OPEN

SUBJECT AREAS:

ENTOMOLOGY

HORMONE RECEPTORS

Received

4 September 2014

Accepted

30 December 2014

Published

2 March 2015

Correspondence and
requests for materials
should be addressed to
X.-F.Z. (xfzhao@sdu.
edu.cn)

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

Di Wang, Wen-Li Zhao, Mei-Juan Cai, Jin-Xing Wang & Xiao-Fan Zhao

Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Jinan 250100, China.

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. armigera*¹³. 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 *EcR*¹³, heterodimeric partner *USP1*, and transcription factors *BR-Z7* and *HHR3*²⁰, decreased (Figure 2E). In HaEpi cells²¹, *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

Figure 1 | Expression profile and hormonal regulation of ErGPCR-2. (A). Western blot with antibody against ErGPCR-2 (specificity of antibody is shown in Supplement Files: Figure S1). (B). Calculation of A according to three independent replicates by ImageJ software (National Institutes of Health, <http://imagej.nih.gov/ij/download.html>). (C). qRT-PCR. 5F: fifth instar 12 h feeding larvae; 5M: fifth instar 36 h molting larvae; 6-0 h, 6-24 h, 6-48 h, 6-72 h, 6-96 h and 6-120 h: sixth instar larvae from 0 h to 120 h; P-0: zero-day-old pupae. F: feeding; M: molting; MM: metamorphic molting; P: pupae. (D). Western blot analysis of hormonal regulation on ErGPCR-2 in larvae. 20E or JH III (500 ng/larva) was injected into the 6th instar 6 h larvae for 1 h to 24 h. Equal volume of DMSO was injected as control. (E). Statistical analysis of (D) according to three independent replicates by ImageJ software. *Significant differences (P values) were statistically analyzed by Student's *t*-test. SDS-PAGE gel in Western blot is 12.5%. β-actin was used as control.

Figure 2 | ErGPCR-2 silencing represses metamorphosis by repressing 20E response gene expression. (A). Phenotypes after ErGPCR-2 knockdown (500 ng/larva, thrice at an 18 h interval) and 20E induction (500 ng/larva). Images were obtained at six instar larvae 120 h according to DMSO control group. Scale bar = 1 cm. (B). Statistical analysis of pupation time from 6th instar 0 h larvae developing to pupae 6th 0 h to pupation in (A). (C). Percentages of the phenotype in (A). (D) and (d). Western blot showing the efficacy of *ErGPCR-2* knockdown, analyzed by ImageJ software. (E) and (F). qRT-PCR showing mRNA levels of 20E response genes after *ErGPCR-2* knockdown in larvae at 6th 72 h in the above treatment and in HaEpi cells (*dsRNA* 2 μ g/mL, 12 h twice, 1 μ M 20E for 12 h). β -actin was used as control. Asterisks indicate significant differences (*P value) using Student's *t*-test based on three replicates, $n = 30 \times 3$ in larvae and $n = 3$ in the cells. Off-target effect was excluded by examination of another GPCR named *ErGPCR-1* (Supplement Files: Figures S2A and B). The HaEpi cell shape was unchanged after incubation with 20E or *ErGPCR-2* knockdown (Supplement Files: Figure S2C).

voltage-gated calcium channel inhibitor flunarizine dihydrochloride (FL)²² and the transient receptor potential calcium 3 (TRPC3) channel inhibitor pyrazole compound (Pyr3)²³ blocked the calcium influx but not the calcium release (Figure 3C). The intracellular Ca^{2+} -ATPases inhibitor thapsigargin (TG), which depletes the stored intracellular calcium²⁴, 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^{2+} influx. However, the receptor tyrosine kinase (RTK) inhibitor SU6668²⁵ affected neither intracellular calcium release nor extracellular calcium influx (Figure 3D). These results suggest that 20E via ErGPCR-2 induces cellular Ca^{2+} increase, and various calcium channels are involved in this process.

