues in the CaSR are C129 and C131 [51,53]. Experiments mutating the two cysteines suggested that disruption resulted in failure to form disulfide bonds, but did not affect the formation of dimer [54]. Other cysteines in the ECD of the CaSR including C60–C101, C358–C395 and C437–C449 form three intramolecular disulfides, which are critical for the CaSR expression and activity [55].

All members of the GPCR family C have a conserved nine-cysteine structure at the end of the C-terminal domain of the ECD and before the seven transmembrane domains except the $GABA_B$ receptor [56]. For the CaSR, the region possesses about 84 residues. Mutations of any of the nine Cys to Ser dramatically impair the surface expression and the function of the CaSR. Deletion of this Cys-rich domain results in the abrogation of the CaSR activity.

The CaSR contains a seven transmembrane domain (7TM) similar to the other proteins in the GPCR superfamily. It is believed that agonist binding to GPCRs will lead to conformational changes of the 7TM α-helices, further inducing alterations in intracellular loops as well as the C-terminal domain, thereby triggering the downstream signaling pathways. The crystal structure of rhodopsin (a GPCR Family A protein), which has a 7TM domain like the GPCRs, has been reported. However, extreme caution is needed when extrapolating the CaSR 7TM structure from rhodopsin as proteins from family C share low sequence identity with proteins from family A. The six prolines in the transmembrane domain are important to maintain the kinks in the transmembrane helices. Mutations of some prolines (P748R, P823A) [57,58] impair receptor function.

The intracellular domain of the CaSR contains 216 amino acids that do not show high conservation among species [59]. Two regions in this intracellular tail are homologous between species: one from 863–925 located at the proximal of C-tail that is pivotal in surface expression; the other from 960–984 that is involved in interacting with downstream proteins [60–62]. Three potential protein kinase C (PKC) phosphorylation sites are present in the intracellular domain: T888, S895 and S915 are involved in regulating receptor activity [63]. Especially, T888 plays a significant role in the negative regulation of the CaSR's activity by PKC.

Along with the PKC phosphorylation sites, two other residues participate in protein kinase A phosphorylation: S899 and S900. Studies of these two sites indicate minor effects of PKA phosphorylation on the modulation of the CaSR activity [64], but the phosphorylation at S899 may regulate the recognition of an arginine-rich region in the proximal carboxyl terminus of the CaSR, which contributes to intracellular retention of the receptor [65].

3 The ligand binding sites on the CaSR

3.1 Prediction of Ca^{2+}-binding sites in the CaSR

The identification of potential Ca^{2+} binding sites on the CaSR is challenging not only because of the lack of a crystal or solution structure of the receptor but also owing to the limitations of current techniques regarding direct measurement of Ca^{2+} binding to the receptor. The fast off rate of Ca^{2+} due to its weak binding affinity also hinders the characterization, as even with proteins that have their crystal structure solved (e.g., mGluR1), the detection of Ca^{2+}-binding sites may still be elusive [66]. Our lab as well as a few other groups have generated homology model structures of the extracellular domain of the CaSR based on the crystal structures of mGluR1, and have identified predicted Ca^{2+}-binding sites using these models [67,68]. One of the predicted Ca^{2+}-binding sites has been proposed by both our group [69] and Silve et al. [67]. This Ca^{2+}-binding site, later referred to as Site 1, is located in the hinge region between lobe I and lobe II of the CaSR ECD, and is composed of residues Ser147, Ser170, Asp190, Tyr218 and Glu297. Mutations of these five residues on the receptor resulted in impaired receptor function demonstrated by both groups. In our first paper about the CaSR, we also identified two additional Ca^{2+} binding sites using grafting approaches. One of them is located in lobe I, containing residues Glu378, Glu379, Thr396, Asp398 and Glu 399, and is referred to as Site 5 in the latter studies. The other one is on lobe II, involving resides Glu224, Glu228, Glu229, Glu231 and Glu232, which is named Site 3. Peptide sequences of the CaSR containing these predicted Ca^{2+} binding site were

