PTH hypersecretion triggered by a GABA_B1 and Ca^{2+}-sensing receptor heterocomplex in hyperparathyroidism

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Molecular mechanisms mediating tonic secretion of parathyroid hormone (PTH) in response to hypocalcaemia and hyperparathyroidism (HPT) are unclear. Here we demonstrate increased heterocomplex formation between the calcium-sensing receptor (CaSR) and metabotropic γ-aminobutyric acid (GABA) B receptor (GABA_B_R) in hyperplastic parathyroid glands (PTGs) of patients with primary and secondary HPT. Targeted ablation of GABA_B_R or glutamic acid decarboxylase 1 and 2 in PTGs produces hypocalcaemia and hypoparathyroidism, and prevents PTH hypersecretion in PTGs cultured from mouse models of hereditary HPT and dietary calcium-deficiency. Co-binding of the CaSR/GABA_B_R complex by baclofen and high extracellular calcium blocks the coupling of heterotrimeric G-proteins to homomeric CaSRs in cultured cells and promotes PTH secretion in cultured mouse PTGs. These results combined with the ability of PTG to synthesize GABA support a critical autocrine action of GABA/GABA_B_R in mediating tonic PTH secretion of PTGs and ascribe aberrant activities of CaSR/GABA_B_R heteromer to HPT.
GPCR-C members to alter CaSR signalling and secretory functions of the glands. We further investigated whether PTCs express the rate-limiting enzymes GAD1/2 to synthesize GABA as an autocrine factor underlying tonic PTH secretion in physiology and diseases.

Results

Expression of GABA<sub>B1</sub>R and GAD1/2 and synthesis of GABA in PTGs. As the CaSR also interacts with other GPCR-C members, including GABA<sub>B1</sub>R<sup>3,23</sup> and metabotropic glutamate receptors 1 and 5 (ref. 24), we performed a general survey for expression of GPCR-C members in normal mouse PTGs to identify potential players that can regulate CaSR signalling by heteromerization. By RNA assessment, we found that the GABA<sub>B1</sub>R is the only GPCR-C member coexpressed with the CaSR in the glands (Fig. 1a, top panel and Supplementary Fig. 1), however, at a lower level (~20%) compared to CaSR expression. The expression of GABA<sub>B1</sub>R<sub>B</sub>, a close dimeric partner of GABA<sub>B1</sub>R in the CNS<sup>3,23</sup>, was undetectable (Fig. 1a). Immunohistochemistry and immunoblotting confirmed the expression of core (~100 kDa) and glycosylated (~130 and 150 kDa) GABA<sub>B1</sub>R proteins in mouse PTGs (Fig. 1b,d, upper panel, and Extended Data Fig. 1a, b).

In the CNS, GAD 1 and 2 (GAD1/2) convert glutamic acids to GABA, which in turn binds and activates GABA<sub>B1</sub>R/GABA<sub>B2</sub>R heteromers<sup>1,25</sup>. A series of molecular biology approaches (quantitative PCR (qPCR), immunohistochemistry and immunoblotting) revealed the expression of GAD1 (67-kD) and GAD2 (65-kD) at the RNA (Fig. 1a, bottom panel) and protein (Fig. 1c,d, bottom panel, and Extended Data Fig. 2a) levels, as well as the presence of their end product GABA in mouse PTGs and/or human parathyroid adenomas (Fig. 1e and Extended Data Fig. 2b,c). While expression patterns of GABA<sub>B2</sub>R and GAD1/2 proteins in PTGs are comparable to those in mouse hippocampi (Fig. 1d, mHIPP), the amount of endogenous GABA in cryopreserved human PTG (10.63 ± 1.88 ng g<sup>−1</sup>) is well below the level that found in mouse brain (198.29 ± 22.1 μg g<sup>−1</sup>) (Fig. 1f and Supplementary Fig. 2), thus indicating an autocrine rather than endocrine action of GABA in PTGs. To further confirm the ability of GAD1/2 to synthesize GABA in PTGs, we incubated human PTG extracts with deuterated d<sub>5</sub>-glutamate, the precursor of GABA, and measured the resulting d<sub>5</sub>-GABA. Using the multiple reaction monitoring (MRM) protocol illustrated in the Supplementary Fig. 3, we detected d<sub>5</sub>-GABA in the reactions by matching its mass spectrometry fragmentation and retention time, and the d<sub>5</sub>-GABA signal was absent in reactions without d<sub>5</sub>-glutamate (Fig. 1g), thus demonstrating the ability of parathyroid GADs to synthesize GABA.

Impact of parathyroid GABA<sub>B1</sub>R and GAD1/2 on PTH secretion. To define the actions of GABA<sub>B1</sub>R, GABA and GAD1/2 in PTGs, we studied the impact of GABA<sub>B1</sub>R agonists, baclofen (Bac), GABA and antagonist CGP54626 (CGP), on the [Ca<sup>2+</sup>]-mediated secretory responses in PTGs freshly isolated from male wild-type C57/B6 (B6:Wt) mice or from mice lacking expression of GABA<sub>B1</sub>R (hereafter referred to as GABA<sub>B1</sub>R-KO or PTGGABA<sub>B1</sub>R<sup>−/−</sup> mice) or GAD1/2 (GAD1/2-DKO) specifically in the glands. We first confirmed the ability of the PTH-Cre driver<sup>26</sup> to excise the Gabb1 genes specifically in PTGs of PTGGABA<sub>B1</sub>R<sup>−/−</sup> mice by PCR analyses of their genomic DNA (Supplementary Fig. 4) and to abrogate GABA<sub>B1</sub>R protein expression by immunoblotting and immunohistochemistry (Extended Data Fig. 1a,b, respectively). The ability of the PTH-Cre to ablate GAD1/2 was also confirmed by the absence of GAD1/2 protein expression in PTG tissue lysates.
Fig. 2 | Impact of GABA<sub>B</sub>1R and GAD1/2 on PTH secretion and mineral and skeletal homeostasis. a, b, PTGs (two or four per group) of 4-week-old C57/B6 wild-type (B6:WT) mice were sequentially incubated with increasing \([\text{Ca}^{2+}]_{i}\) from 0.5 to 3.0 mM (1 h per concentration) in the presence of vehicle (Veh, 0.1% DMSO, red circle), 300 \(\mu\)M GABA<sub>B</sub>1R agonist (GABA, green triangle) or baclofen (Bac, blue square) (a) or 10 \(\mu\)M GABA<sub>B</sub>1R antagonist, CGP54626 (CGP, green triangle) (b). Top panels show changes in the rate of PTH secretion on a per-gland and per-hour basis with raising \([\text{Ca}^{2+}]_{i}\) to compare the PTH-max. Bottom panels show normalized PTH secretion rate to the basal rate at 0.5 mM Ca\(^{2+}\) to better assess changes in the Ca\(^{2+}\)-set-point ([Ca\(^{2+}\)]\(_{i}\) needed to suppress 50\% of [Ca\(^{2+}\)]\(_{i}\)-suppressible PTH secretion). Colour dotted vertical lines indicate Ca\(^{2+}\) set-points for the corresponding treatments. Mean ± s.e.m. of \(n = 4\) groups of PTGs from eight mice for each treatment in a and five (Veh) or six (CGP) pairs of PTGs from five or six mice for b. * \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\) versus vehicle by two-way ANOVA with Sidak’s multiple comparisons test using Prism 8 statistics software.

t-Test. Means, average body weights (B.Wt.) (Fig. 2a), Average B.Wt. (i), sPTH and Ca\(^{2+}\) (sCa) levels (j) and PTG micrographs and quantified volumes (k) of GAD1/2-DKO mice and cont littermates with the n as indicated for each genotype. For k, PTGs were compressed into discs between a pair of glass slide and coverslip with a 120-µm spacer during fixation, washed, stained with blue fluorescent DAPI dye and imaged. Scale bars in k, 0.4 mm. Glandular volumes were calculated as the products of glandular areas ×120 µm and presented in the scatter histograms. Mean ± s.e.m. of \(n = 13\) mice as indicated per genotype. * \(P < 0.01\) versus Cont-Veh; & \(P < 0.01\) KO-Bac versus cont-Veh or cont-Bac by two-way ANOVA with Sidak’s multiple comparisons test using Prism 8 statistics software.

