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Role of cAMP/PKA/CREB pathway and β-arrestin 1 in LH induced luteolysis in pregnant rats

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Abstract

Luteal dysfunction in pregnant women is associated with early pregnancy loss, making the study of structure and function of the corpus luteum (CL) critical. Luteinizing hormone (LH) plays a crucial role in the mammalian female reproduction majorly by regulating luteal development. In rats, the luteotropic roles of LH have been widely investigated but its role in the process of luteolysis has received little attention. In this study, we explored the luteolytic actions of LH during different stages of pregnancy in rats. Repeated administration of LH during the late and mid-stages of pregnancy led to functional luteolysis during both stages, while structural luteolysis was observed only during the late-stage. We analyzed the involvement of cAMP/PKA/CREB pathway, MAP kinases and β-arrestins to elucidate the molecular mechanism of LH-mediated luteolysis. The results indicate that the repeated administration of LH causes LH/CGR desensitization along with an increase in β-arrestin 1 expression, while luteal expression of MAP kinases remained unaffected. Further, siRNA-mediated depletion of β-arrestin 1 in primary luteal-cell cultures prevents initiation of the luteolysis process to some extent during both the stages of pregnancy, underscoring its role in LH mediated-luteolysis. In conclusion, the luteolytic actions of LH appear to involve more than one signaling pathway and cAMP/PKA/CREB pathway appears to be the key regulator. This is the first report to show a positive correlation between β-arrestin 1 and *20α-hsd* expression. These findings have implications for our understanding of the molecular pathways that regulate luteolysis.

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Introduction

In humans, corpus luteum (CL) acts as the principal source of progesterone (P_4) until the placenta takes over (Goyeneche *et al.* 2003, Stouffer *et al.* 2013). Luteal dysfunction during this period is the major cause of implantation failure leading to early phase miscarriages (Shah & Nagarajan 2013). Luteinizing hormone (LH) plays a critical role in the growth and maintenance of CL in mammals. LH has a stage-dependent effect on pregnancy in rats. Inhibiting LH via antiserum administration from days of pregnancy (DOP) 7–12 causes interruption of pregnancy in rats and hence impinge on its luteotropic nature (Loewit *et al.* 1969). LH antisera administration from DOP 12–16 leads to the complete or partial reabsorption of pregnancy whereas administration from DOP 14–18 appears to have no effect on pregnancy (Loewit *et al.* 1969, Stocco & Deis 1998). However, during the later stages of pregnancy, Stocco *et al.* have utilized different methods including intrabursal administration of rat LH antiserum, intrabursal administration of different doses of LH and repeated administration of LH on day 19 of pregnancy to demonstrate its luteolytic effects (Stocco & Deis 1996, 1998).

Intriguingly, in rats, it has been observed that circulating levels of pituitary LH increase prior to parturition (Morishige *et al.* 1973). The physiologic increase in the levels of LH toward the end of pregnancy might be linked to the onset of luteolysis preceding parturition. Neutralization of LH toward the end of pregnancy with a blocking antibody prevents serum P_4 levels from dropping (Stocco & Deis 1996). This further points toward the luteolytic effect of increased LH levels during later stages of pregnancy. Although the luteotropic role of LH has been widely accepted and investigated, its role in the process of luteolysis has received little attention. Therefore, we conducted experiments to understand the molecular mechanism involved in the luteolytic effects of LH. Of the different methods reported (Stocco & Deis 1996), the repeated administration of LH was used to induce luteolysis.

LH mediates its effects by binding to its cognate receptor, lutropin-choriogonadotropic hormone receptor (LH/CGR) and thereby activating the canonical cAMP/PKA/CREB and MAP kinase signaling cascades (Yadav & Medhamurthy 2006, Riccetti *et al.* 2017). Moreover, LH/CGR can also engage multiple G-protein independent pathways including β-arrestin dependent pathways (Ulloa-Aguirre *et al.* 2011, De Pascali & Reiter 2018). As is the case with other GPCR's, repeated activation of LH/CGR by agonists attenuates coupling

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of LH/CGR to Gαs and thereby the activation of cAMP/PKA/CREB signaling cascade leading to desensitization of the receptor (Mukherjee *et al.* 1999a, Choi & Smitz 2014). Physiologically, the desensitization of LH/CGR occurs immediately after mid-cycle gonadotropin surges (Ascoli *et al.* 2002). Initially, β-arrestins were considered to be only involved in the process of GPCR silencing. However, they are now recognized as scaffold proteins that also participate in the regulation of GPCR signaling, trafficking and activation of G-protein independent signaling pathways (Smith & Rajagopal 2016, van Gastel *et al.* 2018). In a recent study involving murine Leydig tumor cell line-1 (mLTC-1), β-arrestins were implicated in P_4 biosynthesis by acting at a site downstream of LH/CGR (Riccetti *et al.* 2017).

Studies have been carried out with a view to elucidate the molecular mechanism/s involved in the initiation of LH-mediated luteolysis in pregnant rats. Since repeated activation of LH/CGR will be a cue for desensitization, a role for β-arrestins in the initiation of luteolysis has been investigated. Another question addressed in this study was whether the repeated administration of the LH method is stage-specific and can it mediate the same effects during different stages of pregnancy. To this end, studies were carried out during the late (DOP 19–20) and mid-stage (DOP 8–10) of pregnancy.

Materials and methods

Reagents

Ovine LH was a kind gift from Professor M R Sairam (University of Montreal, Quebec, Canada). Cetrorelix (Cet) was a kind gift from Asta Medica (Frankfurt, Germany). Trizol (93289), M199 (M4530), DMEM (D5796), FBS (F2442), antibiotic-antimycotic reagent (A5955), DPBS (D5652) and Forskolin (F6886) were purchased from Sigma–Aldrich Co. P_4 RIA kit (IM1188) was procured from Beckman Coulter (CA, USA). Antibody against cyclic AMP, CV-27 pool was obtained from Dr A.F. Parlow, NHPP (CA, USA). PKA assay kit (ADI-EKS-390A) was purchased from Enzo Life Sciences (NY, USA). siGENOME Rat Arrb1 (25387) siRNA-SMARTpool (M-080156-01-0005) and siGENOME Non-targeting siRNA pool#1 (D-001206-13-05) were purchased from Dharmacon. Opti-MEM (#31985062) and lipofectamine 2000 (#11668019) were purchased from Thermo Fisher Scientific. The details of antibodies and siRNA employed are provided in Tables 1 and 2, respectively. All the other reagents were either purchased from Sigma–Aldrich Co. or sourced from local distributors.

Animals

The Institutional Animal Ethics Committee, Indian Institute of Science (Bangalore, India), approved procedures for animal handling and experimentation. *Rattus norvegicus* (Harlan–Wistar strain) were provided with proper light:darkness cycle, temperature, humidity, food and water. To obtain pregnant rats, 2- to 3-month-old female rats were cohabitated with fertile male rats. Vaginal smears were screened daily for the presence of sperm and the day of appearance of sperm was designated as DOP 1. After confirmation of pregnancy status, female rats were separated and housed in individual cages before the initiation of studies. The gestation period in our colony of rats was 23 days.

