G-protein-coupled receptor controls steroid hormone signaling in cell membrane

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G-protein-coupled receptors (GPCRs) are involved in animal steroid hormone signaling, but their mechanism is unclear. In this research, we report that a GPCR called ErGPCR-2 controls steroid hormone 20-hydroxyecdysone (20E) signaling in the cell membrane of the lepidopteran insect Helicoverpa armigera. ErGPCR-2 was highly expressed during molting and metamorphosis. 20E, via ErGPCR-2, regulated rapid intracellular calcium increase, protein phosphorylation, gene transcription, and insect metamorphosis. ErGPCR-2 was located in the cell surface and was internalized by 20E induction. GPCR kinase 2 participated in 20E-induced ErGPCR-2 phosphorylation and internalization. The internalized ErGPCR-2 was degraded by proteases to desensitize 20E signaling. ErGPCR-2 knockdown suppressed the entrance of 20E analog [3H]ponasterone A ([3H]Pon A) into the cells. ErGPCR-2 overexpression or blocking of ErGPCR-2 internalization increased the entrance of [3H]Pon A into the cells. However, ErGPCR-2 did not bind to [3H]Pon A. Results suggest that ErGPCR-2 transmits steroid hormone 20E signaling and controls 20E entrance into cells in the cell membrane.

Animal steroid hormones, such as mammal estrogen1 and insect 20-hydroxyecdysone (20E)2, exert their actions via the genomic pathway, wherein hormones fuse into cells and bind to intracellular nuclear receptors, which then bind to DNA to initiate gene transcription3. Recent studies suggest that animal steroid hormones can activate receptors in the cell membrane to initiate rapid nongenomic interactions, such as rapid cellular calcium increase4. G-protein-coupled receptors (GPCRs) are proposed as membrane receptors of animal steroid hormones. For example, GPCR 30 (GPR30/GPER) in the cell membrane binds estrogen and mediates rapid intracellular calcium mobilization in humans5. In Drosophila, the dopamine receptor DmDopEcR binds the 20E analog and is proposed as a 20E membrane receptor6. In Bombyx mori, 20E via unknown GPCRs increases the intracellular Ca²⁺ level in the anterior silk gland7. In Helicoverpa armigera, 20E induces rapid protein phosphorylation and nuclear translocation of calponin8, cell membrane trafficking of Rab4b9, and nuclear translocation of heat shock cognate protein Hsc7010. Further studies reveal that 20E via GPCRs and Ca²⁺ signaling regulates rapid phosphorylation of cyclin-dependent kinase 10 (CDK10)11. 20E via phospholipase C-gamma-1 (PLCG1) regulates heterodimeric partner (USP1) phosphorylation and connects the GPCR-mediated nongenomic pathway and the nuclear receptor EcRB1-mediated genomic pathway12. A GPCR called ErGPCR (renamed as ErGPCR-1 to distinguish it from ErGPCR-2) participates in 20E signaling in the cell membrane to regulate calcium increase, protein phosphorylation and subcellular translocation, gene transcription, and metamorphosis in H. armigera13. These data suggest the functions of GPCRs in steroid hormone signaling.

GPCRs, which belong to the seven-transmembrane protein family, are involved in signal transduction across cell membranes14. Signal transduction via GPCRs is fundamental for mediating various cellular responses to changes in the extracellular environment15. Different GPCRs exhibit diverse amino acid sequences; however, most GPCRs show similar mechanisms of desensitization by internalization and resensitization by recycling to the cell membrane16. GPCR desensitization17 is regulated by GPCR kinase (GRK) mediating phosphorylation and internalization of GPCRs18. After internalization, GPCRs can be trafficked to lysosomes for degradation or recycled back to the cell surface for resensitization in another round of signaling19. However, animal steroid hormone-induced GPCR internalization remains poorly understood. In this study, we discovered an ecdysone-responsive GPCR (ErGPCR-2), which transmits steroid hormone 20E signaling and controls steroid hormone 20E entrance into the cells. Under 20E stimulation, GRK2 phosphorylates the C-terminus of ErGPCR-2 to regulate ErGPCR-2 internalization. The internalized ErGPCR-2 is then degraded by proteases, which desensitize
20E signaling. ErGPCR-2 participates in 20E signal transmission in the cell membrane for further gene expression and metamorphosis.