inserted into CD2 protein. The latter is known as a non Ca^{2+}-binding protein, and the chimeric proteins were used for verifying their calcium-binding capabilities using various biophysical approaches. Following these findings, our lab further used subdomain approaches to verify the potential Ca^{2+}-binding sites on the ECD, including the three previously identified Ca^{2+}-binding sites and two additional Ca^{2+}-binding sites: Site 2, which is composed of residues Glu215, Leu242, Ser244, Asp248, Gln253; and Site 4, which contains residues Glu350, Glu353, Glu354, Asn386, and Ser388. The three subdomains, comprising residues 132–300, 185–324, and 323–494 were expressed in a bacterial system. Ca^{2+}-induced conformational changes were observed in spectroscopic studies using 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence, intrinsic tryptophan spectra as well as nuclear magnetic resonance [69]. Moreover, the studies using subdomains of the CaSR also suggested multiple cooperative metal-binding processes as revealed by Tb^{3+} luminescence energy transfer studies and mutagenesis work on the putative Ca^{2+}-binding sites [69]. More recently, using mutagenesis, we have shown that mutations in Ca^{2+}-binding site 1, such as Glu297 and Tyr218, not only disrupted the Ca^{2+}-sensing capacity of the CaSR but also had an impact on the positive homotropic cooperative interactions of Ca^{2+} with other Ca^{2+}-binding sites [70]. Moreover, molecular dynamic simulation results revealed that residues located at Site 1 showed strong correlated motions with other residues in the other four sites. These observations demonstrated the molecular connectivity among the five predicted Ca^{2+}-binding sites with Site 1 playing a central role and have provided a description at the atomic level of the cross-talk between different Ca^{2+}-binding sites of the CaSR.

The transmembrane domain of the CaSR (7TM) has been suggested to contain additional Ca^{2+}-binding sites in a few studies [12,71]. Hauache et al. [12] engineered a chimeric receptor with the extracellular domain of rhodopsin linked with the transmembrane domain and intracellular C-tail of the CaSR to show that the chimeric receptor could be expressed at the cell surface and was able to response to extracellular Ca^{2+} in the presence of NPS R-568, a positive allosteric modulator of CaSR. Hu et al. [58] analyzed 12 naturally occurring activating mutations of the CaSR located in the 7TM domain and found that five of them were located at the junction of TM helices 6 and 7 between residues Ile819 and Glu837. They utilized alanine-scanning mutagenesis and revealed that Ile819-Glu837 played a key role in CaSR activation by Ca^{2+}. The same group later found that substitution of three of the five acidic amino acids with alanine in the extracellular loop 2, Asp758, Glu759 and Glu767, increased the activity of the CaSR [71]. These findings indicated that Ca^{2+} could tune the receptor's function partially through the transmembrane domain.

3.2 Amino acid-binding by the CaSR

As a multimodal, multimetabolic sensor, the CaSR regulates whole body calcium homeostasis not only through its responses to extracellular Ca^{2+}, but also by sensing L-amino acids [72]. Under physiological conditions, L-amino acids, especially aromatic amino acids (e.g., L-Phe), as well as short aliphatic and small polar amino acids [73], potentiate the high $[Ca^{2+}]_o$-elicited activation of the CaSR by altering the EC_{50} values required for $[Ca^{2+}]_o$-evoked $[Ca^{2+}]_i$ responses and its functional cooperativity [72,73]. The intracellular Ca^{2+} mobilization induced by L-amino acids at the threshold level of extracellular Ca^{2+} concentration is characterized by slow oscillations with a frequency at around 1–2 peaks min^{-1} [74]. Distinctive signaling pathways triggered by amino acids compared with extracellular Ca^{2+} have been confirmed in quite a few studies [74]. However, the specific binding pocket(s) for amino acids are still unclear due to the lack of structural information of the CaSR and the low binding affinity (in the mmol L^{-1} range) of the ligands.

Sequence alignment of the CaSR with conserved amino acid residues that are involved in the binding of glutamate to mGluRs was applied as an initial approach to identify the potential L-Phe binding site [75]. Zhang et al. [75] demonstrated that mutating three adjacent Ser to Ala eliminated L-Phe potentiated receptor activity measured by cell population assay of changes in Ca^{2+}_i using fluorimetry. In 2004 and 2005, Mun et al. [76] published two papers about L-amino acid-sensing in the CaSR. In their 2004 study, they confirmed the binding site for amino acids was within the VFT domain of the CaSR by utilizing CaSR-mGluR chimeric receptor constructs. The receptor lacking the CaSR ECD exhibited impaired response to L-amino acids. One year later, using mutagenesis and cell population studies, they showed that two residues, Thr145 and Ser170, could be crucial for sensing L-amino acids, since a double mutant of the CaSR, T145A/S170T, selectively failed to respond to L-Phe [77]. Recently, our lab reported an L-Phe binding site near the previously predicted calcium-binding Site 1 based on computational docking results and mutational studies using both single and cell population assays of receptor-induced changes in Ca^{2+}_i [70]. We found that the potential L-Phe-binding site is comprised of residues Leu51, Thr145, Ser170, Tyr218 and Ser272 and was responsible for positive heterotropic cooperativity between extracellular Ca^{2+} and L-Phe [70].