Effect of LH administration on CL function and structure in pregnant rats

Studies were carried out during the late (DOP 19) and mid-stage (DOP 8–10) of pregnancy. Previously published protocol with few modifications (Stocco & Deis 1998) was followed. Briefly, 19 days old pregnant rats (late-pregnant stage) were assigned into two groups (five animals/group) and each group received either four i.p. injections of Veh (100 µL of 1× PBS) or LH (10 µg dissolved in 100 µL of 1× PBS) at 08:00, 09:00, 10:00 and 11:00 h. On DOP 20, rats from both the groups received one i.p. injection of LH (10 µg). During mid-pregnancy, circulating LH levels have been observed to be high and CL has been shown to be dependent on LH during this time period (Loewit *et al.* 1969). In order to rule out the involvement of endogenous LH during repeated exogenous administration of LH, Cet, a specific GnRH receptor antagonist was administered to inhibit endogenous levels of LH. The tested dose and duration of Cet treatment were reported previously (John *et al.* 2016). It was observed that administration of 150 µg/Kg BW of Cet every 12 h on DOP 8–9 of pregnancy resulted in luteolysis and loss of pregnancy (John *et al.* 2016). Rats on DOP 8 were assigned to four different groups with five rats/group. Rats in the first and second groups received four s.c. injections of either Veh (100 µL of 1× PBS) or Cet (150 µg/Kg BW) at 12 h intervals for 48 h beginning on DOP 8, respectively. Twelve hours after the last injection of Veh/Cet, one i.p. injection of Veh was administered on DOP 10. Rats in the third and fourth groups received Cet treatment as per the second group and after the last Cet injection, received one (third group) or four (fourth group) injections of LH (10 µg: at hourly intervals), respectively. The first, second, third and fourth groups will be referred to as Veh, Cet+Veh, Cet+LH and Cet+4LH groups, respectively. Blood and CL were collected from rats during both stages, 40 min after the last Veh/LH injection.

Table 1 List of antibodies used for immunoblotting.

Si. No.	Antibody	Company	Catalog number	Dilution	Secondary antibody
1.	GAPDH D16H11	CST	#5174	1:1000	Anti-rabbit
2.	Beclin 1	CST	#3738	1:1000	Anti-rabbit
3.	LC3I/II D3U4C0	CST	#12741	1:1000	Anti-rabbit
4.	Cleaved caspase 3	CST	#9661	1:1000	Anti-rabbit
5.	pCREB Ser133	CST	#9198	1:1000	Anti-rabbit
6.	CREB 48H2	CST	#9197	1:1000	Anti-rabbit

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Table 2 List of primers used for real-time PCR.

Gene name	Primer sequence (5′ to 3′)	AT (°C)
Rpl19		62
	F- CGTCCTCCGCTGTGGTAAA	
	R- AGTACCCTTCCTCTTCCCTATGC	
*Akr1c18**		64
	F- CTGTAAACCAGGTCGAATGTCAC	
	R- GGGTAGTTCGGGTTCACCC	
Arrb1		64
	F- CAAGATCTCGGTGCGCCAGTATGCAGA	
	R- GTTGGCGCCTTCCCGCAACAGAGT	
Scrambled siRNA pool		
	UAGCGACUAAACACAUCAA	
	UAAGGCUAUGAAGAGAUAC	
	AUGUAUUGGCCUGUAUUAG	
	AUGAACGUGAAUUGCUCAA	
Arrb1 siRNA pool		
	CAACAUUCUGCAAGGUCUA	
	GGGUCUGACUUUUCGCAAA	
	ACGAAGAGGAUGAUGAUGGCAC	
	CGACAAAGGGACACGAGUG	

**20α-hsd.*
AT, annealing temperature.

In-vitro luteal tissue culture

Studies were performed to induce desensitization of LH/CGR/cAMP/CREB pathway utilizing *in-vitro* tissue culture systems as previously reported with few modifications (Hedin & Rosberg 1983). On DOP 10 (mid-stage) or 20 (late-stage) of pregnancy, corpora lutea were harvested, sliced and pre-incubated in media (M199) containing LH (5 µg), which was added every half an hour for 2 h to induce desensitization, this period is referred to as pre-incubation period henceforth. Control experiments were also done where sliced CL tissues were incubated with media alone (Veh) for 2 h in the pre-incubation period. At the end of the pre-incubation period, the luteal tissue slices were washed and stimulated with media containing Veh, LH (10 µg) or forskolin (FSK 10 mM) for 40 min, this period is referred to as the stimulation period henceforth. At the end of the stimulation period, CL tissue slices were either processed for RNA isolation or cAMP lysate preparation. Media collected at the end of the stimulation period was stored at −20°C for P_4 estimation.

Luteal cell primary culture

Ovaries of mid-stage (DOP 8–10) or late-stage (DOP 19–20) pregnant rats were cleared of fat and extensively washed in 1× DPBS in a sterile petri-plate. The protocol followed for the establishment of the luteal cell primary culture was reported previously (Nelson *et al.* 1992). The dispersed luteal cells were counted and ~ 0.6 − 1 × 10^6 cells were seeded in a 12 well plate in a complete DMEM medium. Cells were grown in a complete DMEM medium supplemented with 10% FBS, 1× antimycotic-antibiotic reagent for 48 h before siRNA transfection.

RNA interference

For transfection, a siRNA smart pool consisting of four siRNAs against a single target was used. The siRNA sequences used to target rat β-arrestin 1 (β-arr 1) and scrambled (sc) siRNA with no silencing effect are provided in Table 2. After 48 h of culture, luteal cells were transiently transfected with the respective siRNA (75 nM) following the manufacturer's recommendations. After 6 h of transfection, the medium was changed to complete DMEM and the cells were allowed to grow for another 48 h. Knockdown of β-arrestin 1 expression was determined 48 h after transfection by qPCR expression analysis and Western blotting. For the induction of desensitization of the LH/CGR/cAMP/PKA pathway in the primary culture of luteal cells post selective depletion of β-arrestin 1, the protocol followed was similar to the one described under *in-vitro* luteal tissue culture. The only difference being the media utilized in the siRNA transfected luteal cells was DMEM instead of M199 and FSK was not used during the stimulation period.

Effect of physiologically low and high circulatory levels of LH on LH/cAMP/CREB signaling pathway

Pregnant rats on DOP 18 (*n* = 3) and 21 (*n* = 3) were administered with a single i.p. injection of LH (10 µg). 40 min after LH administration rats were sacrificed to collect the blood (for P_4 level analysis) and CL (for cAMP level analysis).