**Results**

**ErGPCR-2 is upregulated during 20E-regulated molting and metamorphosis.** To study the function of ErGPCR-2, the tissue specificity and developmental expression profiles of ErGPCR-2 were examined. The results showed that ErGPCR-2 was expressed in the midgut, fat body, and epidermis. In these three tissues, ErGPCR-2 protein and mRNA exhibited higher expression levels at the fifth instar molting (5M) and metamorphic stages (sixth instar 72 h to 120 h) than at the fifth instar feeding (5 h to 24 h) and sixth instar feeding stages from 6-0 h to 6-48 h (Figures 1A, 1B, and 1C). The expression level of ErGPCR-2 was upregulated by 20E injection into the sixth instar 6 h larvae but was unaffected by JH III or Dimethyl sulfoxide DMSO injection (Figures 1D and 1E). These results indicate that ErGPCR-2 expression is upregulated by 20E during molting and metamorphosis.

**ErGPCR-2 participates in 20E-induced metamorphosis.** To examine the function of ErGPCR-2 in 20E-regulated metamorphosis, we injected the dsRNA of ErGPCR-2 into the sixth instar 6 h larval hemocoel to knock down ErGPCR-2, followed by 20E induction. The larvae pupated earlier than the DMSO control after injection with 20E alone or dsGFP plus 20E. By contrast, the larvae died before pupation or delayed pupation 37 h after injection with dsErGPCR-2 plus 20E (Figures 2A and 2B). Up to 19% of the larvae died and 81% delayed pupation following ErGPCR-2 knockdown (Figures 2C and 2D). Furthermore, transcript levels of 20E-response genes, including ecdysone nuclear receptor EcRβ1, heterodimeric partner USP1, and transcription factors BR-Z7 and HHRI3, decreased (Figure 2E). In HaEpi cells, ErGPCR-2 knockdown also blocked 20E-induced gene expression (Figure 2F). These results suggest that ErGPCR-2 participates in 20E-regulated gene expression and metamorphosis.

**ErGPCR-2 participates in 20E-induced rapid reactions and gene transcription.** 20E, via GPCRs, induces rapid increase in cellular calcium and phosphorylation of transcription complex proteins USP1 and CDK10 to activate gene transcription12. Thus, the function of ErGPCR-2 in these cascades was detected in HaEpi cells. 20E induced intracellular calcium release and extracellular calcium influx in normal cells (Figure 3A). However, ErGPCR-2 knockdown repressed the 20E-induced intracellular calcium release and the extracellular calcium influx (Figure 3B), suggesting that ErGPCR-2 is involved in 20E-induced calcium increase. The T-type calcium channels were involved in 20E/Pip-induced responses. To confirm this, we used a calcium channel inhibitor, which blocked 20E/Pip-induced responses, while the combination of 20E/Pip and calcium channel inhibitor did not. This suggests that ErGPCR-2 participates in 20E/Pip-induced calcium release and gene transcription.
voltage-gated calcium channel inhibitor flunarizine dihydrochloride (FL) and the transient receptor potential calcium 3 (TRPC3) channel inhibitor pyrazole compound (Pyr3) blocked the calcium influx but not the calcium release (Figure 3C). The intracellular Ca\textsuperscript{2+} ATPases inhibitor thapsigargin (TG), which depletes the stored intracellular calcium\textsuperscript{2+}, repressed the intracellular calcium release and extracellular calcium influx, but did not block extracellular calcium influx in 20E induction (Figure 3C). The GPCR inhibitor suramin blocked both intracellular calcium release and extracellular Ca\textsuperscript{2+} influx. However, the receptor tyrosine kinase (RTK) inhibitor SU6668\textsuperscript{25} affected neither intracellular calcium release nor extracellular calcium influx (Figure 3D). These results suggest that 20E via ErGPCR-2 induces cellular Ca\textsuperscript{2+} increase, and various calcium channels are involved in this process.