Moreover, 20E induced USP1 and CDK10 phosphorylation. By contrast, lambda protein phosphatase (λ PPase) treatment degraded USP1 and CDK10 phosphorylation. *ErGPCR-2* knockdown repressed 20E-induced USP1 and CDK10 phosphorylation

Figure 3 | ErGPCR-2 is involved in 20E-induced rapid mobilization of Ca^{2+} in HaEpi cells. (A). 20E-induced cytosolic Ca^{2+} levels increase. AM ester calcium crimsonTM dye 3 μM , 20E 1 μM , CaCl_2 1 mM, equal volume of DMSO as solvent control. Fluorescence was recorded using a Confocal Microscope at 555 nm and then analyzed using Image Pro-Plus software. F: fluorescence of cells after treatment; F_0 : average fluorescence of cells before treatment. (B). Effect of the *ErGPCR-2* knockdown by dsRNA (2 $\mu\text{g}/\text{mL}$) on the Ca^{2+} levels. (C). Inhibition of 20E-induced increase in cytosolic Ca^{2+} levels. FL: T-type calcium channel blocker FL (50 μM); Pyr3: the TRPC3 channel inhibitor (10 μM); and TG: Thapsigargin (2 μM) were added to the medium 30 min before 20E induction. (D). RTK inhibitor SU6668 (5 μM) and suramin (50 μM) were added to the medium 30 min before 20E induction.

(Figures 4A and 4B), which are essential for the formation of EcRB1/USP1 transcription complex to initiate gene transcription in 20E signaling^{11,12}. These results suggest that ErGPCR-2 is involved in 20E-induced rapid cellular reactions. 20E induced USP1 interaction with EcRB1 to form EcRB1/USP1 transcription complex, thereby initiating gene transcription in 20E signaling³. USP1 and CDK10 are related to 20E-induced transcription¹¹. 20E-induced transcription activity was also decreased by *ErGPCR-2* knockdown and reflected by the expression levels of red fluorescence protein using pIEx-HR3pro-RFP report plasmid¹², which contains 20E-response element (EcRE), the DNA element that EcR binds to initiate gene transcription²⁶, from *Helicoverpa* hormone receptor 3 (HR3) and red fluorescence protein (RFP) as reporter (Figures 4C). Overexpression of 7TM-GFP also significantly increased 20E-induced gene expression (Figure 4D).

ChIP experiments were performed to further examine the mechanism of 20E regulates gene transcription through ErGPCR-2. 20E regulates EcRB1/USP1 heterodimer binding to ecdysone response element (EcRE) to regulate gene transcription²⁶. The 5' regulatory region of *Helicoverpa* HR3 (HHR3), which contains EcRE (GGG-GTCAATGAACTG), was cloned¹¹. The EcRE level was significantly higher by qRT-PCR detection in the immunoprecipitates produced by anti-RFP antibody, which precipitated EcRB1-RFP and the bond EcRE, in 20E induction in the EcRB1-RFP expressing cells (Figure 4E, a). However, in the EcRB1-RFP expressing cells, the qRT-PCR product significantly decreased by 20E induction after knockdown of *ErGPCR-2*, compared with the *dsGFP* treatment control (Figure 4E, b). The RFP expression cells were used as negative control (Figure 4E, c), and the efficacy of *ErGPCR-2* knockdown is showed in Figure 4E, d. These results from ChIP experiments suggest that 20E via ErGPCR-2 regulates EcRB1 binding to EcRE to regulate the 20E-induced gene transcription.

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 (Figure 5B). The increased molecular mass was decreased by lambda protein phosphatase (λPP) (Figure 5C), which probably resulted from protein phosphorylation.

To confirm the 20E-induced internalization of ErGPCR-2, the 7TM (amino acid 359–757, 7TM-GFP), the C-terminus deletion (aa 701–757) of 7TM (7TM^{ΔC-terminal}-GFP), and the second extracellular region deletion (aa 565–595) of 7TM (7TM^{Δe2loop}-GFP) (Figure 5D) were overexpressed and fused with green fluorescence protein (GFP). The overexpressed GFP alone was distributed in all the cells without location variation by 20E induction. By contrast, 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^{Δe2loop}-GFP to lose its cell membrane-locating capability. When the C-terminus of 7TM was deleted, the 7TM^{ΔC-terminal}-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.