RIA of cAMP

For cAMP lysate preparation, CL tissue weighing around 10–15 mg was homogenized in 200–300 µL of 0.1N HCl and centrifuged at 7871 ***g*** for 8 min at 4°C. The supernatant was saved and stored at −80°C until assayed for cAMP levels. To increase the sensitivity of the assay, the supernatant was acetylated by adding 1.5 µL triethylamine, 0.75 µL acetic anhydride and 242.75 µL of sodium acetate buffer to 5 µL of sample. From this, 200 µL of the acetylated sample was used for analysis. The assay protocol followed has been described previously (Shah *et al.* 2014). The antibody (CV-27) at a dilution of 1:30,000 (~ 40% binding) was used for the assay of samples.

qPCR

Total RNA from CL tissues was isolated using TRI reagent according to the manufacturer's instructions. The qPCR analysis was performed as described previously (Shah *et al.* 2014). The list of primers used in the qPCR analysis is provided in Table 2.

Immunoblotting

Immunoblot analysis of the total protein lysates from CL tissue was carried out as per the procedures reported previously (Shah *et al.* 2014). The list of antibodies used in the immunoblotting analysis is provided in Table 1.

PKA assay

The PKA kinase activity assay is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA). PKA activity was determined by employing a commercially available PKA kit as per the manufacturer's instructions. The PKA assay was performed as described previously (Priyanka & Medhamurthy

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2007). The unit represents pmol of phosphate incorporated into substrate/min/ug of the sample, at 30°C.

P_4 assay

Serum P_4 levels were determined by employing a commercially available RIA kit as per the manufacturer's instructions. The inter- and intra-assay coefficients of variation were found to be ≤9%.

Statistical analysis

All data are expressed as mean ± s.e.m. The graphs were plotted and analyzed using GraphPad Prism® 5 software (GraphPad Software, Inc.). Statistical analysis of the results between two groups was done using two-tail paired '*t*'-test with 95% confidence intervals. Comparisons between three or more groups were carried out using one-way ANOVA and Bonferroni post-tests with 95% confidence intervals. A *P* value < 0.05 was considered to be statistically significant.

Results

Effect of repeated administration of LH on CL function and structure in late- and mid-pregnant rats

Repeated administration of LH reduced P_4 levels (162.0 ± 13.19 ng/mL to 100.2 ± 12.29, $P<0.01$) (Fig. 1A, right panel) and elevated luteal *20α-hsd* expression ($P < 0.01$) (Fig. 1A, left panel) during late-pregnancy. During mid-pregnancy, P_4 levels were significantly low in Cet+Veh group (34.33 ± 6.84 ng/mL, $P< 0.001$) and Cet+LH (67.66 ± 8.1 ng/mL, $P< 0.01$) group compared to Veh treated rats (150 ± 11.55 ng/mL) (Fig. 1B, right panel). However, P_4 levels increased in the Cet+4LH group (94 ± 14.18 ng/mL, $P< 0.05$) as compared to Cet+Veh (34.33 ± 6.84 ng/mL) group (Fig. 1B, right panel). Consistent with the reduction in P_4 levels post-Cet treatment, an increase ($P< 0.01$) was observed in *20α-hsd* expression in rats receiving Cet+Veh treatment (Fig. 1B, left panel). However, administration of a single LH injection to Cet-treated rats (Cet+LH group) brought *20α-hsd* to basal levels (Fig. 1B, left panel). A significant increase ($P< 0.05$) in *20α-hsd* expression was observed in Cet treated rats receiving four injections of LH compared to Cet+LH group rats (Fig. 1B, left panel).

Analysis of markers of structural luteolysis revealed an increase in protein levels of cleaved caspase 3 (marker of apoptosis, $P< 0.05$), Beclin 1 (marker of autophagy, non-significant) and LC3 II (marker of autophagy, non-significant) in repeated LH-treated late-pregnant rats (Fig. 1C, D and E). During mid-pregnancy, cleaved caspase 3 protein levels increased non-significantly in Cet+Veh group rats compared to Veh treated rats. However, its expression decreased to undetectable levels in Cet+LH and Cet+4LH groups (Fig. 1F). Expression levels of Beclin 1 and LC3 II did not change across different treatment groups during mid-pregnancy (Fig. 1G and H).

Effect of repeated administration of LH on cAMP/PKA/CREB and MAPK pathway in late- and mid-pregnant rats

LH/CGR has been shown to regulate cAMP/PKA/CREB pathway, MAPK pathway and *Arrb1* expression (Yadav & Medhamurthy 2006, Choi & Smitz 2014, Riccetti *et al.* 2017). Hence, we analyzed their involvement in the initiation of luteolysis. Repeated administration of LH to late-pregnant rats decreased the luteal expression of *Lh/Cgr* ($P< 0.001$), cAMP content ($P< 0.001$), PKA activity ($P< 0.001$) and pCREB protein levels ($P< 0.01$) compared to Veh-treated rats (Fig. 2A, B, C and D). In mid-pregnant rats, Cet+Veh administration decreased *Lh/Cgr* expression ($P< 0.001$) with non-significant changes in cAMP levels, PKA activity and pCREB expression (Fig. 2E, F, G and H). Administration of a single LH injection to Cet-treated rats increased cAMP levels ($P< 0.001$), PKA activity ($P< 0.01$) and pCREB expression ($P< 0.05$) compared to Cet+Veh group rats (Fig. 2F, G and H). However, Cet+4LH group rats showed a decrease in the luteal cAMP ($P< 0.001$), PKA activity ($P< 0.01$) and pCREB levels ($P< 0.05$) compared to Cet+LH group (Fig. 2F, G and H). *Lh/Cgr* levels did not change in Cet+LH and Cet+4LH groups (Fig. 2E). The results of repeated administration of LH during late-stage of pregnancy and Cet-treated mid-pregnant rats suggest desensitization of the pathway.

To confirm if the desensitization of the LH/CGR/cAMP/PKA/CREB pathway was responsible for the initiation of luteolytic events, we conducted experiments in luteal tissue culture. cAMP levels were used as a marker of desensitization. Presence of LH during both pre-incubation and stimulation period reduced cAMP accumulation during late-stage (Veh pre-incubated: 400 ± 41.63 fm/mg CL, LH pre-incubated: 66.67 ± 21.86 fm/mg CL, $P< 0.01$, Fig. 3B) and mid-stage (Veh pre-incubated: 639 ± 24.79 fm/mg CL, LH pre-incubated: 71.67 ± 8.76 fm/mg CL, $P< 0.001$, Fig. 3D) of pregnancy compared to Veh pre-incubated and LH-stimulated luteal tissue cultures. The results indicated that repeated exposure of luteal tissue to LH significantly reduced cAMP levels under *in-vitro* culture conditions. After establishing desensitization of the LH/CGR/cAMP/PKA/CREB pathway upon repeated LH treatment *in-vitro*, we next activated this pathway via FSK in LH-preincubated luteal cells and analyzed its effects on the initiation of LH-mediated luteolysis. Pre-incubation in LH did not reduce the subsequent response of the cAMP system to FSK compared to LH pre-incubated and stimulated luteal tissues during both late-stage ($P< 0.001$, Fig. 3B) and mid-stage ($P< 0.001$, Fig. 3D) of pregnancy. This suggests the desensitization to be at the receptor level. No changes in the expression of *Lh/Cgr* were observed across different treatment groups during both the late (Fig. 3A) and mid-(Fig. 3C) stage of pregnancy.