Moreover, 20E induced USP1 and CDK10 phosphorylation. By contrast, lambda protein phosphatase (\lambda phosphatase) treatment degraded USP1 and CDK10 phosphorylation. ErGPCR-2 knockdown repressed 20E-induced USP1 and CDK10 phosphorylation.
ErGPCR-2 protein was mainly localized in the cell membrane. To observe its rapid response to 20E induction, the subcellular location of ErGPCR-2 was analyzed to initiate gene transcription in 20E signaling. The molecular mass of ErGPCR-2 in the cytoplasm was higher than that in the cell membrane. These results suggest that ErGPCR-2 is involved in 20E-induced rapid mobilization of Ca^{2+} in HaEpi cells. Western blot analysis confirmed the internalization of ErGPCR-2 by 20E induction. The molecular mass of ErGPCR-2 in the cytoplasm was also higher than that in the cell membrane. The increased molecular mass was decreased by lambda protein phosphatase (λPP).

ChIP experiments were performed to further examine the mechanism of 20E regulates gene transcription through ErGPCR-2. 20E regulates EcRBy1/USP1 heterodimer binding to ecdysone response element (EcRE) to regulate gene transcription. The S' regulatory region of Helicoverpa HR3 (HHR3), which contains EcRE, the DNA element that EcR binds to initiate gene transcription, from Helicoverpa hormone receptor 3 (HHR3) and red fluorescence protein (RFP) as reporter (Figures 4C). Overexpression of 7TM-GFP also significantly increased 20E-induced gene expression (Figure 4D).

ErGPCR-2 is localized in the cell membrane and partially internalized by 20E induction. The subcellular location of ErGPCR-2 was analyzed to observe its rapid response to 20E induction. ErGPCR-2 protein was mainly localized in the cell membrane. However, ErGPCR-2 was internalized into the cytoplasm within 15 min of 20E treatment (Figure 5A). Western blot analysis confirmed the internalization of ErGPCR-2 by 20E induction. The molecular mass of ErGPCR-2 in the cytoplasm was also higher than that in the cell membrane. The increased molecular mass was decreased by lambda protein phosphatase (λPP). To elucidate the fate of ErGPCR-2 after internalization into the cytosol, the subcellular location of ErGPCR-2 was examined following anisomycin (protein translation inhibitor) and PMSF (protein degradation inhibitor). ErGPCR-2 was localized in the cell membrane in DMSO control and was internalized in 15 min by 20E induction. However, deletion of the second extracellular loop caused 7TM-GFP to lose its cell membrane-localizing capability. When the C-terminus of 7TM was deleted, the 7TM-GFP was localized in the cell membrane and can be internalized in 15 min by 20E induction. However, deletion of the second extracellular loop caused 7TM-GFP to lose its cell membrane-localizing capability. When the C-terminus of 7TM was deleted, the 7TM-GFP was not internalized from the cell membrane by 20E induction (Figure 5E). These results suggest that the C-terminus of ErGPCR-2 determines the internalization of ErGPCR-2.
GRK2 regulates phosphorylation and internalization of ErGPCR-2.

Given that GRK serves an important function in GPCR phosphorylation and GPCR endocytosis\(^2\), we detected the function of GRK2 in 20E-induced ErGPCR-2 phosphorylation and internalization. Immunocytochemical analysis showed that ErGPCR-2 was internalized after 15 min of 20E induction in dsGFP control cells. However, ErGPCR-2 was not internalized after GRK2 knockdown (Figure 6A). Western blot analysis confirmed that ErGPCR-2 was partially phosphorylated and internalized into the cytoplasm by 20E induction in dsGFP-treated cells; however, ErGPCR-2 was kept nonphosphorylated in the cell membrane and could not be internalized by 20E induction in the dsGRK2-treated