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. With the exception of those located in the cell membrane, the amount of ErGPCR-2 in the cytosol decreased 2 h after withdrawal of 20E, following the incubation of cells in the inhibitor-free medium. By contrast, the amount of ErGPCR-2 in the cytosol was maintained 2 h after withdrawal of 20E when the protein translation inhibitor and proteases were added into the medium (Figure 5F). Western blot confirmed the degradation of ErGPCR-2 in the inhibitor-free medium, as well as its presence, with the addition of the said inhibitors in the medium (Figure 5G). These results suggest that ErGPCR-2 is not recycled in the cell membrane but is degraded after internalization into the cytosol by 20E induction.

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 pIEx-HR3pro-RFP-His plasmid (2.5 μg/mL, 24 h), induced 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 EcRB1 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-EcRB1-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 EcRB1 binding to EcRE. Cells were transfected with pIEx-4-EcRB1-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.

GRK2 regulates phosphorylation and internalization of ErGPCR-2. Given that GRK serves an important function in GPCR phosphorylation and GPCR endocytosis²⁷, 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 5 | 20E regulates ErGPCR-2 phosphorylation and internalization in HaEpi cells. (A). 20E treatment (1 μ M, 15 min). **Green:** ErGPCR-2 protein stained with an anti-ErGPCR-2 and secondary antibody labeled with Alexa 488. **Red:** plasma membrane detected with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA). **Blue:** nucleus stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI). Observed by confocal microscope. (B). and (C). Western blot showing the subcellular distribution and phosphorylation of ErGPCR-2 as the treatment in (A). Gel concentration is 7.5%. **M:** Membrane protein; **Cy:** Cytoplasm protein. **λPP:** λ protein phosphates (5 μ M, 30 min at 30 °C). (D). and (E). Mutation and overexpression of ErGPCR-2. Green and blue are the same as those in (A). (F). Degradation of ErGPCR-2. (a), (b), (c), and (d) indicates **DMSO:** solvent control; **20E 15 min:** 20E 2 μ M for 15 min; **withdrawal 20E:** culture 2 h after withdrawal of 20E; **inhibitors:** anisomycin (10 μ M), proteases inhibitor PMSF (1 mM). Green and blue are the same as those in (A). (G). Western blot showing the relative levels of ErGPCR-2 in membrane and cytoplasm after the treatments in (F). Coomassie Brilliant Blue staining was used as loading control for membrane protein quantity and quality. **M:** membrane; **Cy:** cytoplasm. Scale bar = 25 μ m.

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

To determine whether ErGPCR-2 binds to [³H]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 [³H]Pon A were then co-immunoprecipitated in the cell membrane and in the cytosol with antibodies against ErGPCR-2. However, no difference existed

Figure 6 | Knockdown of *GRK2*-blocked 20E-induced ErGPCR-2 internalization. (A). Cells were treated with *dsGFP* and *dsGRK2* (2 μ g/mL) for 24 h, and then 1 μ M 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 μ m. (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 μ M. DMSO was used as the control. (F). Phosphorylation of 7TM-His after knockdown of *GRK2*. Cells treated with *dsGFP* and *dsGRK2* (2 μ g/mL) for 24 h, and then 1 μ M 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%.

on the [3 H]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 [3 H]Pon A in the cell membrane or in the cytosol. After co-immunoprecipitation (Co-IP), the [3 H]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 [3 H]Pon A, this GPCR determined the entrance of 20E analog into the cells.