3.3 The binding of allosteric modulators to the CaSR

Distinct from L-amino acids, the allosteric modulators of the CaSR, many of which have a phenylalkylamine structure, have been proposed to regulate the CaSR's activity via interacting with its transmembrane domains instead of the extracellular domain [71,78,79]. Hauache et al. [12] made

truncation and deletion mutants of the CaSR and engineered a chimeric protein with the ECD domain of the CaSR and the 7TM of mGluR1. Their results showed that the positive allosteric modulator of the CaSR, R-568, was effective in constructs with the 7TM from the CaSR but not in the ones with the mGluR1 7TM instead. Two years later, the same lab reported that an acidic residue, Glu837, in extracellular loop 3 was involved in the interaction with NPS R-568, as the CaSR mutant E837A abolished the NPS R-568-potentiated receptor activity while maintaining partial Ca^{2+}-sensing capability [71]. Two independent studies have used homology modeling of the 7TM domain of the CaSR based on the X-ray structure of rhodopsin to predict the potential binding site for the allosteric modulators. Miedlich et al. [79] found that mutations on residues Phe668, Arg680, Phe684 and Glu387 altered the CaSR's response to NPS2143, which is a negative allosteric modulator of the CaSR, suggesting their involvement in binding the drug. Petrel et al. [78] independently revealed that the binding pockets for calcimimetics and calcilytics were both located on the 7TM domain. They partially overlap with residues Trp818, Phe218, Glu837 and Il3841, but are not identical to the residues predicted in the other study. The recognition of calcilytics also involved residues in TM3 and TM5.

3.4 Physiological relevance of studying ligand binding *in vitro*

In biological systems, free L-amino acids are essential molecules since they not only serve as the building blocks of proteins but also are the metabolic precursors of crucial substances that serve as ligands for receptors [80]. Evidence for the presence of amino acid-sensing mechanisms in various studies, e.g., regulating insulin secretion from pancreatic B cells, hepatic autophagy, etc., suggest the widespread existence of amino acid sensors, although the identities of these amino acid sensors are in many cases unknown. These findings highlight amino acid-dependent control of cellular signal transduction pathways. Fluctuation of the plasma levels of amino acids can, therefore, regulate the rate of hormone synthesis and secretion as well as Ca^{2+} metabolism, among other processes [80]. The CaSR is present throughout the gastrointestinal tract [17,81], including in gastrin-secreting antral G cells and cholecystokinin-secreting l cells, both of which are known to be activated by both $[Ca^{2+}]_o$ and aromatic amino acids [81]. L-amino acids, especially aromatic amino acids, are known to enhance the sensitivity of the CaSR to $[Ca^{2+}]_o$, which could be one potential explanation for how dietary protein modulates $[Ca^{2+}]_o$ homeostasis in normal individuals as well as in patients with chronic renal failure [80,82].

The amino acid concentrations in plasma before and after a meal are listed in Table 1. All of them are within the μmol L^{-1} range. Even after a steak meal, the peak value for certain amino acids (e.g., Gly) is around 0.5 mmol L^{-1} [83]. However, the reported EC_{50} for activation of the CaSR by L-Phe was 3.5 mmol L^{-1} as measured by intracellular calcium readout from the CaSR-transfected HEK293 cells [73]. The discrepancy for these phenomena relies on the fact that in physiological conditions, the receptors are stimulated by a mixture of amino acids, small peptides as well as proteins instead of a single amino acid. In fact, small peptides, for instance, γ-glutathione and its variants, have lower EC_{50}s (in the range of μmol L^{-1}) compared with L-Phe or L-Trp (Table 2).