**Figure 4** | ErGPCR-2 is involved in 20E-induced rapid reactions and gene transcription in HaEpi cells. (A) and (B). Western blot analysis 20E-induced phosphorylation of USP1-His and CDK10 (1 μM 20E for 1 h). USP1-His-P: overexpressed phosphorylated USP1-His detected using an anti-His-tag antibody; CDK10-P: phosphorylated CDK10 detected using the anti-CDK10. λPP: protein was incubated with 0.5 μM λPPase at 30 °C for 30 min. SDS-PAGE gel in Western blot was 7.5%. (C). Effect of ErGPCR-2 knockdown on 20E-induced transcription activity. Cells were transfected with pEEx-HR3pro-RFP-His plasmid (2.5 μg/mL, 24 h), inducted with 1 μM 20E for 18 h. The images were statistically analyzed by ImageJ software. (D). The effects of ErGPCR-2-7TM overexpression on 20E-induced gene expression, analyzed by qRT-PCR. β-actin was used as quantitative control. (E). ErGPCR-2 regulates EcR1 binding to EcRE during 20E induction. (a). ChIP analysis by qRT-PCR detecting the EcRE fragment from the precipitates by anti-RFP under 20E treatment. Cells were transfected with plasmid of pIEx-4-EcR1-RFP (3 μg/mL), and then treated with DMSO or 20E (1 μM) for 6 h. No antibody was used as the negative control. (b). ErGPCR-2 depletion decreased the 20E-induced EcR1 binding to EcRE. Cells were transfected with pIEx-4-EcR1-RFP (b) or pIEx-4-RFP (c) for 24 h. The cells were treated with dsErGPCR-2 (2 μg/mL) or dsGFP (2 μg/mL) for 12 h, and then induced by 1 μM 20E or DMSO for 6 h. Western blots indicate protein expression levels. (d). qRT-PCR detected the efficacy of ErGPCR-2 knockdown. Input: The positive control of non-immunoprecipitated chromatin. In all experiments, *P value via Student’s t-test based on three replicates.
cells (Figure 6B). GRK2 was also co-precipitated by antibodies against ErGPCR-2 from the 20E-injected larval midgut, but not from DMSO- and JH III-injected larval midgut (Figures 6C and 6D). These results suggest that 20E induces an interaction between ErGPCR-2 and GRK2, resulting in phosphorylation and internalization of ErGPCR-2.

The level of 20E-induced ErGPCR-2 phosphorylation was two phosphates per molecule of 7TM-GFP protein, determined using phosphoprotein phosphate estimation assay kit. When the C-terminus of 7TM was deleted, 7TM-D C-terminal-GFP was not phosphorylated by 20E induction (Figure 6E), suggesting that 20E-induced phosphorylation occurs at the C-terminal of 7TM. When GRK2 was knocked down in the HaEpi cells using dsGRK2, 7TM-His could not be phosphorylated by 20E induction (Figure 6F), suggesting that GRK2 participates in ErGPCR-2 phosphorylation.

ErGPCR-2 determines the entrance of \( ^{[3]H}\)Pon A into cells. To demonstrate the role of ErGPCR-2 in the entrance of 20E into cells, the levels of \( ^{[3]H}\)ponasterone A (\( ^{[3]H}\)Pon A) in the whole cells were assayed. EcRB1 proteins were equally overexpressed in the cells to grasp \( ^{[3]H}\)Pon A upon its entrance. In ErGPCR-2 knockout cells, the \( ^{[3]H}\)Pon A levels decreased significantly compared with those in dsGFP-treated cells (Figure 7A). These results suggest that ErGPCR-2 determines the entrance of \( ^{[3]H}\)Pon A into the cells.

To determine whether ErGPCR-2 binds to \( ^{[3]H}\)Pon A, the cells were treated with 20E for 12 h to increase the expression levels of proteins, including ErGPCR-2 and EcRB1, in the 20E pathway, after ErGPCR-2 knockdown. Both ErGPCR-2 and \( ^{[3]H}\)Pon A were then co-immunoprecipitated in the cell membrane and in the cytosol with antibodies against ErGPCR-2. However, no difference existed
on the [3H]Pon A levels in co-immunoprecipitates from normal cells and dsGFP- or dsErGPCR-2-treated cells (Figure 7B), suggesting that ErGPCR-2 does not bind to [3H]Pon A in the cell membrane or in the cytosol. After co-immunoprecipitation (Co-IP), the [3H]Pon A levels in the supernatants significantly decreased in the dsErGPCR-2-treated cells compared with those in the normal cells or dsGFP-treated cells (Figure 7C). These data confirm that although ErGPCR-2 did not bind to [3H]Pon A, this GPCR determined the entrance of 20E analog into the cells.