**Impact of GABAB1R and GAD1/2 on PTH secretion and mineral and skeletal homeostasis.** a, b, PTGs (two or four per group) of 4-week-old C57/B6 wild-type (B6:WT) mice were sequentially incubated with increasing \([\text{Ca}^{2+}]_{i}\) from 0.5 to 3.0 mM (1 h per concentration) in the presence of vehicle (Veh, 0.1% DMSO, red circle), 300 \(\mu\)M GABA<sub>B</sub>1R agonist (GABA, green triangle) or baclofen (Bac, blue square) (a) or 10 \(\mu\)M GABA<sub>B</sub>1R antagonist, CGP54626 (CGP, green triangle) (b). Top panels show changes in the rate of PTH secretion on a per-gland and per-hour basis with raising \([\text{Ca}^{2+}]_{i}\) to compare the PTH-max. Bottom panels show normalized PTH secretion rate to the basal rate at 0.5 mM Ca\(^{2+}\) to better assess changes in the Ca\(^{2+}\)-set-point ([Ca\(^{2+}\)]\(_{i}\) needed to suppress 50\% of [Ca\(^{2+}\)]\(_{i}\)-suppressible PTH secretion). Colour dotted vertical lines indicate Ca\(^{2+}\) set-points for the corresponding treatments. Mean ± s.e.m. of \(n = 4\) groups of PTGs from eight mice for each treatment in a and five (Veh) or six (CGP) pairs of PTGs from five or six mice for b. * \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\) versus vehicle by two-way ANOVA with Sidak’s multiple comparisons test using Prism 8 statistics software.

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term panel, green triangle) without a significant change in PTH-max (Fig. 2a, top panel, green triangle). In contrast, bocca, a selective GABA\(_B\)R agonist, markedly (P < 0.01) enhanced PTH-max to −580 pg.h\(^{-1}\) per gland (Fig. 2a, top panel, blue square) in the lower range of [Ca\(^{2+}\)], without significantly changing the Ca\(^{2+}\) set-point (Fig. 2a, bottom panel, blue square). These results indicate the ability of GABA\(_B\)R agonists to stimulate acute PTH secretion, particularly in the lower range of [Ca\(^{2+}\)] encountered in hypocalcaemic states. As homoceric CaSRs are anticipated to be inactive at low [Ca\(^{2+}\)], our data reveal an additional GABA\(_B\)R-mediated mechanism, other than the simple inactivation of homoceric CaSR, in promoting tonic PTH secretion.

Further supporting the GABA\(_B\)R action was the ability of a specific GABA\(_B\)R antagonist CGP54626 (CGP) to significantly reduce PTH-max (Fig. 2b, top panel, green triangle) and left-shift the Ca\(^{2+}\) set-point from 1.22 ± 0.01 to 0.87 ± 0.03 mM (Fig. 2b, bottom panel, green triangle) in B6 PTGs. These actions of CGP on secretory properties (that is, PTH-max and Ca\(^{2+}\) set-point) could be replicated in PTGs isolated from GABA\(_B\)R-KO mice with Cre/loxP-mediated deletion of both Gabb1 genes in their PTCs (see Methods for the generation of the mice). These knockout (KO) PTGs showed a reduction in tonic PTH secretion (PTH-max) (Fig. 2c, top panel, green triangle), a left-shifted Ca\(^{2+}\) set-point from 1.35 ± 0.07 mM in control (cont) PTGs to 1.15 ± 0.08 mM (Fig. 2c, bottom panel, GBR1-KO-Veh versus Cont-Veh) and inability of Bac to increase PTH-max in the GABA\(_B\)R-KO versus control PTGs (Fig. 2c, top panel, GBR1-KO-Bac versus Cont-Bac). These data not only support a critical role of GABA\(_B\)R in promoting tonic PTH secretion at low [Ca\(^{2+}\)], but also specify the receptor as a target of Bac in PTGs.

We next tested whether production of endogenous GABA in PTGs is required for PTH secretion by examining the secretory properties of PTGs lacking GAD1/2. As we anticipated, PTGs from the mice with concurrent ablation of both Gad1 and Gad2 genes in PTCs (GAD1/2-DKO) displayed a left-shifted Ca\(^{2+}\) set-point (from 1.29 ± 0.06 in control (cont) PTGs to 0.95 ± 0.08 mM in DKO) (Fig. 2d, bottom panel). However, they showed an increased PTH-max (Fig. 2d, top panel) instead of a reduced PTH-max as seen in GABA\(_B\)R-deficient PTGs (Fig. 2c, top panel). It is plausible that the differential effects of GABA versus GABA\(_B\)R KO on the secretory properties of PTG are due to the ability of GABA\(_B\)R to interact with other ligands made in PTGs to drive tonic PTH secretion in the absence of GABA. Nevertheless, given the higher efficacy and selectivity of Bac for GABA\(_B\)R in PTGs, we used Bac in subsequent experiments to better define GABA\(_B\)R actions of in PTCs.

Impact of parathyroid GABA\(_B\)R and GAD1/2 on growth and systemic homeostasis. We next examined systemic impacts of parathyroid GABA\(_B\)R and GAD1/2 by comparing changes in growth, mineral, hormonal and skeletal homeostasis in the PTG-GABA\(_B\)R\(^{-}\) (or GABA\(_B\)R-KO) and PTG-GAD1/2\(^{-}\) (or GAD1/2-DKO) mice versus their control littermates. Both male and female mice were studied at 3 months of age and did not show notable sex differences. We, therefore, reported data from only the male mice. The GABA\(_B\)R-KO mice showed smaller body sizes (Fig. 2e) and left-shifted Ca\(^{2+}\) set-points (from 1.29 ± 0.06 in control (cont) PTGs to 1.15 ± 0.08 mM in DKO) (Fig. 2f, bottom panel, GBR1-KO-Veh versus Cont-Veh) and inability of Bac to increase PTH-max in the GABA\(_B\)R-KO versus control PTGs (Fig. 2f, top panel, GBR1-KO-Bac versus Cont-Bac). These data not only support a critical role of GABA\(_B\)R in promoting tonic PTH secretion at low [Ca\(^{2+}\)], but also specify the receptor as a target of Bac in PTGs.