To address whether ErGPCR-2 internalization brings [3 H]Pon A to the cells, we detected the [3 H]Pon A levels in the co-immunopre-

cipitates produced by anti-ErGPCR-2 and the supernatants after Co-IP, which blocked ErGPCR-2 internalization by *GRK2* knockdown. The [3 H]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 [3 H]Pon A. However, the [3 H]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^{AC-terminal}-GFP were overexpressed in HaEpi cells to examine the entrance of [3 H]Pon A into the cells. The [3 H]Pon A

Figure 7 | Binding assay of ErGPCR-2 to $[^3\text{H}]$ Pon A in HaEpi cells. (A). $[^3\text{H}]$ Pon A levels in the whole cells, *dsGFP* or *dsErGPCR-2* (2 $\mu\text{g}/\text{mL}$), for 12 h. The cells ($1\text{--}100 \times 10^4$) were then incubated with 0.1 nM $[^3\text{H}]$ Pon A (5740 cpm) in 200 μL binding buffer at 27°C for 1 h. (B). Co-immunoprecipitation of ErGPCR-2 and $[^3\text{H}]$ Pon A with the antibody against ErGPCR-2 after knockdown of *ErGPCR-2*. The cells were incubated with 1 μM 20E for 12 h, followed by the treatment used in (A). (C). $[^3\text{H}]$ Pon A levels in the supernatant after co-immunoprecipitation in (B). (D). Co-immunoprecipitation with the antibody against ErGPCR-2 after *GRK2* knockdown, similar to the method in (B). (E). $[^3\text{H}]$ Pon A levels in the supernatant after co-immunoprecipitation in (D). (F). $[^3\text{H}]$ Pon A levels in the whole HaEpi cells overexpressing GFP, 7TM-GFP, and 7TM $\Delta\text{C-terminal}$ -GFP, and then treated with the same method used in (A). **cpm**: counts per minute of $[^3\text{H}]$ Pon A. Asterisk indicates significant differences (**P* value) between two compared samples by Student's *t* test based on three independent experiments. Pictures on the overexpression of EcRB1, ErGPCR-2 and its mutants, and *ErGPCR-2* and *GRK2* knockdown are shown in Supplement Files: Figure S3.

levels in the whole cells were increased by overexpression of 7TM-GFP compared with the GFP control. The $[^3\text{H}]$ Pon A levels in the whole cells also increased more when 7TM $\Delta\text{C-terminal}$ -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 $[^3\text{H}]$ Pon A, and ErGPCR-2 internalization is unnecessary for the entrance of $[^3\text{H}]$ Pon A into the cells.

Discussion

Animal steroid hormones clearly transmit signals via GPCR²⁸. 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 $[^3\text{H}]$ Pon A entrance into the cells. However, ErGPCR-2 does not bind to $[^3\text{H}]$ Pon A. These results provide evidence that steroid hormone via GPCR transmits signals to direct gene transcription.

Some GPCRs can be internalized to desensitize signaling¹⁹ or transmit signals inside the cells continuously²⁹. The mechanism comprises GRK phosphorylation^{30,31}, interaction with β -arrestin, and internalization into the cytoplasm³². The internalized GPCR is either degraded by proteases in the lysosome or recycled in the cell membranes¹⁶. *H. armigera* ErGPCR-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 $[^3\text{H}]$ Pon A. These data suggest that the internalization of ErGPCR-2 does not bring $[^3\text{H}]$ 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 odorants¹⁴. Some GPCRs bind to animal steroid hormones; for example, GPR30 binds estrogen in humans³³ and DmDopEcR binds $[^3\text{H}]$ Pon A in *Drosophila*⁶. 20E (<http://en.wikipedia.org/wiki/Ecdysterone>) and $[^3\text{H}]$ Pon A (<http://www.scbt.com/zh/datasheet-202768-ponasterone-a.html>) are both ecdysones. 20E is a 20E-hydroxyecdysone, whereas $[^3\text{H}]$ Pon A is a 25-deoxy-20-hydroxyecdysone. $[^3\text{H}]$ Pon A is used to detect the binding of GPCR to steroid hormone 20E³⁴. However, we did not detect binding of ErGPCR-2 to $[^3\text{H}]$ Pon A by Co-IP of ErGPCR-2 and $[^3\text{H}]$ Pon A from the cell membrane or cytosol, which suggests that ErGPCR-2 does not bind or does not tightly bind to $[^3\text{H}]$ 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 $[^3\text{H}]$ 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 $[^3\text{H}]$ Pon