To address whether ErGPCR-2 internalization brings [3H]Pon A to the cells, we detected the [3H]Pon A levels in the co-immunoprecipitates produced by anti-ErGPCR-2 and the supernatants after Co-IP, which blocked ErGPCR-2 internalization by GRK2 knockdown. The [3H]Pon A levels in the co-immunoprecipitates did not increase after GRK2 knockdown compared with those in the normal cells and dsGFP-treated cells (Figure 7D). This finding suggested that cell membrane-arrested ErGPCR-2 does not bind to [3H]Pon A. However, the [3H]Pon A levels in the supernatants did not decrease but increased in the Co-IP after GRK2 knockdown compared with those in the normal cells or dsGFP-treated cells (Figure 7E). The 7TM-GFP and 7TM<sub>ΔC-terminal</sub>-GFP were overexpressed in HaEpi cells to examine the entrance of [3H]Pon A into the cells. The [3H]Pon A

Figure 6 | Knockdown of GRK2-blocked 20E-induced ErGPCR-2 internalization. (A). Cells were treated with dsGFP and dsGRK2 (2 μg/mL) for 24 h, and then 1 μM 20E for 15 min, respectively. Green: ErGPCR-2 protein stained with an anti-ErGPCR-2 and secondary antibody labeled with Alexa488. Blue: nucleus stained with DAPI. Scale bar = 25 μm. (B). Western blot of samples in (A). (C). DMSO, 20E, or JH III (500 ng/larva) was injected to 6th 6 h larvae; midgut protein was examined. Input: immunoprecipitates by anti-ErGPCR-2, β-actin was used as control; Output: co-immunoprecipitates. 12.5% gel in SDS-PAGE. (D). Statistical analysis of (C) according to three independent replicate experiments by ImageJ software. Significant differences (*P value) in two samples were statistically analyzed by Student’s t-test. (E). Number of moles of phosphorus per mole of 7TM-GFP analyzed by a phosphoprotein phosphate estimation assay kit. 20E concentration was 1 μM. DMSO was used as the control. (F). Phosphorylation of 7TM-His after knockdown of GRK2. Cells treated with dsGFP and dsGRK2 (2 μg/mL) for 24 h, and then 1 μM 20E for 15 min, respectively. Statistical significance (*P Value) was based on three biologically independent repeats and analyzed by the Student t test. Gel concentration was 7.5%.
levels in the whole cells were increased by overexpression of 7TM-GFP compared with the GFP control. The [3H]Pon A levels in the whole cells also increased more when 7TM-arrestin-C-terminal-GFP was overexpressed compared with those upon overexpression of 7TM-GFP (Figure 7F). These results suggest that the function of ErGPCR-2 in controlling the entrance of [3H]Pon A, and ErGPCR-2 internalization is unnecessary for the entrance of [3H]Pon A into the cells.

Discussion

Animal steroid hormones clearly transmit signals via GPCR. However, the mechanism underlying steroid signal transmission by GPCRs remains unclear. We reveal that ErGPCR-2 is located in the cellular surface and internalized by 20E induction. GRK2 participates in 20E-induced phosphorylation and internalization of ErGPCR-2. The internalized ErGPCR-2 is then degraded, thereby desensitizing 20E signaling. ErGPCR-2 determines [3H]Pon A entrance into the cells. However, ErGPCR-2 does not bind to [3H]Pon A. These results provide evidence that steroid hormone via GPCRs transmit signals to directly gene transcription.