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Heteromerization between GABA\(_B\)R and CaSR in PTGs and in HEK293 cells. To test our hypothesis that GABA\(_B\)R interacts with CaSR to regulate PTH secretion, we first confirmed the formation of native CaSR/GABA\(_B\)R heteromers in mouse PTGs (Fig. 3a, left panels) and human parathyroid adenomas (Fig. 3b, left panels) by using a proximity ligation assay (PLA) with selective antibodies against the N termini of these receptors. These PLA signals were considered specific as they were absent in PTGs from PTG-GABA\(_B\)R\(^{-}\) (or GABA\(_B\)R-KO) mice (Fig. 3a, right panels) or human PTGs incubated with anti-CaSR antibody only (Fig. 3b, right panels). The formation of GABA\(_B\)R/CaSR heteromer in PTGs was further supported by specific coimmunoprecipitation of the CaSR with the GABA\(_B\)R from human parathyroid adenomas (Fig. 3c), as this signal was profoundly reduced in samples coimmunoprecipitated with non-immune sera (Extended Data Fig. 1e).

We further evaluated the propensity of GABA\(_B\)R and CaSR in heteromerization by a series of fluorescence resonance energy transfer (FRET) experiments using cultured human embryonic kidney (HEK)-293 cells expressing recombinant receptors. Specific intermolecular FRET was detected between CaSR and GABA\(_B\)R, which were C-terminally tagged with cyan and yellow fluorescent proteins (CFP and YFP), respectively (Fig. 3d), with an efficacy similar to the FRET signals recorded for the well-established GABA\(_B\)R/CaSR heteromer (Fig. 3e). The specificity of this CaSR/GABA\(_B\)R interaction is supported by minimal FRET signals between the plasma membrane-tagged CFP and YFP (CFPm and YFPm, respectively), or between CFP and YFP fused to the C terminus of GABA\(_B\)R (GABA\(_B\)R\(^{CFP}\)) and PTH receptor (PTH\(^{YFP}\)), respectively (Fig. 3e). We further corroborated the selectivity of CaSR/GABA\(_B\)R heteromer through a bimolecular fluorescence complementation (BiFC) assay. This assay is based on the generation of functionally complemented fluorescent protein by physical association of two halves of N- and C- of fluorescent proteins that are brought in close proximity by specific interactions of two molecules tagged with the complementary fluorescent protein fragments.

Specifically, HEK293 cells were transfected with an equal molar ratio of complementary DNA to express simultaneously (1) the CaSR C-terminally fused to the N terminus fragment of CFP (residues 1–158) (CaSR\(^{N-CFP}\)), (2) the GABA\(_B\)R C-terminally fused to the N terminus fragment of YFP (GB1RN-YFP) and (3) the GABA\(_B\)R C-terminally fused to the C terminus fragment of CFP (residues 158–239) (GABA\(_B\)R\(^{C-FP/CYFP}\)), which is identical to and functionally exchangeable with the C terminus fragment of YFP. The relative amount of CaSR\(^{N-CFP}/GABA\(_B\)R\(^{C-YFP}\) heteromer versus GABA\(_B\)R\(^{N-YFP}/GABA\(_B\)R\(^{C-YFP}\)) homomer was then determined by recording BiFC-CFP versus BiFC-YFP fluorescence, respectively. We observed only a saturable CFP (CaSR/GABA\(_B\)R) signal (Fig. 3f,g) and minimal YFP (GABA\(_B\)R/GABA\(_B\)R) signal, indicating a preference in the formation of CaSR/GABA\(_B\)R heteromer over GABA\(_B\)R/GABA\(_B\)R homomer. The propensity of CaSR/GABA\(_B\)R heteromerization was again comparable to that of GABA\(_B\)R/GABA\(_B\)R heteromerization as indicated by their comparable BiFC signals (Fig. 3f). Taken together, our data reveal strong propensity of CaSR and GABA\(_B\)R to form heteromers in transfected cells and PTCs.

Impact of GABA\(_B\)R/CaSR heteromer on signalling responses of homoceric CaSRs. Given the ability of CaSR to chaperone GABA\(_B\)R to cell surface in the form of a heteromer in transfected HEK293 cells, we tested whether GABA\(_B\)R traffics with homoceric CaSR to the cell surface and interfaces with signalling responses of the last receptor there, particularly in response to binding to GABA\(_B\)R agonists. As previous studies suggested that...
homomeric CaSR mediates the inhibitory action of extracellular Ca\(^{2+}\) on PTH secretion through Gq/G11 and Gi activation, we first confirmed this notion by assessing PTH secretion in PTGs cultured from mice with PTG-specific deletion of G\(\alpha\)q in the background of germline G\(\alpha\)q KO (\(\text{Gq}^\text{−/−}/\text{G11}^\text{−/−}\)) and in wild-type PTGs treated with pertussis toxin (PTX) to block Gi activation, respectively. We showed that ablation of Gnaq and Gna11 genes in PTGs blocked the ability of raising [Ca\(^{2+}\)], to suppress PTH secretion, as indicated by the right-shifted Ca\(^{2+}\) set-points in a gene-dosage-dependent manner (Extended Data Fig. 3), while PTX enhanced PTH secretion, particularly in the lower range of [Ca\(^{2+}\)], in which baclofen promotes PTH secretion (Extended Data Fig. 4). In PTGs cultured from mice lacking G\(\alpha\)q and G\(\alpha\)11, we observed increases in PTH secretion with raising [Ca\(^{2+}\)], in the lower range of [Ca\(^{2+}\)], (0.5 to 1.5 mM) (Extended Data Fig. 3). The last effects were, however, absent in PTGs cultured from mice lacking both alleles of Casr genes (Extended Data Fig. 3). These data not only confirmed the requirement of both Gq/11 and Gi action in mediating the high [Ca\(^{2+}\)]-induced suppression of PTH, but also unveiled a Gq/11-independent action of Ca\(^{2+}\)/Casr, probably by activating the CaSr/GABAB\(_R\) heteromer to promote PTH secretion.