Conigrave et al. [73] have investigated the physiological relevance of their *in vitro* findings regarding the amino acid concentration dependence of the CaSR. They mixed 20 common L-amino acids at similar concentration compared to those present in fasting human plasma and showed that the mixture could reproduce the effects of high concentrations of single amino acids. These findings suggest that the CaSR sensing L-amino acid is a universal property and the amino acid composition of human plasma has a pronounced effect on the activity of the CaSR compared to single amino acids.

The metabolism of both Ca^{2+} and amino acids will be re-appraised in the context of protein ingestion and dietary supplements, since L-amino acid and Ca^{2+} can potentiate the sensing capability by the CaSR for each other. It was also emphasized by Conigrave et al. [73] that the CaSR should

Table 1 Plasma amino acid concentrations before and during intravenous amino acid infusion and after an oral steak meal (μmol L^{-1})

Amino acid infused IV	Basal	0–60 min	61–120 min	121–180 min	181–240 min	Steak meal
Phe	51±5	68±8	90±7*	103±14*	198±37*	85±7
Trp	49±5	66±6	97±8*	140±16*	244±38*	94±9
Gly	283±50	291±49	315±50*	417±75*	559±98	386±53
Ala	316±16	345±15	353±25	326±10	365±31	549±50
His	80±14	104±8	130±10	172±19	316±25	100±11

a) *, Time period when gastric acid secretion was significantly greater than saline control. Mean (±SE) plasma amino acid concentrations of phenylalanine, tryptophan, glycine, alanine, and histidine before (basal) and in response to intravenous infusion of each individual amino acid and after the steak meal. Each amino acid was infused in 125 mL for 1 h in increasing stepwise manner; doses of Phe, Gly, Ala, and His were 0.0125, 0.025, 0.05, and 0.1 mol L^{-1}, respectively, and doses of Trp were 0.005, 0.01, 0.02, and 0.04 mol L^{-1}. The steak meal contained 49 g protein and 405 kcal (table adopted from [83]).

Table 2 Potencies of γ-glutamyl peptides for $[Ca^{2+}]_i$ mobilization in CaSR-expressing HEK-293 cells

γ-Glutamyl peptide	EC_{50} for peptide (μmol L^{-1})
S-Methylglutathione	1.7±0.5 (*n*=4)
Glutathione	3.9±0.7 (*n*=4)
γ-Glu-Cys	4.7±0.9 (*n*=3)
γ-Glu-Ala	4.8±0.7 (*n*=3)

a) HEK-293 cells that stably express the CaSR were loaded with fura-2AM and assayed for receptor-dependent intracellular Ca^{2+} mobilization by microfluorimetry. The data were obtained from cells perfused with physiological saline solution in the presence of 2.5 mmol L^{-1} $[Ca^{2+}]_o$. In the accompanying experiments, the EC_{50} for L-Phe was 1.1±0.5 mmol L^{-1} (*n*=4) (table adopted from [84].

be reassessed as a potential target of aromatic L-amino acids under certain toxic metabolic conditions. For instance, the CaSR expressed in the CNS might be involved in contributing to the toxic effects resulting from the elevated levels of L-Phe in phenylketonuria or in hepatic encephalopathy.

4 Proteins interacting with the CaSR

In addition to heterotrimeric G proteins, the CaSR needs to recruit a number of other proteins to ensure that signaling networks are fully functional. Recently, a few of these proteins have been identified using the yeast two-hybrid system and/or co-immunoprecipitation studies. The absence of these proteins would, in turn, influence the CaSR signaling.

Filamin A has been demonstrated to interact with the C-terminal of the CaSR in 2001 by two individual groups [61,62]. Later studies using truncation and deletion mutants revealed that the binding took place in a two β-strands region from amino acid 962 to 981 of the CaSR. Blocking the interaction between Filamin A and the CaSR attenuated CaSR-induced ERK activity [61]. The binding of the two proteins also exhibited importance in CaSR-mediated Rho signaling [85,86] as well as JNK activation [87]. Studies in a cell line deficient in Filamin A suggested that Filamin A might protect the CaSR from degradation [88].

Potassium channels (Kir4.2 and Kir4.1) have been reported to colocalize with the CaSR in HEK293 cells stably expressing the CaSR as well as in endogenous rat kidney tissues [89]. However, how and whether their function can be regulated by the CaSR still needs to be elucidated. It is possible that Filamin A may act as a bridge that links the CaSR with either Kir4.1 or Kir4.2.