Some GPCRs can be internalized to desensitize signaling or transmit signals inside the cells continuously. The mechanism comprises GRK phosphorylation, interaction with β-arrestin, and internalization into the cytoplasm. The internalized GPCR is either degraded by proteases in the lysosome or recycled in the cell membranes. H. armigera ErRGPCR-2 is identified as a methuselah-like-2 protein by basic local alignment search tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi), with several phosphorylation sites predicted at its C-terminus (Supplement Files: Figure S4). ErGPCR-2 was internalized via GRK by 20E induction, and the C-terminus of ErGPCR-2 is critical. The internalized ErGPCR-2 is degraded within 2 h. Whether the internalized ErGPCR-2 still transmits signals inside the cells need further study. Blocking the internalization of ErGPCR-2 by deleting the C-terminus of ErGPCR-2 or by GRK2 knockdown did not block the entrance of [3H]Pon A. These data suggest that the internalization of ErGPCR-2 does not bring [3H]Pon A into the cells but desensitizes 20E signaling after 20E entered the cells. This condition may explain the 20E-upregulating ErGPCR-2 expression, which can compensate for the internalized ErGPCR-2 on the cell membrane. This finding may be ascribed to the different GPCRs that initiate varied signaling on the cell membrane. The activation of GRK2 by 20E induction needs further study.

GPCRs can bind various ligands, such as peptides, lipids, ions, light, and odorants. Some GPCRs bind to animal steroid hormones; for example, GPR30 binds estrogen in humans and DmDopEcR binds [3H]Pon A in Drosophila. 20E (http://en.wikipedia.org/wiki/Ecdysterone) and [3H]Pon A (http://www.scbt.com/zh/datasheet-202768-ponasterone-a.html) are both ecdysteroids. 20E is a 20E-hydroxyecdysone, whereas [3H]Pon A is a 25-deoxy-20-hydroxyecdysone. [3H]Pon A is used to detect the binding of GPCR to steroid hormone 20E. However, we did not detect binding of ErGPCR-2 to [3H]Pon A by Co-IP of ErGPCR-2 and [3H]Pon A from the cell membrane or supernatant of ErGPCR-2 and [3H]Pon A, which suggests that ErGPCR-2 does not bind or does not tightly bind to [3H]Pon A.

The structure of ErGPCR-2 by Swiss model (http://swissmodel.expasy.org/) showed that ErGPCR-2 appeared as a tubaeform toward the outside of the cell membrane with a wide cave and vertical hole at the center of the structure (Supplement Files: Figure S5). Whether [3H]Pon A enters the cave, passes the hole of ErGPCR-2, and enters the cells by conformational change without tightly binding to ErGPCR-2 need further clarification. The possibility that ErGPCR-2 maintains the cell membrane structure for the entrance of [3H]Pon A is possible.
A should also be studied when the appropriate methods become available.

Another GPCR called ErGPCR-1 participates in 20E signal transduction in H. armigera without binding to [3H]Pon A10. Both ErGPCR-1 and ErGPCR-2 belong to methuselah-2 GPCR in the class B secretin family and are located in the cell membrane. However, ErGPCR-1 contains 489 amino acids with a 19-amino acid signal peptide, whereas ErGPCR-2 contains 757 amino acids without signal peptide as predicted by theoretical analysis (http://smart.embl-heidelberg.de) (Supplement Files: Figure S4). Both ErGPCR-1 and ErGPCR-2 transmit 20E signals in the cell membrane, including regulation of calcium increase, protein phosphorylation, gene transcription, and metamorphosis. However, ErGPCR-2 was internalized by 20E induction to control 20E entrance into the cells, which was not observed in ErGPCR-1 in a previous study13. However, the 20E-mediated internalization of ErGPCR-2 was independent from ErGPCR-1 because ErGPCR-1 knockdown did not affect 20E-mediated ErGPCR-2 internalization. Suramin blocked 20E-mediated internalization of ErGPCR-2, but SU6668 did not. This finding indicates that the GPCR pathway (not RTK pathway) is involved in 20E-mediated internalization of ErGPCR-2 (Supplement Files: Figure S6). These data suggest that various GPCRs are involved in 20E signaling, which initiates signaling coordinately. Whether ErGPCR-1 signaling depends on ErGPCR-2 is undetermined because of the shortage of direct readout on ErGPCR-1 after 20E induction.