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 [3 H]Pon A¹³. 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 EcRb1²⁰, which then interacts with USP1 to form EcRb1/USP1 transcription complex, thereby initiating gene transcription in 20E signaling³. The transcription factors include *BR-Z7*, *HHR3*, *E74*, and *E75*, which initiate insect metamorphosis³⁵. 20E mediates CDK10 phosphorylation to enhance formation of the EcRb1/USP1 transcription complex³⁶. 20E via GPCR-, PLC-, Ca²⁺-, and PKC-signaling mediates USP1 phosphorylation for gene transcription¹². At the downstream of ligand activated-GPCR, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG)³⁷. 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 PKC³⁸. 20E via PKC regulates CDK10¹¹ and USP1 phosphorylation to form the EcR/USP1 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 study¹¹, 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).

Figure 8 | Diagram illustrating the 20E signal transmission mechanism of ErGPCR-2. ErGPCR-2 controls 20E entering the cells. 20E via ErGPCR-2 regulates rapid increase of cytosolic calcium, CDK10 and USP1 phosphorylation, gene transcription, and metamorphosis. ErGPCR-2 is internalized by GRK2-regulated phosphorylation and degraded to desensitize the 20E signal. Pathway Builder Tool 2.0 was used to draw the figure.

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 $26 \pm 1^\circ\text{C}$ 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-2RNAiF and ErGPCR-2RNAiR (Supplement Files: Table S1). The dsRNA was synthesized using MEGA-script RNAi Kit (Ambion Inc, Austin, TX, USA). The dsRNA purity and integrity were determined by agarose gel electrophoresis. The dsRNA was 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 thrice 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 *Helicoverpa* 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 $\mu\text{g}/\text{mL}$, 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 2×10^6 based on the above protocol, the cells were incubated with 3 μM acetoxymethyl (AM) ester calcium crimson™ dye (Invitrogen, Carlsbad, CA, USA) in Dulbecco's phosphate-buffered saline (DPBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , and 8 mM Na_2HPO_4) 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 at 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), flunarizine 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 λ PP degradation as described by Song and Gilbert (1998). Briefly, 40 μ L of protein (2 μ g/ μ L) was incubated with 0.5 μ L of λ PP, 5 μ L of buffer, and 5 μ L of MnCl_2 (50 μ L total) at 30°C for 30 min according to the manufacturer's specifications (Millipore, Temecula, CA, USA). The sample was boiled for 10 min after adding the SDS sample buffer and then subjected to SDS-PAGE and Western blot analysis. To detect the levels of ErGPCR-2 phosphorylation, 7TM domain was overexpressed in HaEpi cells by transfection with the pEX-7TM-GFP-His and pEX-4-7TM-His plasmids and then purified by His-Bind resin (50 μ L of resin) after different treatments. The levels of purified 7TM-GFP phosphorylation were analyzed in a 96-well microplate using a phosphoprotein phosphate estimation assay kit (Sangon Biotechnology, Shanghai, China) based on the alkaline hydrolysis of phosphate from seryl and threonyl residues in phosphoproteins. The released phosphates were then quantified using malachite green and ammonium molybdate in accordance with the manufacturer's instructions.

Overexpression of ErGPCR-2 and its mutants. Proofreading DNA polymerase (Tiangen, Beijing) was used to amplify ErGPCR-2 or its mutants via PCR with various primers (Supplementary Table S1). All the fragments were inserted into the pEX-4 plasmid (Merck, Darmstadt, Germany) and fused with GFP at the C-terminus. The plasmid (5 μ g/mL) was transfected into HaEpi cells with Cellfectin following the protocol of the supplier (Invitrogen, Carlsbad, CA, USA) in Grace's medium without FBS at 27°C for 24 h. After 2 d of culture, 1 μ M 20E was added to the cells. An equal volume of DMSO was used as solvent control for 20E.

Isolation of cell membrane and cytosol proteins. Membrane and cytoplasmic protein extraction kit (Beyotime, Haimen, China) was used to separate the membranes and cytosolic proteins with phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany) in accordance to the manufacturers' instructions.