We then measured time courses of G-protein (Gq or Gi) activation using FRET-based biosensors\(^{12,13}\) in HEK293 cells expressing the recombinant CaSr and/or GABAB\(_R\). Activation of Ca\(^{2+}\) to single cells expressing the CaSr alone induced a fast increase in FRET ratios reflecting Gq (Fig. 4a) or Gi (Fig. 4d) activation. Coexpression of GABAB\(_R\) with the CaSr suppressed the maximal activation of Gq (Fig. 4b) and Gi (Fig. 4e) mediated by Ca\(^{2+}\) by \(\sim 30–50\%\) (Fig. 4c,f). These inhibitory effects were further exacerbated by addition of baclofen (Fig. 4b,c), thus indicating reduced homomeric CaSr signalling when CaSr/GABAB\(_R\) complexes are activated by baclofen (Fig. 4c,f). Baclofen did not affect Gq or Gi
activation via CaSR alone (Fig. 4a, d), nor did Ca²⁺ affect G-protein activation via GABAB₃R when each receptor was expressed alone in HEK293 cells. Similar inhibitory action of baclofen on Ca²⁺-mediated Gq activation were obtained in rat parathyroid-derived PTH-C1 cells (Extended Data Fig. 5a) with (left panel) or without (right panel) supplementation of exogenous CaSR and GABAB₃R cDNAs. The ability of baclofen to inhibit CaSR- or Gq activation, when GABAB₃R was coexpressed, was also observed at
the second-messenger level. Specifically, we studied effects of high [Ca\(^{2+}\)], on receptor-mediated activation of total inositol phosphate turnover (IP\(_{\text{total}}\)) and inhibition of cAMP in cells pretreated with cholera toxin (CTx) that induces persistent activation of the Go/adenylate cyclase/cAMP pathway as downstream readouts of Gq and Gi signalling, respectively. In cells expressing CaSR alone, high [Ca\(^{2+}\)], stimulated IP\(_{\text{total}}\) in a concentration-dependent manner (Fig. 4g, black circles). This effect of high [Ca\(^{2+}\)], decreased markedly when GABA\(_B1\)R was coexposed, and baclofen further decreased this effect by ~15% (Fig. 4g, blue circles, and 4h). Pretreatment of PTH-C1 cells expressing CaSR alone with CTx increased the basal level of cAMP, and high [Ca\(^{2+}\)], markedly decreased the production of cAMP (Extended Data Fig. 5b and Fig. 4i). Coexpression of GABA\(_B1\)R decreased the efficacy of high [Ca\(^{2+}\)], to block cAMP accumulation (Fig. 4j), and baclofen further prevented the inhibitory action of high [Ca\(^{2+}\)], (Fig. 4k). The observed effect of baclofen probably resulted from a direct binding to GABA\(_B1\)R because addition of a competitive GABA\(_B1\)R antagonist, CGP54626, reversed the action of baclofen and restored the cAMP response to a level similar to that caused by high [Ca\(^{2+}\)], in the absence of baclofen (Fig. 4k). Baclofen did not modulate IP\(_{\text{total}}\) or cAMP production by itself, thus indicating that these effects were dependent on the coactivation of both CaSR and GABA\(_B1\)R by their respective ligands. These signaling data together suggest that following baclofen binding, a conformational change transmitted from the GABA\(_B1\)R to the homomeric CaSRs reduces efficacy of Gq, and Gi activation (extent and duration of G-protein signalling) of the latter receptors as a molecular basis to promote PTH secretion.

Increased GABA\(_B1\)/CaSR heteromers in hyperplastic PTGs from patients with 1° and 2° HPT. We reasoned that reduced CaSR expression in PTG, a well-established characteristic of both 1° and 2° HPT, renders a receptor stoichiometry favouring the formation of CaSR/GABA\(_B1\)R heteromers, thus promoting PTH secretion. To test this hypothesis, we compared the expression of CaSR and GABA\(_B1\)R by immunohistochemistry and expression of CaSR/GABA\(_B1\)R heteromer by PLA in PTGs surgically removed from patients who were clinically diagnosed with 1° or 2° HPT (Supplementary Table 1) to the levels in normal PTG tissues that were excised during independent pathway.

To test whether the parathyroid GABA\(_B1\)R is involved in PTH hypersecretion resulting from chronic Ca\(^{2+}\)-deficiency, we subjected the GABA\(_B1\)-KO mice and their cont littersmates to a continuous low Ca\(^{2+}\) diet (0.02 versus 1% in the normal diet) for 4 weeks. Our data showed that the ability of this diet to increase serum PTH levels (by ~50%) in cont littersmates was completely lost in the GABA\(_B1\)-KO mice (Fig. 6g, left panel), despite the fact that their serum [Ca\(^{2+}\)] remained lower than cont mice (Fig. 6g, right panel), supporting a role for the GABA\(_B1\)R in mediating hypocalcaemia-induced PTH hypersecretion.

Discussion

Our data unveil a biological action of the GABA/GABA\(_B1\)R system in mediating PTH secretion to maintain mineral balance and normal skeletal development and a pathological role in enabling PTH hypersecretion in HPT states. Our demonstrations of GAD1/2 expression and GABA biosynthesis in PTGs and the ability of GAD1/2 KO to produce hypoparathyroidism further support an autocrine scheme in which PTGs release GABA to alter PTH secretion by attenuating homomeric CaSR signalling through allosteric interactions between the CaSR and GABA\(_B1\)R receptors (Fig. 7). We speculate that inherent declines in CaSR expression in 1° and 2° HPT disease conditions decreases levels of CaSR homomers and at the same time increases the likelihood for GABA\(_B1\)/CaSR heteromer formation. However, concurrent reductions of GABA\(_B1\)R and CaSR expression in PTGs of patients with 1° HPT and 2° HPT suggest that mechanisms other than simple stoichiometric ratio changes in receptor expression...
(that is, \(\text{GABA}_{\beta 1}\)R over \(\text{CaSR}\)) could also be involved to alter the propensity of \(\text{GABA}_{\beta 1}\)R/CaSR heteromerization. A notable observation is the distinct pharmacological profiles of \(\text{Bac}\) and \(\text{GABA}\) in mediating PTH secretion. The former tends to increase PTH-max, while the latter shifts the \(\text{Ca}^{2+}\) set-points (Fig. 2a). Given that the only structural difference between \(\text{GABA}\)- and \(\text{baclofen}\)-occupied \(\text{GABA}_{\beta 1}\)R is the orientation adopted by its N-terminal extracellular residue W276 (refs. 36,37), the distinct efficacy and potency, by which \(\text{GABA}\) and \(\text{baclofen}\) act on \(\text{GABA}_{\beta 1}\)R/CaSR heteromer to interfere with the homomeric CaSR, might reflect distinct conformational change of the \(\text{GABA}_{\beta 1}\)R/CaSR heteromer when it interacts with \(\text{GABA}\)- versus \(\text{baclofen}\)-bound \(\text{GABA}_{\beta 1}\)R. These differential signalling responses are also reflected by the different phenotypic manifestations between \(\text{GABA}_{\beta 1}\)R-KO and GAD1/2-DKO mice. \(\text{GABA}_{\beta 1}\)R-KO mice appear to produce more severe growth defects than the GAD1/2-DKO on the basis of their body weights (Fig. 2f versus 2i). \(\text{GABA}_{\beta 1}\)R KO also had more profound impact on reducing PTH-max than GAD1/2 DKO in the PTGs (Fig. 2g versus 2j).

Several questions remain to be answered to further understand the mechanism of the process described here. Among them are the following:

How does the CaSR heteromerize with the \(\text{GABA}_{\beta 1}\)R? While covalent disulfide bonding in the N-terminal extracellular domain (ECD) of the CaSR is involved in its homodimerization, the ability of CaSR-ECD (a.a. 1–579) alone to complex with the \(\text{GABA}_{\beta 1}\)R-
lacks a contributing cysteine-rich domain in its ECD, however, supports critical noncovalent interactions between the ECDs of the receptors in CaSR/GABA B1 R heteromerization. Given that a polar interface, instead of hydrophobic action seen in the dimerization of mGlURs, is critically involved in GABA B1 R/GABA B2R heterodimerization38 and that GABA B1 R and GABA B2R mutually compete for heterodimerization with CaSR22, it is an attractive hypothesis for heterodimerization with CaSR22, it is an attractive hypothesis that formation of ionic salt bridges between the ECDs of CaSR and GABA B1 R as well as the helix–helix interactions in the hydrophobic transmembrane domains are needed to mediate the complex formation and, possibly, the respective ligands (GABA and Ca2+). Mapping of the interaction sites between these two receptors permit new strategies for blocking the formation and/or functions of CaSR/GABA B1 R heteromers as a new pharmacological means to prevent PTH hypersecretion.