The region between amino acids 880–900 of the CaSR has been identified to interact with the region 660–838 of an E3 ubiquitin ligase, dorfin [90]. Overexpression of dorfin is believed to increase the ubiquitination of the CaSR, resulting in accelerated degradation [90].

Associated molecule with SH3 domain of STAM (AMSH) is an ubiquitin isopeptidase that regulates the sorting of the receptor EGFR [91]. The CaSR C-tail region from 895-1075 was found to bind to AMSH [92]. Increasing AMSH expression level or stimulating the CaSR with calcium could reduce CaSR expression in HEK293 cells transfected with these two proteins [92,93].

β-arrestins play an important role in modulating the desensitization and internalization of GPCRs through collaborating with G protein receptor kinases (GRK) [94]. Overexpression of β-arrestins negatively regulates the inositol phosphate signaling mediated by the CaSR in HEK293 cells. A PKC inhibitor or mutation of the CaSR PKC phosphorylation sites reduces the effect of overexpressing β-arrestins.

RAMP is an abbreviation for receptor activity-modifying protein, which has been reported to influence receptor trafficking, glycosylation as well as second messenger production [95]. Experiments in COS7 cells demonstrated that RAMP1 and RAMP3, but not RAMP2, can increase the surface expression of the co-transfected CaSR [96]. Moreover, RAMP3 had a greater influence on modulating the cell surface expression of the receptor.

5 Trafficking of the CaSR in cells

Like many other GPCRs, the trafficking of the CaSR is a complex process that involves interacting with various proteins that determine the final destination of the receptor. Once synthesized in the endoplasmic reticulum (ER), CaSR that is improperly folded will fail the quality control process and be shunted toward the proteasome for degradation. Receptors passing the quality control process will traffic to the plasma membrane or other cellular compartments via interaction with chaperones and small GTP-binding proteins [97–100].

Several proteins interact with the CaSR when the receptor exits from the ER, including p24A (transmembrane emp24 domain trafficking protein 2(TMED2)) [101]; the small GTP-binding protein Sar1 [102]; receptor-activity-modifying proteins (RAMPs) [96]; Rab1 [103]; calmodulin (CaM) [63]; 14-3-3 [104] and Dorfin [90]. p24A binds predominantly to the immature form of the CaSR and cycles between the ER, the ER-Golgi intermediate compartment (ERGIC) and the cis-Golgi membranes [105,106]. Through its interaction with p24A, the stability of the CaSR and its plasma membrane targeting will be increased, possibly via an increase in total receptor number [101]. Sar1 is another protein that is required for releasing the receptor from the ER [101,102]. RAMPs and Rab1 are small GTP-binding proteins that localize to the cytoplasmic face of organelle membranes. Both of them are found to facilitate the CaSR trafficking from ER to Golgi [96,103]. The 14-3-3 protein is predicted to interact with the arginine-rich domain of the CaSR (^{890}RRxxxxRKR^{898}) and may lead to the retention of the CaSR in the ER [65,104,107]. There is also a calmodulin-binding site in the C-tail of the CaSR comprising residues 874–895 [47]. However, the function of CaM still

needs to be revealed. It is reported that CaM binds to both immature and mature forms of the CaSR, suggesting its role in modulating anterograde trafficking of the receptor [63]. On the other hand, CaM may stabilize the surface receptor on the cell membrane [47].

Its interactions with several different proteins contribute to the subcellular localization of the CaSR. The association of intracellular loops 1 and 3 with caveolins keeps the CaSR highly enriched in invaginations of plasma membrane called caveolae in certain types of cells, like parathyroid chief cells and cardiac myocytes [19,108–110]. Filamin A, a scaffold/adaptor protein, has also been shown to interact with the C-terminal of the CaSR, targeting the receptor to sites of Rho activity [85,86,111]. The CaSR also interacts with integrins, and the latter may contribute to the regulation of cell migration [7].

The surface expressed CaSR may undergo endocytosis initiated by phosphorylation by G protein-coupled receptor kinases (GRKs) or protein kinase C [112,113], involving β-arrestins and facilitated by Rab7, Rab11a and adaptor protein-2 (AP2) [93,114,115]. The endocytosed receptors are either recycled to the cell membrane, thereby contributing to the receptor resensitization, or translocated to lysosomes for degradation [116,117].