Chromatin immunoprecipitation assay. Based on a previous procedure³⁹, chromatin immunoprecipitation (ChIP) assay was implemented. The cells were cultured in a six-well plate. First, the cells were transfected with pEX-4-EcRB1-RFP and the controls with pEX-4-RFP for 24 h. Then, the cells were transfected with *dsErGPCR-2* and the controls with *dsGFP* for 24 h. Finally, the cells were treated with 20E and DMSO for 6 h. Through the above process, the cells were cross-linked with 1% formaldehyde at 37°C for 10 min. Up to 0.125 M glycine was then added at 25°C for 10 min to terminate the cross-linking. The cells were washed twice with 1 $\times$ PBS and then suspended with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), followed by sonication to obtain the average DNA fragments of 200 bp to 1000 bp. After centrifugation, 100 μ L of supernatant was used to detect the effect of sonication. Other supernatants were added to the Protein A resin and incubated at 4°C for 1 h to pre-treat nonspecific binding. After centrifugation, one supernatant was used as a negative control sample for qRT-PCR. Other supernatants were incubated with anti-RFP antibody or without antibody (negative control) at 4°C overnight. Protein A resin was added into the immunoprecipitated protein-DNA complexes and incubated at 4°C for 2 h. The complexes were then washed with elution buffer (1% SDS, 0.1 M NaHCO_3). The DNA proteins were reversely cross-linked at 65°C overnight, followed by RNase and proteinase K treatments. The DNA was purified by phenol/chloroform extraction and subjected to qRT-PCR analysis using HHR3F/R primers (Table S1).

[³H]Pon A-binding assays. Ecdysone receptor B1 (EcRB1) fused with histidines (EcRB1-His) was overexpressed in HaEpi cells with pEX-4-His vector to bind [³H]Pon A. The cells were incubated twice with *dsGFP* or *dsErGPCR-2* (2 μ g/mL) for 12 h as described above. The cells (1×10^4 to 100×10^4) were collected and then incubated with 0.1 nM [³H]Pon A (5740 cpm) in 200 μ L of binding buffer (20 mM HEPES, 100 mM NaCl, 6 mM MgCl_2 , 1 mM EDTA, and 1 mM EGTA) at 27°C for 1 h. Subsequently, the cells were subjected to [³H]Pon A detection. For Co-IP with antibodies against ErGPCR-2, the cells were incubated with 1 μ M 20E for 12 h to increase the expression of ErGPCR-2 and EcRB1, followed by incubation with 2 μ g/mL of *dsGFP*, *dsErGPCR-2*, and *dsGRK2* twice for 12 h each. The cells were collected and incubated with 0.1 nM [³H]Pon A in 200 μ L binding buffer at 27°C for 1 h. The cells were lysated in RIPA (Radio immunoprecipitation Assay) buffer, after which 40 μ L of anti-ErGPCR-2 and 50 μ L of the Protein A resin was added at 4°C for 4 h to co-immunoprecipitate (CoIP) ErGPCR-2 in the membrane and cytosol which possible binding to [³H]Pon A. The precipitates were washed twice with RIPA buffer. After Co-IP, [³H]Pon A was detected in the supernatant. For the overexpression of the seven-transmembrane (7TM) domain of ErGPCR-2 and its mutants, the plasmids of pEX-4-GFP, pEX-4-7TM-GFP, OVERGPCR-2-7TM^{AC-terminal}-GFP, and OVERGPCR-2-7TM^{Δ2loop}-GFP (5 μ g/mL) were transfected into the HaEpi cells with Cellfectin following the protocol of the supplier (Invitrogen, Carlsbad, CA, USA) in Grace's medium without FBS at 27°C for 24 h. Then, the cells were collected and incubated with 0.1 nM [³H]Pon A (5740 cpm) in 200 μ L binding buffer at 27°C for 1 h. After all the above mentioned treatments, particular cell proteins were collected on glass fiber filters. After air drying in the dark, the different filters were then added into 5 mL of scintillation fluid. Radioactivity was measured using SN-6930 liquid scintillation counter (Shanghai Hesuo Rihuan Photoelectric Instrument Co., Ltd.,

China). The efficacy of RNA interference of ErGPCR-2 and GRK2 was examined via Western blot.