How does GABA B1 R interfere with the coupling of homomeric CaSRs to downstream Gq/11 and Gi signalling responses? This interaction may take place in an acute dynamic fashion between two or more complexes (that is, CaSR/GABA B1 R heterodimers and CaSR/CaSR homodimer) immediately after binding to their respective ligands. However, given that simply coexpressing GABA B1 R (without Bac) is sufficient to reduce coupling of CaSR to downstream signalling responses, we prefer the model involving a pre-assembled higher-order receptor complex (for example, trimer, tetramer and so on) containing both CaSR/GABA B1 R heterodimers and CaSR/CaSR homodimer with a predetermined stoichiometric composition. In transfected HEK cells, we observed colocalization of recombinant CaSR and GABA B1 R as early as in the perinuclear endoplasmic reticulum38, suggesting that this pre-assembled complex may take place immediately after protein synthesis.

Can additional parathyroid GPCRs be upregulated in pathological conditions and interact with CaSR and/or GABA B1 R? This is particularly pertinent given that GPR64, an orphan adhesion GPCR whose expression is upregulated in parathyroid adenomas39, has been proposed to physically interact with the CaSR.

Can the GABA B1 R regulate PTH secretion independently of the CaSR? While our present results demonstrate the interaction between CaSR and GABA B1 R as a means to regulate PTH secretion from PTGs, the robust PTH secretion in the absence of CaSR in the PTGs of the PTGCaSR KO indicates ablation of ~90% of CaSR in the PTGs of the PTGCaSR KO mice (Fig. 6f, top panel, brown circles) and profound abrogation of this hypersecretory activity in PTGs of PTGCaSR KO mice (Fig. 6f, top panel, yellow squares) raise the possibility of a CaSR-independent action of GABA B1 R in promoting PTH secretion. Alternatively, a minute amount (~10%) of CaSR due to incomplete Casr gene excision could readily interact with GABA B1 Rs to promote PTH secretion, as shown previously that PTH-Cre permits ablation of ~90% of CaSR in the PTGs of the PTGCaSR R/− mice46.

Fig. 6 | Impacts of GABA B1 R KO on PTH secretory functions and mineral and hormonal status in mouse models of hereditary HPT and chronic Ca2+-deficiency. a, Pictures of 3-month-old male mice with heterozygous or homozygous GABA B1 R KO in the background of heterozygous or homozygous CaSR KO in their PTCs and their cont littermates and 3-week-old mice with homozygous CaSR KO, which usually die between 3–4 weeks of age. b–d, Average body weights (b), sPTH (c) and Ca2+ levels (d) in mice with PTG-specific heterozygous (+/−) or homozygous (−/−) CaSR and heterozygous (+/−) or homozygous (−/−) GABA B1 R KO and cont littermates carrying floxed-alleles without PTH-Cre expression. Mean ± s.e.m. of n mice for each group as indicated below the genotype. *P < 0.05, **P < 0.01 between groups by one-way ANOVA with Sidak’s test. e, Assessment of PTH secretory properties (PTH-max and Ca2+ levels) in mice with GABA B1 R KO and cont littermates carrying floxed-alleles without PTH-Cre expression. Mean ± s.e.m. of n (>1) mice as indicated below the genotype. f, Serum PTH and Ca2+ levels in 16-week-old male PTGCaSR KO and cont littermates after feeding with normal (1%) or low Ca2+ (0.02%) diets for 4 weeks. Mean ± s.e.m. of n = 8–12 mice as indicated, P values between groups as indicated were assessed by one-way ANOVA with Sidak’s test.
experiments, 12-week-old male PTGGABAB1 R sex differences. We, therefore, only reported data from male mice. For dietary Palmiter (University of Washington) and obtained from Q. Wu (Baylor College of regulation of CaSR signalling and function by the GABAB1 R also neurons and many vital peripheral tissues, we anticipate that the and female CaSRflox/wt//GABAB1 Rflox/wt mice, which were then used to produce and/or Floxed-CaSR mice were bred to obtain male PTGCaSR complex is speculative and remains to be determined.

hPT states. The presented stoichiometric composition of the receptor been used to treat neurological disorders. effects of more generalized pharmaceutics such as baclofen that has effectiveness of disease treatment and prevent the unwanted side- effects of more generalized pharmaceutics such as baclofen has been used to treat neurological disorders.

While future studies are required to address these questions, our results unveil the unexpected and determinant role of GABA and GABAB1 R in regulating CaSR signalling and PTG functions. Considering that the GABA R and CaSR, are expressed in central neurons and many vital peripheral tissues, we anticipate that the regulation of CaSR signalling and function by the GABA R also impacts biological processes beyond mineral and skeletal homeostasis. Development of targeted pharmaceuticals against cell and/or subcellular domain-specific heteromers could also improve the effectiveness of disease treatment and prevent the unwanted side-effects of more generalized pharmaceutics such as baclofen that has been used to treat neurological disorders.

Methods

Detailed information on experimental design and reagents included in the study is attached in the affiliated Reporting Summary document.

Mice. All animal procedures were approved by the Institutional Animal Care and Use Committee of the San Francisco Department of Veteran Affairs Medical Center (Protocol numbers 2012-035, 2015-029 and 2015-003). Wild-type C57/B6 and PTH-Cre mice48 (Stock no. 005989) were purchased from the Jackson Laboratory. Floxed-GABA R mice were provided by B. Beterli (University of Basel, Switzerland). Floxed-GAD1/mice were generated by R. Palmer (University of Washington) and obtained from Q. Wu (Baylor College of Medicine). Floxed-CaSR mice were made previously. PTH-Cre, Floxed-GABA R and/or Floxed-CaSR mice were bred to obtain male GABA R−/− and female GABA R−/− mice, which were then used to produce GABA R−/−/CaSR−/−, GABA R−/−/CaSR−/−, GABA R−/−/CaSR−/−/− and GABA R−/−/CaSR−/−/− mice and cell litters. The last litters carry one or two alleles of floxed-CaSR and/or floxed-GABA R without PTH-Cre transgene. PTH-Cre and Floxed-GAD1/LGAD2 mice were bred to obtain GAD1−/−/GAD2−/−/− and female GAD1−/−/−/− and GAD2−/−/−/− mice that were then crossed to produce GAD1−/−/−/− and double KO (or GAD1−/−/− and GAD1/2-DKO) mice and cell litters. Cell litters were carried fixed and/or analyzed by PCR using primers for genotyping and for CaSR, GABA B1 R, GABA B2 R or GABA B3 R. PCR products were sequenced. Ex vivo PTG cultures. Mouse PTGs were isolated, dissected free of thyroid and surrounding fibrous tissues, and cultured to assess PTH-max and Ca2 + levels. Serous cells were grown in selection medium (DMEM, 10% FBS, 0.135 mg ml−1). Human PTG cultures. Deidentified parathyroid tissues (nine 1° HPT, seven 2° HPT and five normal controls; see Supplementary Table 1), were obtained by endocrine surgical collaborators in the Department of Surgery, University of California San Francisco (UCSF) as a routine patient treatment procedure, after obtaining a general informed consent. The PTG samples were later retrieved by Khanafsahr from the archives of Department of Pathology, University of California San Francisco under a human research protocol (no. 15-17253) approved by UCSF Institutional Review Board on the basis of the following clinical criteria verified by medical record review by E. Khanafsahr, D.M. Shoback and A. Herberger. Criteria used for selection of the tissues for study were the following. 1° HPT: (1) serum Ca2 + ≤2.6 mg dl−1; (2) serum PTH levels in setting of hypercalcæmia and (3) the absence of a known genetic basis for the 1° HPT (for example, MEN1). 2° HPT: (1) CKD stage 5 on dialysis; (2) elevated serum PTH levels and (3) symptoms of uremic 2° HPT necessitating surgical intervention as determined by referring nephrologists. Normal PTGs: (1) glands inadvertently removed during thyroid operations identified in the pathologic specimens; (2) no known abnormality in serum Ca homeostasis and (3) PTGs classified as histologically normal by attending pathologists. The PTGs were fixed in 4% PFA, embedded in paraffin, sectioned into thicknesses of 5 μm and used for immunohistochemical detection for CaSR and/or GABA B1 R, as described in the Immunohistochemistry section.