There are two mechanisms involved in degradation of the CaSR. One is mediated by the activity of the E3 ubiquitin ligase family member, dorfin. The CaSR will be targeted to the proteasome after being ubiquitinated by dorfin [118]. The CaSR can also be degraded in lysosomes. A deubiquitinating enzyme specific for k63-linkages (AMSH, which is short for associated molecule with the SH3 domain of STAM) has been reported to be involved in this process [93]. Moreover, the PEST-like sequence in the C-tail of the CaSR, which is rich in proline, glutamine, serine and threonine, can also lead the receptor to lysosomes [115].

In the continuous presence of agonist stimulation, those secretory pathways as well as the endocytosis mechanisms mentioned above work collaboratively, causing a net increase in CaSR expressed on the plasma membrane. This phenomenon was named Agonist-Driven Insertional Signaling (ADIS) [104]. However, a number of questions raised by the ADIS mechanism remained to be explored.

6 The disease-associated CaSR mutations

The discovery of the CaSR and its role in maintaining physiological calcium level led to the identification of disorders of calcium homeostasis that are related to abnormal receptor activity. There are more than 200 mutations of the CaSR that have been catalogued in the online database at http://www.casrdb.mcgill.ca/. The CaSRdb is the primary attribution for the CaSR-related disorders [119]. Autoimmune antibodies against the CaSR are another cause for receptor dysfunction [120–122].

Mutations of the CaSR may lead to an inactive receptor or an overactive receptor. The majority of these disease-associated mutations are missense mutations, with single amino acid substituted. Amino acids insertion, deletion, open reading frame shift and splice-site mutations have also been reported [119].

The disorders associated with inactive receptors include cases of familial hypocalciuric hypercalcemia (FHH), which in more severe cases can be associated with dominant negative activity of the mutant CaSR [123] as well as a more serious disorder known as neonatal severe hyperparathyroidism (NSHPT), most commonly due to inactivating mutations in both copies of the CaSR gene [120]. Mild to moderate hypercalcemia (no more than 20% above the upper limit of normal of the serum calcium level) and unusually high renal tubular reabsorption of calcium are the key clinical features of FHH [119]. On the other hand, NSHPT is a rare disorder characterized by marked symptomatic hypercalcemia and usually occurs in children younger than 6 months. NSHPT can cause multiple fractures, neurodevelopmental disorders and even death.

The disorders associated with the CaSR activating mutations include autosomal dominant hypocalcemia (ADH) [124] and Bartter syndrome type V [125]. Patients with ADH usually do not show symptoms but have mild reductions in calcium concentration in the blood. Seizures may happen to younger patients, often occurring during febrile episodes. Serum PTH levels are generally within or near the lower limit of the normal range. Calcium concentration in the urine is higher than in hypoparathyroid patients of other etiologies. Bartter's syndrome subtype V is associated with severe heterozygous activating mutations in the CaSR. Patients with Bartter syndrome type V show symptoms like hypokalemic metabolic alkalosis and hyperaldosteronism along with hypocalcemia [125].

Hannan et al. [126] have reported that the majority (>50%) of their newly identified CaSR mutations in patients with FHH, NSHPT and autosomal dominant hypocalcemic hypercalciuria are missense substitutions within the ECD of the CaSR. Combining their study with previously reported disease-associated the CaSR mutations, here we show that the bilobed VFTD plays a pivotal role in interacting with Ca^{2+} and regulating the function of the CaSR, as more than half of the mutations occurred in the ECD (Figure 3C). By analyzing the homology modeled structure of the CaSR, our studies further revealed that >50% of these mutations are situated within 10 Å of one or more calcium-binding sites [126]. Moreover, since a substantial percentage (>50%) of the mutations near calcium-binding sites are located at Site 1, the importance of VFTD cleft is highlighted. The rest of the calcium-binding sites can possibly play an auxiliary role by building molecular connectivity between Site 1 as well as other calcium-binding sites and thereby enhancing positive cooperativity [70].