- Gruber, C. J., Tschugguel, W., Schneeberger, C. & Huber, J. C. Production and actions of estrogens. *N. Engl. J. Med.* **346**, 340–352 (2002).
- Lezzi, M. et al. Ligand-induced heterodimerization between the ligand binding domains of the *Drosophila* ecdysteroid receptor and ultraspiracle. *Eur. J. Biochem.* **269**, 3237–3245 (2002).
- Riddiford, L. M., Hiruma, K., Zhou, X. & Nelson, C. A. Insights into the molecular basis of the hormonal control of molting and metamorphosis from *Manduca sexta* and *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **33**, 1327–1338 (2003).
- Losel, R. M. et al. Nongenomic steroid action: controversies, questions, and answers. *Physiol. Rev.* **83**, 965–1016 (2003).
- Maggiolini, M. & Picard, D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J. Endocrinol.* **204**, 105–114 (2010).
- Srivastava, D. P. et al. Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. *The J. Neurosci.* **25**, 6145–6155 (2005).
- Manaboon, M., Iga, M., Iwami, M. & Sakurai, S. Intracellular mobilization of Ca^{2+} by the insect steroid hormone 20-hydroxyecdysone during programmed cell death in silkworm anterior silk glands. *J. insect physiol.* **55**, 123–129 (2009).
- Liu, P. C., Wang, J. X., Song, Q. S. & Zhao, X. F. The participation of calponin in the cross talk between 20-hydroxyecdysone and juvenile hormone signaling pathways by phosphorylation variation. *PLoS one* **6**, e19776 (2011).
- Hou, L., Cai, M. J., Liu, W., Song, Q. & Zhao, X. F. Small GTPase Rab4b participates in the gene transcription of 20-hydroxyecdysone and insulin pathways to regulate glycogen level and metamorphosis. *Dev. Biol.* **371**, 13–22 (2012).
- Zheng, W.-W. et al. Hsc70 binds to ultraspiracle resulting in the upregulation of 20-hydroxyecdysone-responsive genes in *Helicoverpa armigera*. *Mol. Cell. Endocrinol.* **315**, 282–291 (2010).
- Liu, W., Cai, M.-J., Wang, J.-X. & Zhao, X.-F. In a non-genomic action, steroid hormone 20-hydroxyecdysone induces phosphorylation of cyclin-dependent kinase 10 to promote gene transcription. *Endocrinology* **155**, 1738–1750 (2014).
- Liu, W., Cai, M.-J., Zheng, C.-C., Wang, J.-X. & Zhao, X.-F. Phospholipase $\text{C}\gamma 1$ connects the cell membrane pathway to the nuclear receptor pathway in insect steroid hormone signaling. *J. Biol. Chem.* **289**, 13026–13041 (2014).
- Cai, M.-J. et al. G-protein-coupled receptor participates in 20-hydroxyecdysone signaling on the plasma membrane. *Cell Communication and Signaling* **12**, 9 (2014).
- Marinissen, M. J. & Gutkind, J. S. G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol. Sci.* **22**, 368–376 (2001).
- Venkatakrishnan, A. J. et al. Molecular signatures of G-protein-coupled receptors. *Nature* **494**, 185–194 (2013).
- Seachrist, J. L. & Ferguson, S. S. Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases. *Life Sci.* **74**, 225–235 (2003).
- Tsao, P. & von Zastrow, M. Downregulation of G protein-coupled receptors. *Curr. Opin. Neurobiol.* **10**, 365–369 (2000).
- Ferguson, S. S. & Caron, M. G. G protein-coupled receptor adaptation mechanisms. *Semin. Cell Dev. Biol.* **9**, 119–127 (1998).
- Ritter, S. L. & Hall, R. A. Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat. Rev. Mol. Cell. Biol.* **10**, 819–830 (2009).
- Koelle, M. R. et al. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**, 59–77 (1991).
- Shao, H.-L. et al. Establishment of a new cell line from lepidopteran epidermis and hormonal regulation on the genes. *PLoS One* **3**, e3127 (2008).
- Terland, O. & Flatmark, T. Drug-induced parkinsonism: cinnarizine and flunarizine are potent uncouplers of the vacuolar H^+ -ATPase in catecholamine storage vesicles. *Neuropharmacology* **38**, 879–882 (1999).
- Kiyonaka, S. et al. Selective and direct inhibition of TRPC3 channels underlies biological activities of a pyrazole compound. *P. Natl. Acad. Sci. U. S. A.* **106**, 5400–5405 (2009).
- Thastrup, O. Role of Ca^{2+} -ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+} -ATPase inhibitor, thapsigargin. *Agents and actions* **29**, 8–15 (1990).
- Abdollahi, A. et al. SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. *Cancer Res.* **63**, 3755–3763 (2003).
- Lan, Q., Hiruma, K., Hu, X., Jindra, M. & Riddiford, L. M. Activation of a delayed-early gene encoding MHR3 by the ecdysone receptor heterodimer EcR-B1-USP-1 but not by EcR-B1-USP-2. *Mol. Cell Biol.* **19**, 4897–4906 (1999).
- Claing, A., Laporte, S. A., Caron, M. G. & Lefkowitz, R. J. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and β -arrestin proteins. *Prog. Neurobiol.* **66**, 61–79 (2002).
- Lösel, R. & Wehling, M. Nongenomic actions of steroid hormones. *Nature Reviews Mol. Cell Biol.* **4**, 46–55 (2003).
- Irannejad, R. & von Zastrow, M. GPCR signaling along the endocytic pathway. *Curr. Opin. Cell Biol.* **27**, 109–116 (2014).
- Hausdorff, W., Caron, M. & Lefkowitz, R. Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB J.* **4**, 2881–2889 (1990).