The effect of LH/CGR desensitization *in-vitro* on the markers of luteolysis was examined. Presence of

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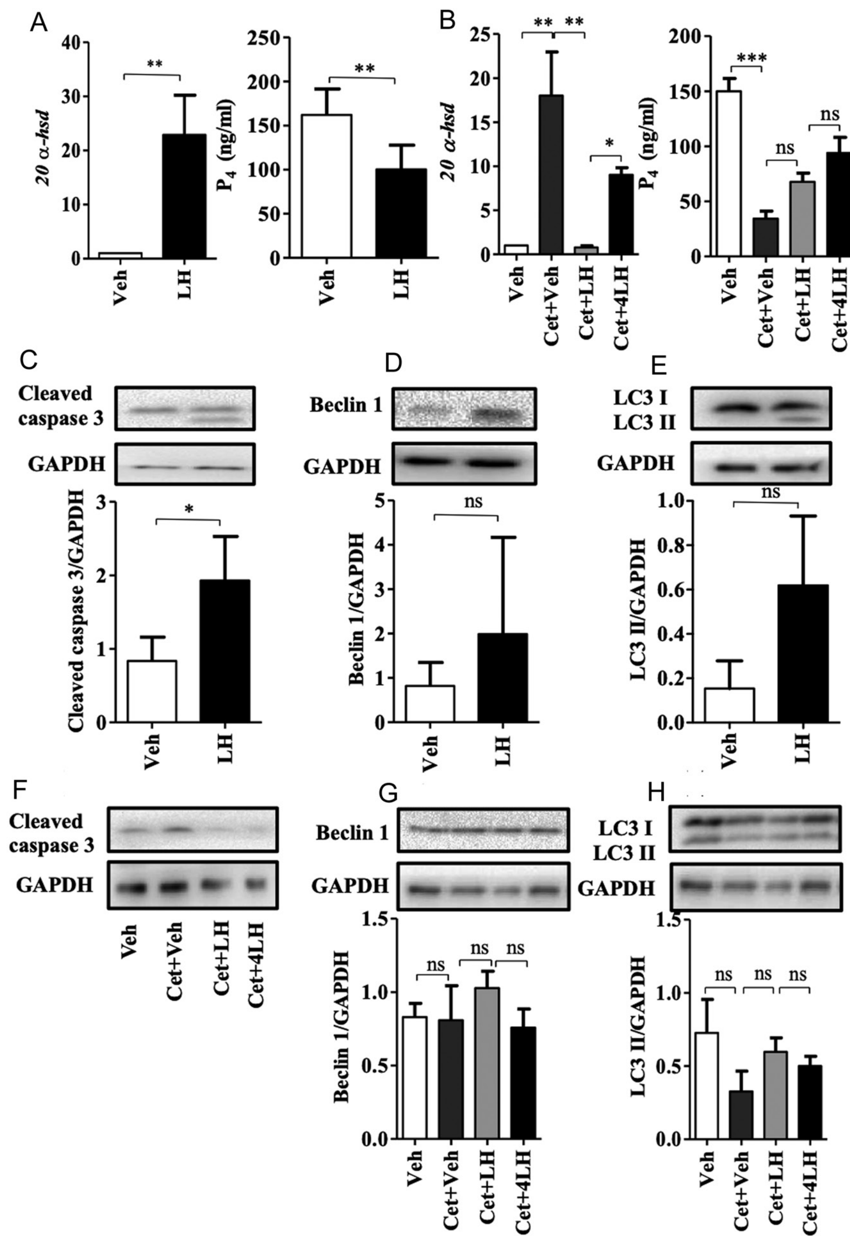

Figure 1 Effects of multiple injections of exogenous LH on CL function and structure in late- and mid-pregnant rats. The mid-pregnant rats were also treated with Cet to inhibit endogenous LH secretion prior to administration of single or multiple injections of Veh/LH. Luteal 20α-hsd mRNA expression and serum P_4 levels in late- (A) and mid (B)-pregnant rats. Representative immunoblot image and quantitation data of structural luteolysis markers such as cleaved caspase 3, Beclin 1 and LC3 I/II from CL of late- (C, D and E) and mid (F, G and H)-pregnant rats, respectively. L19 mRNA expression and GAPDH protein levels were used as an internal control for qPCR and immunoblot analyses, respectively. The data represent mean ± s.e.m of 3–5 rats/group. *P < 0.05; **P < 0.01 and ***P < 0.001.

LH during both pre-incubation and stimulation period increased the expression of *20a-hsd* (late: P < 0.001, Fig. 3E, left panel; mid: P < 0.001, Fig. 3F, left panel) and decreased P_4 levels (late: P < 0.05, Fig. 3E, right panel, mid: non-significant, Fig. 3F, right panel) compared to Veh pre-incubated and LH-stimulated luteal tissue. During both stages of pregnancy, the stimulation of LH-pre-incubated luteal tissue with FSK restored the expression of *20a-hsd* and P_4 levels similar to the levels where LH is present only in the stimulation media (Fig. 3E and F). The results of *in-vitro* studies indicate that the repeated exposure of luteal tissue results in desensitization of the LH/CGR which hinders its ability to activate the cAMP/PKA/CREB pathway which in turn leads to activation of luteolytic events. During both the stages of pregnancy, no significant change was observed in the expression of markers of the MAPK pathway: pERK, pp38 and pJNK (data not shown).

Role of β-arrestins in LH-mediated activation of luteolytic events

During the late-stage of pregnancy, the repeated administration of LH resulted in a non-significant increase in *Arrb1* mRNA and protein levels (Fig. 4A and B) with no change in pARRB1 levels (Fig. 4C). During mid-pregnancy, the luteal mRNA levels of *Arrb1* did not change in Cet+Veh or Cet+LH group; however,