The genomic pathway of 20E has been well studied. 20E binds its nuclear receptor EcrB112, which then interacts with USP1 to form EcrB1/USP1 transcription complex, thereby initiating gene transcription in 20E signaling1. The transcription factors include BR-Z7, HHR3, E74, and E75, which initiate insect metamorphosis16. 20E mediates CDK10 phosphorylation to enhance formation of the EcrB1/USP1 transcription complex17. 20E via GPCR-, PLC-, Ca2+- , and PKC-signaling mediates USP1 phosphorylation for gene transcription18. At the downstream of ligand-activated-GPCR, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG)19. IP3 binds to its receptor in the endoplasmic reticulum membrane to drive the release of intracellular calcium ions, whereas DAG and Ca2+ bind to the protein kinase C (PKC) to activate PKC20. 20E via PKC regulates CDK1011 and USP1 phosphorylation to form the Ecr/USP transcription complex12. In the current study, the larvae died before pupation or delayed pupation after knockdown of ErGPCR-2, suggesting that ErGPCR-2 is involved in gene transcription and metamorphosis in the 20E pathway. 20E-induced transcripts of genes, including EcrB1, USP1, and HR3, were not up-regulated within 15 minutes of 20E induction in the previous study14, therefore, the 20E induced rapid internalization of ErGPCR-2 and Ca2+ signaling are independent of gene transcription and protein expression.

ErGPCR-2 knockdown decreased the entrance of 20E into the cells. The 20E-induced calcium increase and phosphorylation of USP1 and CDK10 were blocked, which repressed the formation of the 20E transcription complex for gene transcription. Therefore, ErGPCR-2 transmits 20E signaling in the cell membrane to regulate gene transcription and metamorphosis.

Conclusions
ErGPCR-2 is a key control factor for the entrance of 20E into cells. Such control is not exerted by directly or tightly binding to 20E. 20E via ErGPCR-2 regulates rapid intracellular Ca2+ increase and phosphorylation of USP1 and CDK10, which induce gene transcription in the 20E pathway, thereby regulating metamorphosis. After performing the task for 20E entrance, ErGPCR-2 is phosphorylated and internalized via GRK2 for degradation to desensitize 20E signaling (Figure 8).

Methods
Insect. Cotton bollworms (H. armigera) were obtained from the Henan Agricultural University in China and were raised on an artificial diet composed of wheat germ and soybean powder with various vitamins and inorganic salts. The insects were kept in an insectarium at 26 ± 1 °C with 60% to 70% relative humidity and under the light/dark cycles of 14 h/10 h.

RNA interference in larvae and cells. DNA fragment of ErGPCR-2 was amplified as template for dsRNA synthesis by the primers ErGPCR-2-RNAiF and ErGPCR-2-RNAiR (Supplement Files: Table S1). The dsRNA was synthesized using MEGAscript RNAiKit (Ambion Inc, Austin, TX, USA). The dsRNA purity and integrity were determined by agarose gel electrophoresis. The dsRNA were quantified using a spectrophotometer (GeneQuant; Amersham Biosciences). The dsRNA (dsErGPCR-2, dsGFP) was injected using a micro-syringe into the larval hemocoel of the sixth instar larvae at 6, 24, and 42 h at 500 ng/larva. After injection with dsRNA thrice for 12 h, 500 ng of 20E (Sigma, St. Louis, MO, USA) was injected into each larva. Dimethyl sulfoxide (DMSO) was used as control. The phenotypes and developmental rates of the larvae were recorded. The mRNA was isolated from the larvae when the control group grew at the sixth instar for 72 h. The Heterochoerus epidermal cell line (HaEpi) was cultured in Grace’s medium with 10% fetal bovine serum (FBS, MDgenics, St. Louis, MO, USA) 2 d before dsRNA transfection. The cells were transfected with dsRNA and RNAfectin transfection reagents (Tiangen, Beijing, China) in Grace’s medium without FBS at 2 and 4 µg/mL, respectively. After about 12 h, the cells were re-fed in a fresh medium with FBS for 12 h. Then repeated transfection once using the same dsRNA and RNAfectin concentrations and duration. Finally, the cells were re-fed in a fresh medium with FBS containing 20E at a final concentration of 1 µM at a different time. The controls were treated with equivalent volume of DMSO. Total RNA was isolated and reverse-transcribed for further experiments.