Immunohistochemistry. Mouse PTGs microdissected free of thyroid and other soft tissues from wild-type C57/B6 mice and GABA B1 R+/−/−, GABA B1 R−/−, GABA B2 R−/− and GABA B3 R−/− and cont litters were embedded in paraffin, and sectioned into thicknesses of 5 μm. Immunohistochemical detection of CaSR, GABA B1 R, GABA B2 R, GABA B3 R, mouse and/or human PTG sections were performed with a rabbit custom-made rabbit polyclonal anti-N-CaSR (VA609, ADDDDYGRPGIEKREAAERDI) (1 μg ml−1); a guinea pig anti-GABA B1 R (CPSPEPDRDLSDSSGRVHLYLK) (1 μg ml−1); a rabbit anti-GABA (catalogue no. A2052, SIGMA) or a rabbit anti-GAD1/2 (catalogue no. Ab49832, Abcam), and corresponding peroxidase–or fluorescein isothiocyanate-conjugated secondary antibodies. All results were obtained with the 3.3 ‘di-amino-benzidine (DAB)–substrate as previously described. The specificity of anti-GABA B1 R, anti-N-CaSR, anti-GAD1/2 and anti-GABA antibodies was confirmed by the lack of immunoreactivity in protein lysates (Extended Data Fig. 1a,c) or tissue sections (Extended Data Figs. 1d and 2a,b).
were performed as previously described45. In brief, cells grown on coverslips were stained for mouse monoclonal antibody against the N terminus of GABAB1 R (Abcam ab55051, 20 μg/mL) and anti-N-CaSR (VA609, 4 μg/mL) at 4°C overnight, followed by sense and antisense oligonucleotide-conjugated secondary antibodies at 37°C for 1 h. DNA ligation was performed with DNA ligase (25 U/μL) at 37°C for 1 h. After incubation with red fluoro-conjugated DNA probes and washing, the sections were mounted with DAPI-containing mounting medium, imaged with a Zeiss Axio Imager 2 Microscopes (Carl Zeiss), and quantified using automated TissueQuest Analysis software (TissueGnostics). Total PLA activities in the regions of interest were divided by total DAPI-positive cell number in the regions of interest to obtain mean PLA activity per cell. The specificity of anti-N-GABAB1 R (Abcam ab55051) was demonstrated by the lack of PLA signalling in the GAGAB1 −/− mice (Fig. 3a).

Immunoprecipitation and immunoblotting. Membrane proteins (400 μg) extracted from human parathyroid adenoma were immunoprecipitated with 7.5 μg of mouse monoclonal antibody against CaSR C-terminal tail (1C7E4-1B, CKNSDEBFQQERQKQ) or guinea pig polyclonal antibody against GABAB1 R or pre-immune guinea pig IgG as described previously16. Immunoprecipitated and naive membrane protein (50 μg) were electrophoresed on polyacrylamide gels, transferred onto polyvinylidine difluoride membrane and immunoblotted with a guinea pig polyclonal antibody against GABABR1 (1:1,000 dilution) or a rabbit polyclonal antibody against CaSR (VA609, 1:1,000 dilution) as previously described16. Protein reactivity was detected using the Clarity Western ECL substrate (BIO-RAD laboratories) and visualized by ChemiDoc XRS+ (TILL Photonics). Samples were excited with a xenon lamp from a polychrome V 10 nm and a beam splitter dichroic long-pass of 505 nm.

To determine agonist induced changes in FRET, cells were continuously superfused with the HEPES buffer and the agonist was applied using a computer-assisted, solenoid-valve controlled rapid superfusion device (ALA-VM8, ALA Scientific Instruments; solution exchange 5–10 ms). Signals detected by avalanche photodiodes were digitalized using an analog-to-digital converter (Digidata1322A, Axon Instruments) and stored on a PC using Clampex 9.0 (Axon Instruments). Data were analysed using OriginPro 8.0 and plotted in GraphPad Prism 7.0.

Photobleaching experiments. FRET between CFP and YFP in cells expressing the receptor constructs was also determined by donor recovery after acceptor bleaching27. The emission intensity of CFP was first recorded at 436-nm excitation (CFPbefore), followed by direct illumination of YFP at 500 nm for 3–5 min. Subsequently, the emission intensity of CFP was recorded again (CFPafter). FRET efficiency was calculated according to equation (3).

\[ \text{FRET efficiency} = 1 - \frac{\text{CFP}_{\text{after}}}{\text{CFP}_{\text{before}}} \] (3)

Skeletal analyses by micro-computed tomography. To compare bone mineral content and structural parameters, we performed micro-computed tomography (μCT) scans at two sites: distal femur for trabecular ( Tb) bone and tibio-fibular junction for cortical ( Ct) bone as described18. Briefly, femurs and tibias were isolated, fixed in 10% neutral buffered formalin for 24 h, stored in 70% ethanol and scanned by a SCANTOM viva CT 40 scanner (SCANCO Medical) with 10.5 μm voxel size and 55-μV X-ray energy. For Tb bone in the distal femoral metaphysis, 100 serial cross-sectional slices (1.05 mm) of the secondary spongyosa were obtained from the end of the growth plate extending proximally. For Ct bone, 100 serial cross-sections (1.05 mm) of the tibia were obtained from the tibio-fibular junction extending proximally. A threshold of 420 mg hydroxyapatite/m3 was applied to segment total mineralized bone matrix from soft tissue. Linear attenuation was calibrated using a μCT hydroxyapatite phantom. Three-dimensional image reconstructions and analyses were performed using the manufacturer’s software to obtain the following structural parameters: Tb tissue volume, Tb bone volume, Tb bone fraction (%), Tb number, Tb connectivity density, Tb thickness, Tb spacing, Ct tissue volume and Ct bone volume.

Live cell cAMP signalling. PTH-C1 cells were transiently transfected with CaSR, GABAB1 R and the intramolecular cAMP FRET sensor (epac1-CFP/YFP) with Lipofectamine 3000 (Thermo Fisher). Cells were next plated on 25 mm glass coverslips coated with collagen and mounted in Attolux fluor cell chambers (Life Technologies), maintained in HEPES buffer and transferred on a Nikon Ti-E equipped with an oil immersion 1.30 Plan Apo objective and a moving stage (Nikon Corporation). FRET measurement was monitored as previously described (NCB 2017). Cells pretreated with cholera toxin (Ctx) for 1 h to elevate basal cAMP then changes in cAMP dynamics were monitored following the addition of CaSR and/or GABAB1 R ligands.