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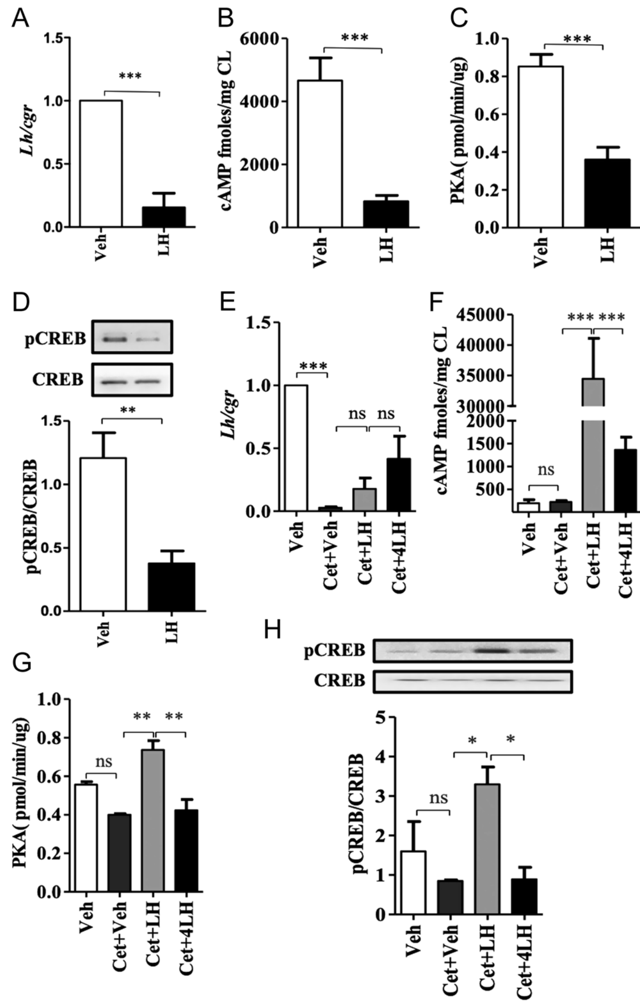

Figure 2 Effect of repeated administration of LH on various components of cAMP signal transduction pathway in CL of late- and mid-pregnant rats. The mid-pregnant rats were also treated with Cet to inhibit endogenous LH secretion prior to administration of single or multiple injections of Veh/LH. The data for late-pregnant rats are presented from A, B, C and D, and for mid-pregnant rats from E, F, G and H. mRNA expression of *Lh/Cgr* (A and E), cAMP levels (B and F), PKA activity in pmol/min/microgram (C and G) in luteal tissues of late- and mid-pregnant rats. Representative immunoblot image and quantitation data for pCREB and CREB (D and H) in the luteal tissues of late- and mid-pregnant rats. *L19* mRNA expression and GAPDH protein levels were used as an internal control for qPCR and immunoblot analyses, respectively. Data represent mean ± s.e.m of 3–5 rats/group. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

a non-significant increase in its mRNA level was observed in Cet+4LH group (Fig. 4D). Protein levels of ARRB1 were lower in the Cet+LH group compared to Cet+Veh group rats (Fig. 4E). However, the levels were significantly ($P < 0.01$) high in Cet+4LH compared to Cet+LH treated rats (Fig. 4E). The pARRB1 levels did not change significantly across treatment groups during mid-pregnancy (Fig. 4F).

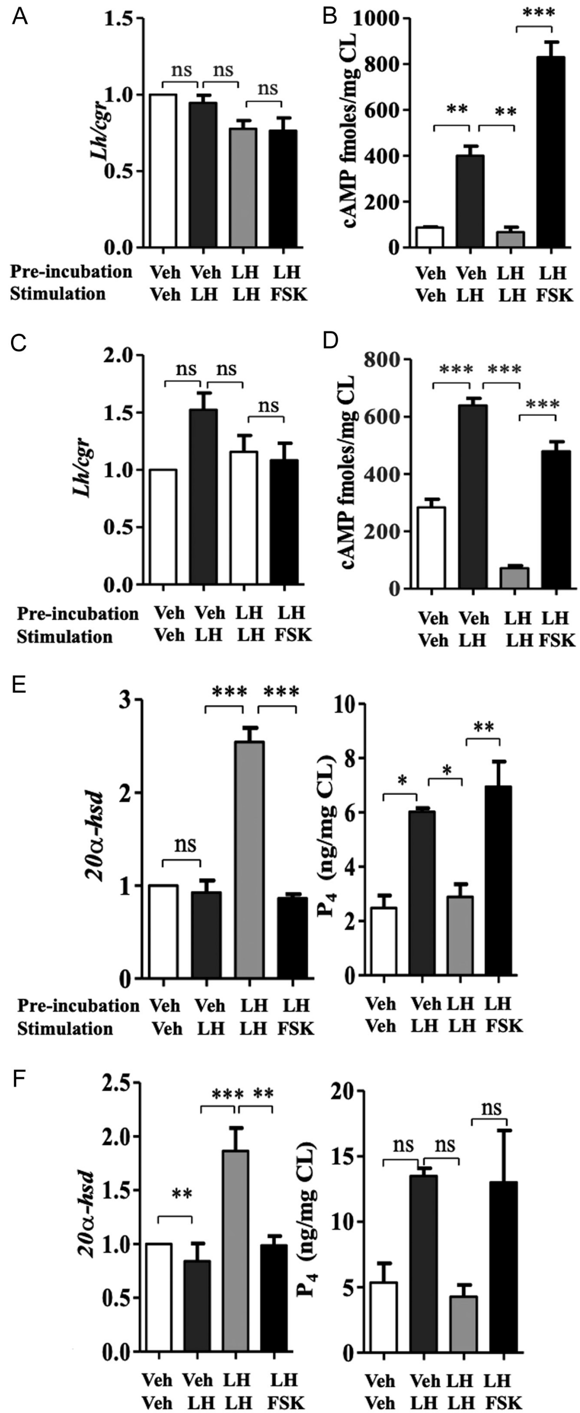

Figure 3 Effect of repeated exposure of LH and FSK treatment on cAMP levels and luteolysis markers in luteal tissue cultures. mRNA expression of *Lh/Cgr* and cAMP levels in luteal tissue cultures exposed to Veh, LH and FSK treatments (A and B: late-pregnant luteal tissue cultures; C and D: mid-pregnant luteal tissue cultures, respectively). mRNA expression of *20α-hsd* and serum P_4 levels in luteal tissue culture studies (E and F; late- and mid-pregnancy luteal tissue cultures, respectively) after different treatments. *L19* mRNA expression was used as an internal control in qPCR analysis. All the experiments were repeated thrice. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.

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Effect of selective depletion of ARRB1 on repeated LH administration-induced luteolytic events in mid- and late-pregnant rats.

We conducted additional experiments utilizing the siRNA approach to study the effect of depletion of endogenous levels of ARRB1 in luteal cells isolated from pregnant rats. The efficiency of ARRB1 downregulation in luteal cells transfected with *Arrb*1 siRNA is shown in Fig. 5A, B, C and D. Transfection with β-arr1 siRNA depleted ARRB1 at both mRNA (Fig. 5A: mid-pregnancy- $P < 0.001$, Fig. 5B: late-pregnancy- $P < 0.05$) and protein levels (Fig. 5C: mid-pregnancy: $P < 0.05$, Fig. 5D: late-pregnancy- $P < 0.05$) compared to luteal cells transfected with sc siRNA. The efficiency of knockdown was more pronounced at the mRNA level during both stages of pregnancy.