31. Benovic, J. L., Strasser, R. H., Caron, M. G. & Lefkowitz, R. J. Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *P. Natl. Acad. Sci. U. S. A.* **83**, 2797–2801 (1986).
32. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G. & Lefkowitz, R. J. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* **248**, 1547–1550 (1990).
33. Gurevich, V. V. *et al.* Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, beta 2-adrenergic, and m2 muscarinic cholinergic receptors. *J. Biol. Chem.* **270**, 720–731 (1995).
34. Elmogy, M., Iwami, M. & Sakurai, S. Presence of membrane ecdysone receptor in the anterior silk gland of the silkworm *Bombyx mori*. *Eur. J. Biochem.* **271**, 3171–3179 (2004).
35. Hiruma, K. & Riddiford, L. M. The molecular mechanisms of cuticular melanization: The ecdysone cascade leading to dopa decarboxylase expression in *Manduca sexta*. *Insect Biochem. Mol. Biol.* **39**, 245–253 (2009).
36. Liu, C. Y., Liu, W., Zhao, W. L., Wang, J. X. & Zhao, X. F. Upregulation of the expression of prodeath serine/threonine protein kinase for programmed cell death by steroid hormone 20-hydroxyecdysone. *Apoptosis* **18**, 171–187 (2013).
37. Kadamur, G. & Ross, E. M. Mammalian phospholipase C. *Annu. Rev. Physiol.* **75**, 127–154 (2013).
38. Rosse, C. *et al.* PKC and the control of localized signal dynamics. *Nat. Rev. Mol. Cell Biol.* **11**, 103–112 (2010).
39. Carey, M. F., Peterson, C. L. & Smale, S. T. Chromatin immunoprecipitation (ChIP). *Cold Spring Harbor Protocols* **2009**, pdb. prot5279 (2009).

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 31230067), the National Basic Research Program of China (973 Program, No. 2012CB114101), and the Ph.D. Programs Foundation of the Ministry of Education of China (No. 20120131110025).

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>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Wang, D., Zhao, W.-L., Cai, M.-J., Wang, J.-X. & Zhao, X.-F. G-protein-coupled receptor controls steroid hormone signaling in cell membrane. *Sci. Rep.* **5**, 8675; DOI:10.1038/srep08675 (2015).

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/4.0/>