Inositol phosphate signalling. Levels of total inositol phosphates (InsPs) including all isomers of InsP3, InsP2, and InsP1, as an index of PLC activation were determined in HEK293 cells transiently transfected with CaSR and GABAB1 R DNA individually or in combination after incubating the cells with different [Ca2+] (0.5–10 mM) with or without baclofen (300 μM) for 60 min as reported previously19. Prelabeling of membrane phosphophosphates with [γ-3H]myo-inositol was done before stimulating the cells.

Detection of GABA by mass spectrometry. Sample preparation. Human PTG and mice brain tissues were homogenized individually by RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 1% sodium...
deoxycholate, 0.1% SDS) with freshly added phenylmethylsulfonyl fluoride to 1 mM and with freshly added aprotinin and leupeptin to 5 μg/ml just before use. Additionally, 50 μM d-glucamic acid and 10 μM pyridoxal-5-phosphate hydrate were added into the lysate to synthesize d-glutamate. GABA. Proteins were precipitated by acetonitrile with 0.5% TFA. The supernatant was dried in vacuum and resuspended in acetonitrile and water (50:50, v/v) for C18-desalting. Finally, the desalted product was dried and stored at −80 °C until the liquid chromatography–tandem mass spectrometry LC–MS/MS analysis.

GABA measurement by using LC–MS/MS. Calibration standards for GABA were prepared at concentrations of 5, 10, 25, 50, 100, 250, 500, 1,000 and 2,500 nM acetonitrile and water (50:50, v/v). Quality control samples were prepared independently, including low, medium and high concentrations (10, 500 and 5,000 nM). All samples were stored at −80 °C until the LC–MS/MS analysis. Liquid chromatography was achieved by an ACQUITY ultrahigh-performance liquid chromatography (UPLC) System (Waters) and separation was carried out using a BEH HILIC column (2.1 × 50 mm, 1.7 μm) at 30 °C. Acetonitrile was used as the mobile phase, and 0.3% formic acid in water was used as the aqueous phase. The flow rate was set at 0.4 ml/min, and the injection volume was 5 μl. The UPLC system was coupled to a Waters Xevo TQD mass spectrometer (Waters). The electrospray ionization mode was positive ionization with the following parameters: capillary voltage, 3,055 V; nebulizer gas (N₂) flow rate, 10.81 ml/min; gas temperature, 450 °C. A triple quadrupole mass spectrometer in MRM mode was employed to identify and quantify GABA (transition m/z 104.02 → M + [H] → 87.1, collision energy 3 eV) and β (d5-GABA) (transition m/z 110.02 → M + [H] → 93.1). The Q1 mass analyser first filters the parent ion of GABA (m/z 104.02). Only ions whose m/z value is 104.02 enter the collision cell for fragmentation. The transition of m/z 104.02 → 87.05 is followed for GABA detection and quantitation in the Q3 mass analyser. All data were analysed using the Masslynx software package (Waters).

Statistics. Comparisons between groups were subjected to statistical analysis using a two-tailed Student’s t-test for two groups, or one-way or two-way analysis of variance (ANOVA) followed by Sidak’s for multiple comparisons using Prism 8 (GraphPad Software, Inc.). Animal, organ culture and cultured cell–sample sizes were determined by a power analysis using the following parameters: standard deviation of 5–10% depending on the assay, two-sided test, P = 0.05, power of the test was 0.8. Data from two groups were represented as mean ± standard error of the mean (s.e.m). Significance was assigned for P < 0.05.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All materials, data, animal models and associated protocols will be made available to all qualified investigators from the corresponding authors on reasonable request or with a simple institutional material transfer agreement. Source Data for Figs. 1 and 3, and Extended Data Fig. 1 are available online.

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Author contributions
W.C., A.H., D.M.S., C.-L.T. and J.-P. V. designed the study. W.C., A.H., C.-L.T., J.H., H.H., A.L., Z.C. and J.-P. V. conducted the study; W.C., A.H., A.D.W., F.J.-A., C.-L.T., J.H., H.H., A.L., Z.C. and J.-P. V. collected data. Q.-Y.D., W.S., and I.S. evaluated the patients, obtained informed consent and conducted the surgeries for all human PTG studies. E.K., D.M.S. and A.H. reviewed clinical and pathology records related to human PTG samples. K.K. provided expertise with mass spectroscopy and performed experiments with the support of H.L. and D.W. W.C., A.H., C.-L.T. and J.-P. V. performed data analyses. W.C., A.H., E.K., D.M.S., C.-L.T. and J.-P. V. interpreted the data. W.C. and J.-P. V. wrote the manuscript with the support of A.H., C.-L.T., D.M.S. and H.K. W.C. and J.-P. V. take responsibility for the integrity of the data analysis.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Expression of CaSR and GABA_{A,R} in mouse and human PTGs. 

**a, b.** Membrane protein lysates (50 µg/lane) (**a**) and tissue sections (**b**) of PTGs from \(^{+/+}\) (control) and \(^{-/-}\) (KO) mice were probed with anti-GABA_{A,R} antibody for expression of GABA_{A,R} as described in On-line Methods. In panel (**a**), a \(\approx 100\) kD (unglycosylated) GABA_{A,R} and \(\approx 130\) kD and \(\approx 150\) kD (presumably glycosylated) were detected in the control, but not KO, PTGs. \(n=2\) batches of PTGs from a total of 20 mice/genotype. 

**c, d.** Membrane protein lysates (50 µg/lane) (**c**) and tissue sections (**d**) of PTGs from \(^{+/+}\) (control) and \(^{-/-}\) (KO) mice were probed with anti-N-CaSR (VA609) antibody for expression of CaSR. In panel (**c**), a \(\approx 120\) kD unglycosylated CaSR and \(\approx 140\) kD and \(\approx 160\) kD glycosylated (arrowheads) and larger aggregates were detected in the control, but not KO PTGs. \(n=2\) batches of PTGs with a total of 16 mice/genotype. Panels b and d show brown DAB staining, indicating immunoreactivity of GABA_{A,R} and CaSR, respectively, and blue/purple haematoxylin counterstaining in mouse PTGs. 

**e.** Membrane proteins (400 µg) extracted from human parathyroid adenomas were subjected to immunoprecipitation (Imppt) with CaSR antibodies or non-immune IgG and immunoblotted (IB) along with non-Imppt controls (input, 50 µg) with either CaSR or GABA_{A,R} antibodies. Left panels demonstrate the ability of CaSR antibody to pull down \(\approx 140\) and 150 kD glycosylated CaSR (arrowheads) and large aggregates (*) along with \(\approx 100\) kD unglycosylated and \(\approx 130\) kD glycosylated GABA_{A,R} (open arrow). Two right panels demonstrate the ability of GABA_{A,R} antibody to pull down \(\approx 100\) kD unglycosylated and \(\approx 130\) kD glycosylated GABA_{A,R} (open arrow) along with the \(\approx 140\) kD glycosylated CaSR (arrowhead) and large aggregates (*). \(n=3\) human PTG lysates.
Extended Data Fig. 2 | Expression of GAD1/2 and GABA in mouse and/or human PTGs.  

**a**, Sections of PTGs from control (Cont) and GAD1/2-DKO mice were probed with anti-GAD1/2 antibody and FITC-conjugated secondary Ab and counterstained with blue fluorescent DAPI nuclear dye (a) or probed with anti-GABA antibody and HRP-conjugated secondary Ab and counterstained with hematoxylin (b) as described in On-line Methods. Inserts show digitally enlarged views of the white box areas. n = 12 PTGs from 6 mice for each genotype.  