The results of β-arr1 siRNA transfection experiments in luteal cells of mid-pregnant rats are presented in Fig. 5E, F and G. In untransfected luteal cells, *Arrb1* mRNA expression was higher ($P < 0.001$) in LH pre-treated cells (Fig. 5E). Transfection of luteal cells with β-arr1 siRNA significantly ($P < 0.001$) decreased *Arrb1* mRNA expression in Veh/LH pre-treated cells compared to sc siRNA and untransfected cells (Fig. 5E). Further, pre-treatment of either sc/β-arr 1 siRNA transfected cell with LH did not alter *Arrb1* expression (Fig. 5E). In untransfected luteal cells, pre-incubation with LH increased *20α-hsd* expression (35.53 ± 3.02-fold, $P < 0.001$, Fig. 5F) compared to Veh pre-treated luteal cells. This finding correlated with decreased P_4 levels ($P < 0.05$) in the media collected from LH pre-incubated untransfected cells (Fig. 5G). Pre-treatment of sc/β-arr1 siRNA transfected cells with LH increased *20α-hsd* expression significantly (sc: $P < 0.001$, β-arr1: $P < 0.05$) compared to Veh pre-treated luteal cells (Fig. 5F). However, the overall *20α-hsd* expression levels were relatively lower in Veh/LH pre-incubated β-arr1 siRNA transfected luteal cells compared with Veh/LH pre-incubated sc siRNA transfected luteal cells (Fig. 5F). The results suggest the involvement of ARRB1 in *20α-hsd* expression regulation. P_4 levels were lower in cells receiving LH both during pre-incubation and stimulation periods compared to cells pre-treated with Veh and stimulated with LH in both sc/β-arr1 siRNA transfected cells (Fig. 5G). However, the overall levels were relatively higher in Veh/LH pre-incubated β-arr1 siRNA transfected luteal cells compared to Veh/LH pre-incubated sc siRNA transfected luteal cells (Fig. 5G).

The results of β-arr1 transfection experiments in luteal cells of late-pregnant rats are presented in Fig. 5H, I and

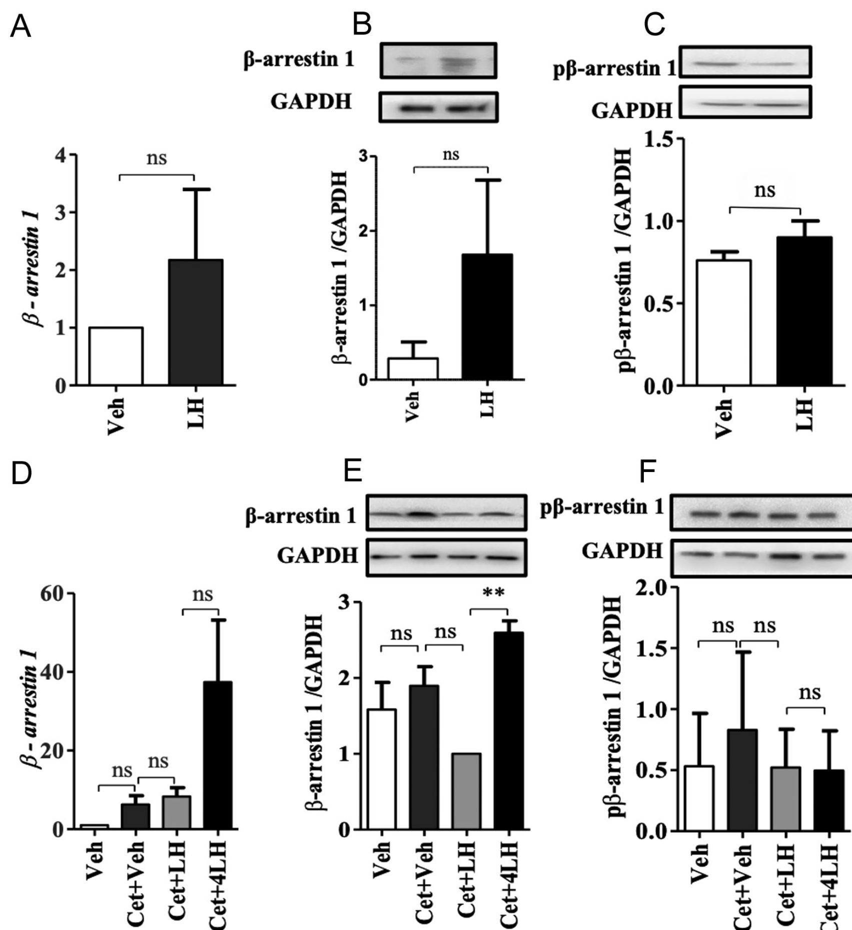

Figure 4 Expression of ARRB1 in the CL of pregnant rats. mRNA/protein expression of ARRB1, protein expression of pARRB1 in CL of late (A, B and C) and mid (D, E and F) pregnant rats. L19 mRNA expression and GAPDH protein levels were used as an internal control for qPCR and immunoblot analyses, respectively. Values are mean ± s.e.m of three rats/group. *$P < 0.05$ and **$P < 0.01$.

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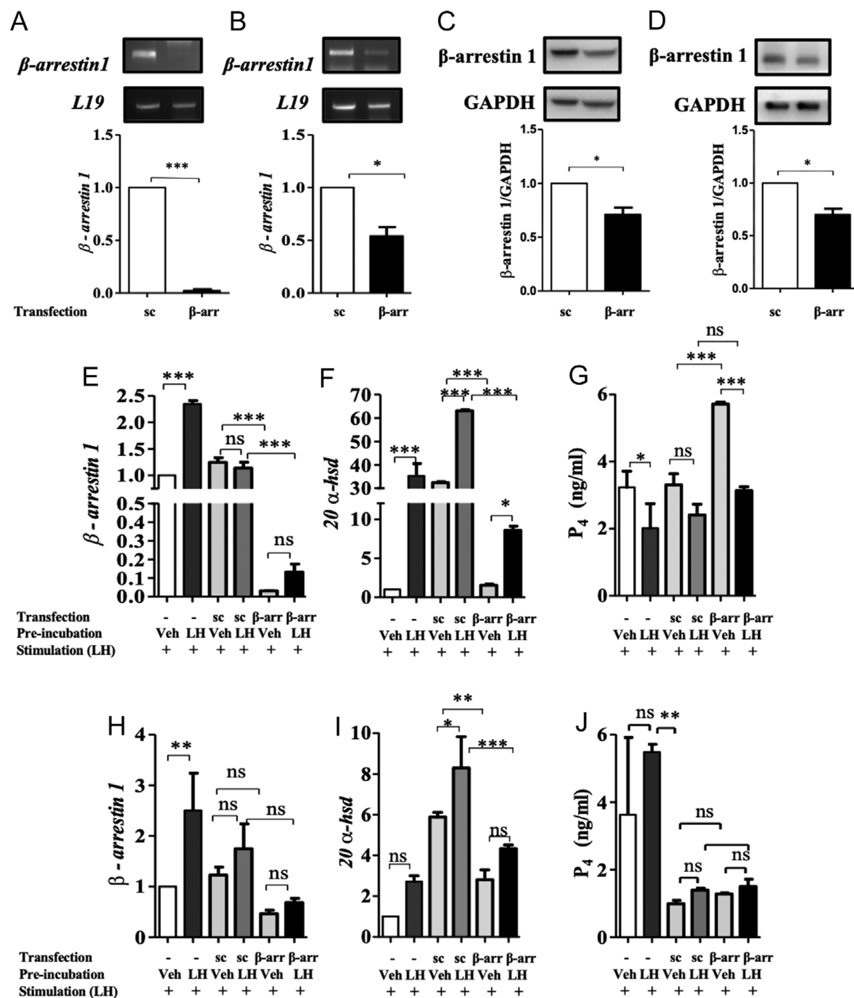