Examination of cellular calcium ions. Upon reaching a density 2 × 106 based on the above protocol, the cells were incubated with 3 µM acetoxymethyl (AM) ester calcium crimson14 dye (Invitrogen, Carlsbad, CA, USA) in Dulbecco’s phosphate-buffered saline (DPBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM Na2HPO4) for 30 min at 27 °C. Cells were washed thrice with DPBS without calcium ions and were exposed at 1 µM 20E to detect the intracellular calcium flux. Calcium chloride was then added to the medium to 1 mM. Fluorescence was detected at 555 nm every 6 s for 360 s using Carl Zeiss LSM 700 laser scan confocal microscope (Thornwood, NY, USA). The data were analyzed using Image Pro-Plus software (Media Cybernetics, United States). After opening the file for editing, the picture was converted into grayscale 8 with automatic counting measurements, and the measurement parameters were selected. Automatic statistics were then repeated. Finally, the file was imported into Excel. The Excel file was then submitted for statistical analysis. For the inhibition experiments, the cells were pretreated with different inhibitors for 30 min at 27 °C before washing thrice with DPBS without calcium ions and stimulation with 20E. Suramin (Sigma, St. Louis, MO, USA), RTK
inhibitor SU6668 (Selleckchem, Houston, TX, USA), pyrazole compound (Sigma, St. Louis, MO, USA), fluoramine dihydrochloride (Sigma, St. Louis, MO, USA), and thapsigargin (Sigma, St. Louis, MO, USA) were used as inhibitors.

**Protein phosphorylation.** Protein phosphorylation was examined by molecular mass variation on 7.5% low gel of SDS–PAGE and λPP degradation as described by Song et al. [31]. A 20 μL of protein was subjected to electrophoresis on 7.5% SDS–PAGE gels and transferred to nitrocellulose membranes and probed with antibodies specific to the phosphorylated forms of proteins, followed by horseradish peroxidase conjugated secondary antibodies. The bands were visualized by Image Quant software (Amersham, Piscataway, NJ, USA).

**Isolation of cell membrane and cytosol proteins.** Membrane and cytosol proteins were isolated from HaEpi cells. The cells were collected by centrifugation, washed with ice-cold PBS, and resuspended in 1% NP-40 lysis buffer at 4°C. The cell lysates were centrifuged at 13,000 g for 10 min to terminate the cross-linking. The supernatants were added to the Protein A resin and incubated at 4°C overnight. The DNA was purified by phenol/chloroform extraction and subjected to qRT-PCR analysis using SYBR Green R primer (Table S1).

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assay was performed using a ChIP-IT kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

**Overexpression of ErGPCR-2 and its mutants.** The expression of ErGPCR-2 and its mutants was overexpressed in HaEpi cells by transfection with the pX3-7TM-GFP-His and pX3-7TM-His plasmids and then purified by His-bind resin (50 μL of resin) after different treatments. The levels of purified 7TM-GFP phosphorylation were analyzed in a 96-well microplate using a phosphoprotein estimation assay kit (Sangon Biotechnology, Shanghai, China). The efficacy of RNA interference of ErGPCR-2 and GRK2 was examined via qRT-PCR analysis.

**Endogenous gene transcription.** The cross talk between 20-hydroxyecdysone and juvenile hormone signaling pathways by phosphorylation variation. Plos one 6, e19776 (2011).

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**Author contributions**

Di Wang performed the major work and drafted the manuscript. Mei-juan Cai detected the Ca\(^{2+}\) levels. Wen-Li Zhao supplied the antibodies and dsRNA of GRK2. Jin-Xing Wang directed the research. Xiao-Fan Zhao designed the studies and edited the manuscript. All authors reviewed the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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