**b**, PTG sections from B6:Wt mice (top panels) and patients with 1o hPT (bottom panels) were probed with anti-GABA antibody (left panels) or non-immune IgG (right panels), followed by horseradish peroxidase (HRP)-conjugated secondary Ab. For panels (b) and (c), brown immunoreactivity signals were developed by immersing the sections with 3,3’-diaminobenzidine (DAB) substrate and counterstained with blue hematoxylin as described in On-line Methods. n = 8 PTGs from 4 mice and 4 human PTGs.
Extended Data Fig. 3 | PTH secretion from PTGs lacking Gq and G11 or CaSR. Secretory properties of PTGs from 8-wk-old male \( \text{PTGGq}^{+/+}/\text{G11}^{++} \) (\( n = 12 \) pairs PTGs from 12 mice), \( \text{PTGGq}^{-/-}/\text{G11}^{++} \) (\( n = 15 \) pairs PTGs from 15 mice), and \( \text{PTGGq}^{-/-}/\text{G11}^{-/-} \) (\( n = 3 \) pairs PTGs from 3 mice) mice, which carry PTG-specific Gnaq and/or germ-line Gna11 gene KO alleles, 4-wk-old \( \text{PTCCaSR}^{-/-} \) mice, which carry PTG-specific Casr gene KO alleles (\( n = 5 \) pairs PTGs from 5 mice), and control littermates (\( n = 7 \) pairs PTGs from 7 mice), which carry floxed-Gnaq and wild-type Gna11 without Cre expression, were assessed by incubating the glands with a series of media containing increasing \([\text{Ca}^{2+}]_{o}\) (from 0.5 to 3 mM). PTH secretory rates were normalized to the rate of basal secretion rate at 0.5 mM \( \text{Ca}^{2+} \) to calculate the \( \text{Ca}^{2+} \) set-points, indicated by vertical dashed lines. Mean ± s.e.m.
**Extended Data Fig. 4 | Effect of pertussis toxin on PTH secretion from PTGs.** PTGs (2 per group) from wild-type C57/B6 were sequentially incubated with increasing \([\text{Ca}^{2+}]_e\), from 0.5 to 2.0 mM (1 hr for each concentration) in the presence of vehicle (0.1% DMSO) or baclofen (Bac, 300 µM) with or without preincubation with pertussis toxin (PTx, 100 µg/ml, 3 hrs). Mean ± s.e.m. of n pairs of PTGs from n mice as indicated. P values vs Vehicle controls were assessed by 2-way ANOVA with Sidak’s test.
Extended Data Fig. 5 | Signaling responses to Ca\textsuperscript{2+} and/or baclofen in parathyroid-derived PTH-C1 cells. 

\textbf{a}, Time-course of Gq activation. Representative FRET experiments showing stimulatory effect of Ca\textsuperscript{2+} (10 mM) which is suppressible by baclofen (300 µM) in PTH-C1 cells coexpressing the FRET-based Gq sensor (Gq\textsuperscript{Tuirq/YFP}) without (-) or with (+) coexpression of recombinant (Recom) CaSR and GABA\textsubscript{R}R. The change in FRET (ΔFRET) was calculated according to equation \#2 (see Online Methods) with the initial value at t = 0 set to 1. Similar results were obtained from 2 independent experiments.

\textbf{b}, Averaged time courses of cAMP in PTH-C1 cells expressing CaSR without (control in blue) or with pretreatment with cholera toxin (CTX in black). Cells were continuously perfused with buffer without or with extracellular Ca\textsuperscript{2+} or forskolin (horizontal bar). Data were normalized to control with the initial value at t = 0 set to 1 and represent the mean ± SEM of n = 45 cells from 3 separate experiments.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: pCLAMP 10.0 Molecular Devices for FRET photometric microscope recordings; NIS-Element Nikon software live cell microscopy; Xcalibur software version 1.4.0 from Thermo Fisher (Waltham, MA, USA) to collect mass spectrometry data in a data dependent acquisition mode.

Data analysis: GraphPad Prism version 7.00 and 8.0 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. Thermo Scientific Proteome Discoverer software platform (Thermo Scientific, version 2.2) for general database search of MS-based data.

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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We have included the following statement. “Data Availability: All materials, data, animal models, and associated protocols will be made available to all qualified investigators from the corresponding authors upon reasonable request or with a simple institutional material transfer agreement.”
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Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- Sample size: for cell signaling experiments: usually N = or > 3, with n > 5 cells per experiments.
  For animal studies: N= or > 3 mice/group.

- Data exclusions: no data excluded from Cell signaling experiments.
  Some mouse serum data (<5%) with variations > 2 SD were excluded from analyses.

- Replication: N independent cell signaling experiments = or >3.
  For animal studies, mice are from at least 2 independent cohorts from different breeding.

- Randomization: Mice are randomly assigned to each treatment after separation by sexes.

- Blinding: blinding N/A for cell signaling experiments.
  All mice were coded with ear tags with their genotypes blinded to all operators performing assays until their final statistical analyses.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | Involved in the study | n/a | Involved in the study |
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| ☐ | Paleontology | ☐ | MRI-based neuroimaging |
| ☐ | Animals and other organisms | ☐ | |
| ☐ | Human research participants | ☐ | |
| ☐ | Clinical data | ☐ | |

Antibodies

Antibodies used:
Rabbit Anti-CalR-N (N-terminus; VA609, ADDDYGRPGIEKFREEASERDI, custome-made); guinea pig anti-GABA-1R-C (CPSEPPRDRSLSSGSRVIWLYK; custome-made); mouse anti-GABA-1R-N (N-terminal; Abcam ab55051); rabbit anti-GAD1/2 (Cat.Ab49832, Abcam); rabbit anti-GABA, (Cat.# A2052, SIGMA)

Validation:
Specificity of all antibodies were validated in this study by the lack of immunoreactivity in tissue lacking the expression of the receptors and/or by manufacturer [for commercial antibody] or in our previous reports cited in the manuscript.

Eukaryotic cell lines

Policy information about: cell lines

Cell line source(s)
HEK-293 cells from ATCC; PTH-C1 cells obtained from Dr. Maria Luisa Brandi.

Authentication:
G-protein signaling

Mycoplasma contamination:
mycoplasma negative
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
All mice were in C57bl6 background and studied at 3 months of age, except those died at early ages. Both male and female mice were studied but analyzed separately. As similar observations were made in male and female mice, we only reported data from the male mice.

Wild animals
N/A

Field-collected samples
N/A

Ethics oversight
N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
N/A

Recruitment
Deidentified parathyroid tissues were obtained by endocrine surgical collaborators in the Department of Surgery, University of California San Francisco as a routine patient treatment procedure, after obtaining a general informed consent. The parathyroid glands (PTGs) samples were later retrieved by Dr. Khanafshar from the archives of Department of Pathology, University of California San Francisco under a human research protocol approved by UCSF Institutional Review Board based on the following clinical criteria verified by medical record review by Drs. Khanafshar, Shoback, and Herberger. Criteria for selection is described in the manuscript

Ethics oversight
ethics committee approved the study protocol; consent was obtained

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
N/A

Study protocol
N/A

Data collection
N/A

Outcomes
N/A