Recently, studies have appeared showing that disorders

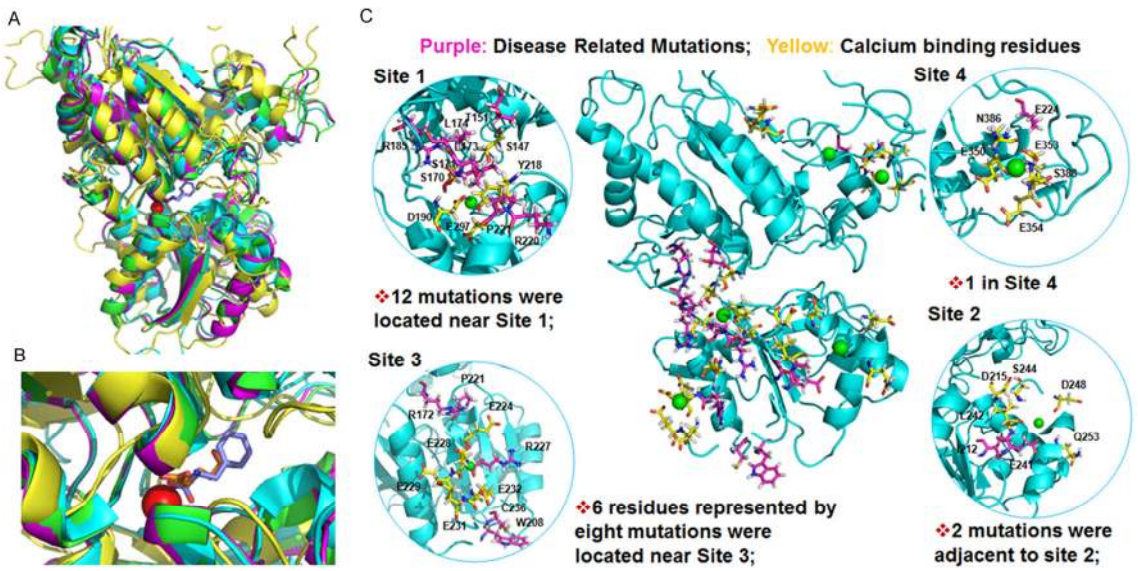

Figure 3 Mapping the disease-associated mutations on the modeled CaSR ECD. A, Overlay of modeled structure of Ca^{2+}-sensing receptor with crystal structures of mGluR1, mGluR3 and mGluR5. The modeled CaSR extracellular domain (ECD), highlighted in yellow, is based on mGluR1 (pdb: 1EWK). mGluR1 (pdb:1EWK) is highlighted in green, mGluR3 (pdb:2E4U) is highlighted in cyan, mGluR5 (pdb:3LMK) is highlighted in violet. Calcium is labeled in red, L-Phe is in purple [68]. B, The zoom in view of the hinge region. Purple: L-Phe; orange: glutamate. C, Disease-related CaSR mutations on ECD. Cyan: the modeled structure of the CaSR ECD; purple: residues involved in disease-associated mutations reported in the recent studies by Hannan et al.; yellow: residues involved in predicted calcium binding pockets.

with an FHH or ADH phenotype may not directly involve mutations of the CaSR, but can be associated with mutations of other proteins that participate in CaSR-mediated signaling [114,127]. Familial hypocalciuric hypercalcemia type 2 (FHH2) and familial hypocalciuric hypercalcemia type 3 (FHH3) are genetically distinct, autosomal dominant disorders of extracellular calcium homeostasis in the absence of mutations in the CaSR. The first form of FHH identified, caused by mutations of the CaSR gene on chromosome 3q21.1, is now called familial hypocalciuric hypercalcemia type 1 (FHH1). The loci for FHH2 and FHH3, in contrast, are located on chromosomes 19p and 19q13.3 respectively [114]. Loss-of-function mutations of G-protein subunit-α11 (GNA11) cause FHH2, and loss-of-gain mutations of the sigma subunit of adaptor protein-2 (AP2), a central component of clathrin-coated vesicles (CCVs), decrease the sensitivity of CaSR to extracellular calcium changes, in association with reduced CaSR endocytosis, and lead to FHH3 [114,127]. Conversely, in addition to the first form of ADH identified, now called ADH type 1, which is due to activating mutations of the CaSR as noted above, ADH type 2 is caused by gain-of-function mutations of G-protein subunit-α11 (GNA11). Gain-of-function mutations of adaptor protein-2 sigma subunit (AP2σ2) represent a theoretically possible cause of ADH, but have not been identified as of yet [127].

7 Conclusion and future perspectives

Throughout this review, the molecular characteristics that contribute to the structural biology of the CaSR in $[Ca^{2+}]_o$-sensing and Ca^{2+} signaling have been summarized. On the way to providing advanced information about the structural features related with this receptor as well as other proteins in GPCR family C, researchers will continue to face several challenges.