Figure 5 Effect of *Arrb1* siRNA transfection on the steroidogenic function of primary luteal cells obtained from pregnant rats. mRNA expression of *Arrb1* during mid (A) and late-stage (B) of pregnancy in primary culture of luteal cells transfected with scrambled (sc) or Arrb1 (β-arr) siRNA. Representative image for *Arrb1* knockdown during mid- (C) and late-stage (D) pregnancy at the protein level. mRNA expression of *Arrb1* and *20α-hsd* in primary luteal cells of mid- (E and F) and late (G and H)-pregnant rats transfected with sc siRNA or β-arr1 siRNA exposed to Veh or LH during pre-incubation and stimulation period. P_4 concentration in media of luteal cultures collected after stimulation period (G and J; mid- and late-pregnancy luteal cell cultures, respectively). All experiments were repeated thrice. *L19* mRNA expression was used as internal control in qPCR analysis and GAPDH protein was used as internal control for immunoblot analysis. *P < 0.05; **P < 0.01 and ***P < 0.001.

J. *Arrb1* mRNA levels were higher (Fig. 5H: P < 0.01) in untransfected cells pre-incubated and stimulated with LH. However, *Arrb1* expression in cells transfected with sc/β-arr1 siRNA was lower compared to untransfected cells (Fig. 5H). Transfection with β-arr1 siRNA reduced *Arrb1* expression unremarkably compared to sc siRNA transfected luteal cells (Fig. 5H). Further, pre-treatment of either sc/β-arr 1 siRNA transfected cell with LH did not alter *Arrb1* expression (Fig. 5H). Expression of *20α-hsd* was non-significantly higher in untransfected luteal cells exposed to LH both during pre-incubation and stimulation periods (Fig. 5I). Pre-treatment of sc/β-arr1 siRNA transfected cells with LH increased *20α-hsd* expression (sc: P < 0.05, β-arr1: not-significant) compared to Veh pre-treated luteal cells (Fig. 5I). However, the overall levels were relatively lower in Veh/LH pre-incubated β-arr1 siRNA transfected luteal cells compared with Veh/LH pre-incubated sc siRNA transfected luteal cells (Fig. 5I). Media P_4 concentration in untransfected cells was non-significantly high, but after sc/β-arr1 siRNA transfection, P_4 concentration remained low and did not change in any treatment group (Fig. 5J).

Analysis of cAMP/PKA/CREB pathway during low and high levels of LH during pregnancy

In rats, circulating LH levels fluctuate throughout the gestation period. The levels are minimum on DOP 18 and are maximum on DOP 21–22 (Linkie & Nisweder 1972). It was of interest to test the responsiveness of luteal tissue to exogenous LH treatment on periods when the endogenous LH levels are low (DOP 18) or high (DOP 21) in concentration. Administration of exogenous LH on DOP 18 increased luteal cAMP accumulation and pCREB protein levels compared to DOP 21 (Fig. 6A and B).

Discussion

Studies from Stocco *et al.* reported that the administration of LH at the end of pregnancy in rats induces the process of luteolysis (Stocco & Deis 1996, 1998). The present study was done with an aim to elucidate the mechanism involved in LH-induced luteolysis and to analyze if LH can induce luteolysis during any other stage of pregnancy. We systematically

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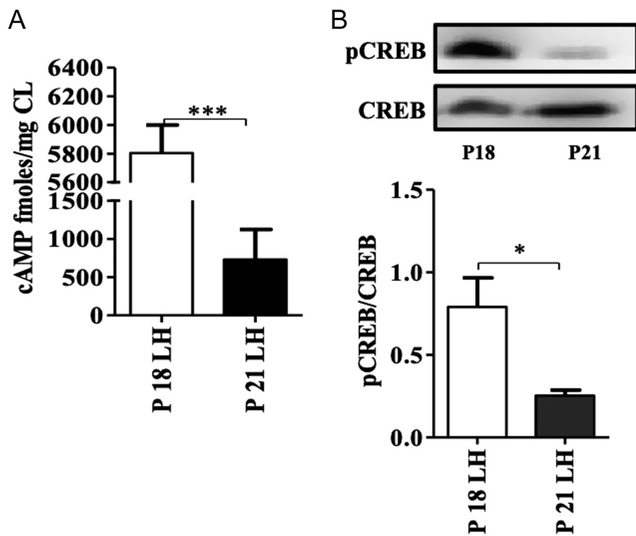

Figure 6 cAMP/PKA/CREB pathway during physiologically low and high levels of LH during pregnancy in rats. cAMP levels in the luteal tissue (A). Representative immunoblots and quantitation for pCREB and CREB (B). GAPDH protein levels were used as an internal control for immunoblot analysis. Values are mean ± s.e.m of three rats/group. $^*P < 0.05$ and $^{***}P < 0.001$.

examined the effects of repeated administration of LH on luteolysis during the late and mid-stage of pregnancy in rats.

We observed that the repeated administration of LH during the late-stage of pregnancy initiates functional luteolysis, as an increase in the expression of *20α-hsd* and a decrease in circulating levels of P_4 was observed. A similar profile of 20α-hsd expression and P_4 levels in late-pregnant rats was observed by Stocco and Deis (1996). The present and previous studies demonstrate the involvement of apoptosis during the process of structural luteolysis (Choi & Smitz 2014). We observed an increase in the expression of cleaved caspase 3 upon LH administration during late-pregnancy. An increase in the activity of caspase 3 has been associated with apoptosis in rodent ovarian cells (Carambula *et al.* 2002) and bovine luteal cells (Yadav *et al.* 2005). Tang and co-workers suggested a role of LC3 II in luteal regression (Tang *et al.* 2017). In the present study, we observed a non-significant increase in the levels of LC3 II upon LH administration during the late-stage of pregnancy. Taken together, our results suggest a correlation between repeated LH administration, luteal cell apoptosis and autophagy in CL during the late-stage of pregnancy for the first time in rodents.