First, the CaSR is a membrane protein, so the challenges associated with studying membrane proteins may also occur with the CaSR. Membrane proteins are difficult to separate from the cells due to their hydrophobic transmembrane domains. Secondly, although X-ray crystallography has been used as one of the major tools to study the structure of proteins, application of this technique to the crystallization of glycosylated proteins exhibits its own limitations. Like other glycosylated proteins, the complex form of the glycans on the CaSR can prevent crystallization of this protein because the chemical and conformational heterogeneity of the glycoproteins usually inhibit crystallization. Thirdly, due to the limitation of the conditions for crystallization as well as the fast on and off rate of Ca^{2+} when its interaction belongs to weak binding, even in some proteins that have had their crystal structure solved like mGluR1, the detection of Ca^{2+}-binding sites in those proteins may be still elusive. Moreover, the selectivity of the CaSR for different amino acids is not clear and the interaction between amino acids and the CaSR are relatively weak (in mmol L^{-1} range). Fourthly, currently, methods for direct measurement of Ca^{2+} and amino acid binding to the CaSR have not yet been well established, nor are there methods for directly monitoring ligand induced conformational changes of the proteins. Ap-

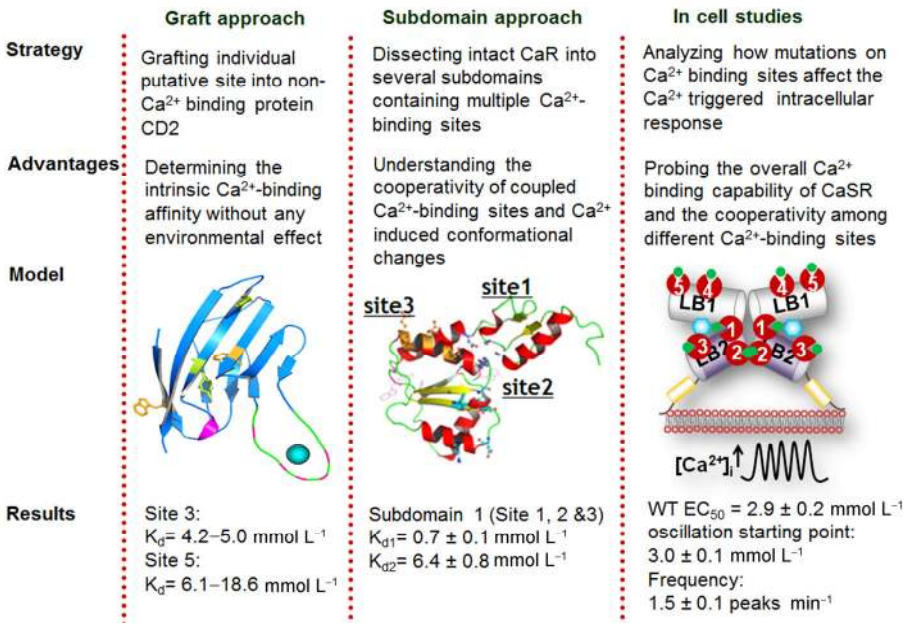

Figure 4 Summary of research approaches.

proaches for addressing these questions rely on indirect measurements of calcium-induced intracellular signaling changes in living cells or utilizing the fluorescence resonance energy transfer (FRET) assay for monitoring ligand-generated fluorescence changes in isolated proteins. Last but not least, the large size of the protein (extracellular domain 612 amino acids; whole protein 1078 amino acids) as well as the fact that the CaSR functions as a dimer contribute to the difficulties in studying the protein, especially when it possesses a high Hill coefficient which suggests possible multiple binding sites for ligands.

However, these challenges can be overcome by innovative experimental designs and advanced technologies. In our lab, we have utilized a grafting approach, subdomain approach and intact cell studies to probe the potential calcium-binding sites on the extracellular domain of CaSR and the functional cooperativity among them (Figure 4). Amino acid-selective isotopic labeling of proteins will be applicable to structural studies of proteins that are not amenable to traditional NMR studies [128]. Various Ca^{2+} sensors can be employed to monitor intracellular calcium signaling and further understand how different allosteric modulators as well as drugs for the CaSR regulate the receptor function. A better understanding of the molecular properties of the CaSR using cutting edge technologies is in demand to provide greater therapeutic approaches since the receptor is involved in various physiological functions.

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