During mid-pregnancy, the repeated administration of LH initiates functional luteolysis. Interestingly, the steroidogenic capacity of CL in terms of P_4 synthesis was restored to control levels when a single injection of LH was administered to Cet-treated rats. However, we observed no further change in P_4 levels upon repeated administration of LH despite an increase in the expression of the gene *20α-hsd*. Studies carried out in monkeys from our laboratory suggest that a decrease in P_4 levels could be seen within 12 h of Cet treatment (John *et al.* 2016). Replacement of LH through i.v. injection increases P_4 levels by as early as 2 h and the levels continue to remain high at least until 24 h (John *et al.* 2016). In rats, replacement with LH after Cet treatment also increased P_4 levels by 4 h (earliest interval measured in the study), and the levels reached 60% of its initial value by 24 h of LH replacement (John *et al.* 2016). Hence, our failure to detect a down regulation in the P_4 levels post repeated injections of LH can be attributed to the fact that increased P_4 levels due to first (maybe second) LH stimulation may continue to persist in the circulation. Although subsequent injections of LH may not increase P_4 secretion. During the mid-stage of pregnancy, we did not observe any change in the expression of markers of structural luteolysis. This suggests that during the mid-stage, for the onset of luteolysis to the full extent (initiation of both functional and structural), the repeated stimulation of LH alone is inadequate.

LH-induced positive contribution of cAMP/PKA/CREB pathway in the regulation of steroidogenesis has been demonstrated before (Stocco *et al.* 2005). We show for the first time desensitization of LH/CGR upon repeated stimulation of LH during the late and mid-stage of pregnancy. However, Hedin *et al.* have demonstrated desensitization of adenylyl cyclase on repeated LH stimulation in pre-pubertal rats (Hedin & Rosberg 1983). To analyze if indeed the inability of LH/CGR as a result of desensitization to activate cAMP/PKA/CREB pathway initiates luteolytic events, we employed FSK – a specific activator of the pathway. FSK stimulates adenylate cyclase and then increases intracellular cAMP levels without involving the receptor or G-protein (Hedin & Rosberg 1983). Unlike LH, stimulation of luteal cells pre-incubated in LH with FSK increased the cAMP levels and restored the steroidogenic capacity of luteal cells. Similar decline in the cAMP levels has been reported by others in pre-pubertal rats upon pre-incubation with LH on steroidogenic machinery (Hedin & Rosberg 1983). Restoration in P_4 levels upon stimulation with FSK highlights the significance of cAMP/PKA/CREB pathway in the regulation of steroidogenesis. FSK has been shown to stimulate P_4 production in immature rat ovaries (Hedin & Rosberg 1983). Steroid production has been associated with the cAMP/PKA pathway in other species as well (Christenson & Devoto 2003). In rats, both induced luteolysis and spontaneous luteolysis are associated with a decline in the levels of P_4 (Stocco & Deis 1996, John *et al.* 2016). Hence, we suggest that desensitization of the LH/CGR makes it unavailable for activation of the cAMP/PKA/CREB pathway. It results in the decreased steroidogenic potential of luteal cells which in turn initiates luteolytic events.

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β-arrestins are multifunctional proteins and have been implicated in the desensitization of the gonadotropin receptor-mediated signaling (Riccetti *et al.* 2017). ARRB1 has been shown to participate in agonist-dependent desensitization of the LH/CGR (Mukherjee *et al.* 1999*b*). Cytosolic ARRB1 is constitutively phosphorylated at Ser-412. However, dephosphorylation of ARRB1 is required for targeting the receptor to clathrin-coated vesicles for internalization (Lin *et al.* 2002). Our studies demonstrate that repeated administration of LH leads to a non-significant increase in the expression of total ARRB1 with no change in pARRB1 expression levels during both stages of pregnancy. These results suggest a positive correlation between LH/CGR desensitization and an increase in the active form of ARRB1 (dephosphorylated form) post repeated administration of LH. To analyze the role of ARRB1 in the initiation of LH-mediated luteolytic events, we depleted the expression of ARRB1 using siRNA approach. During the mid stage of pregnancy, pre-treatment of β-arrestin 1 siRNA transfected luteal cells with LH could still activate the process of luteolysis to some extent. These results suggest the involvement of multiple LH-dependent pathways in the regulation of luteolysis. During the late stage, we observed a loss of steroidogenic potential of luteal cells post-transfection. It can be due to aging of luteal cells and the transfection process itself, which is known to cause apoptosis and induce changes in gene expression patterns (Fiszer-Kierzkowska *et al.* 2011).

Recently, Riccetti and co-workers (Riccetti *et al.* 2017) have demonstrated that the depletion of β-arrestins leads to a partial reduction in P_4 synthesis on LH stimulation. In our study, we observed an opposite effect wherein depletion of ARRB1 prevented the LH-mediated decrease in P_4 levels. The opposite effects of β-arrestins depletion on P_4 synthesis could, however, be due to the difference in the cell model employed. Another report demonstrates that depletion of endogenous β-arrestins increased the secretion of P_4 in the hGL5 cell line (Casarini *et al.* 2016). ARRB1 in AZG cells has been shown to upregulate StAR expression (Lymperopoulos *et al.* 2009). Our study demonstrates the role of ARRB1 for the first time in the regulation of P_4 biosynthesis in the primary culture of pregnant rat luteal cells.

In rats, the circulating LH serum levels have a unique pattern; they increase from DOP 2–4 of pregnancy and then are relatively constant until DOP 10 (Linkie & Nisweder 1972). From DOP 10–18, the levels gradually reduce and reach the minimum on day 18 and remain low until day 20, but thereafter increase and reach the maximum level by day 22 (Morishige *et al.* 1973). The physiologic increase in the levels of LH toward the end of pregnancy might be linked to the onset of luteolysis preceding parturition pointing toward the luteolytic effect of increased LH level during later stages of pregnancy. We conducted some experiments to check the responsiveness of the luteal tissue on DOP 18 and 21 where physiological levels of LH are low and high, respectively. We observed that CL collected from rats on DOP 21, when levels of LH are physiologically high, is less responsive to single exogenous LH stimulation in terms of cAMP accumulation and CREB phosphorylation. The luteal tissue at physiologically high LH serum levels behaves similarly to the luteal tissue collected after repeated external LH administration in terms of activation of cAMP/CREB pathway. Hence, this experimental model is similar to the repeated LH administration model to some extent.

In summary, LH/CGR desensitization occurs post repeated LH stimulation during both the late and mid-stage of pregnancy. The cAMP/PKA/CREB appears to be the major regulator of luteolytic events initiated by repeated administration of LH during both stages of pregnancy. Involvement of cAMP/PKA/CREB pathway in LH-mediated luteolysis has been shown for the first time in late- and mid-stage pregnant rats. A positive correlation appears to exist between *Arrb1* and *20α-hsd* expression during both stages of pregnancy. Studies evaluating the involvement of β-arrestins with steroidogenesis in ovarian tissues remain scarce (Casarini *et al.* 2016, Riccetti *et al.* 2017) and our study shows an association between ARRB1 and the process of steroidogenesis in luteal tissue.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R Medhamurthy conceived and conceptualized the research program, designed experimental strategies and supervised all experiments. A Vashistha and R Medhamurthy designed the experiments. A Vashistha and R Khan collected samples. A Vashistha performed data acquisition, data analysis and wrote the draft of the manuscript. All authors contributed to the preparation and finalization of the manuscript and they approved submission of the manuscript.

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