Chapter III

FSH Signaling in various postnatal ages of rats
Introduction

FSH is a member of the glycoprotein hormone family that includes LH, hCG and TSH. These hormones are disulfide-rich heterodimers that share a common α subunit but have unique β subunits that impart hormone specificity. The peptide is essential for receptor binding with the receptor and the oligosaccharide moiety is important for the downstream signaling. FSH transmits its signals via a 75 kD (675 amino acids) FSH receptor (FSH-R) that is present only in testicular Sc. FSH-R is a G protein-coupled receptor (GPCR) that spans the membrane seven times with seven conserved alpha helixes. The gene encoding the FSH-R consists of 10 exons. The first 9 exons encode the extracellular domain (ECD) and the last exon encodes the trans-membrane domain (TMD) and a short intracellular/cytosolic tail (C-terminal tail). A Leucine-rich repeat (LRR) in the ECD is essential for ligand binding. TMD is important for the transmitting of the signal and the C-terminal tail is known to be involved in membrane localization and stability of the receptor itself. On binding of the ligand, structural changes occur in the seven membrane-spanning domain that elicit guanine nucleotide exchange in receptor associated Gαs (stimulatory subunit) or Gαi (inhibitory subunit) proteins. Recruitment of Gαs or Gαi proteins depends upon the degree and nature of glycosylation pattern of the ligand (FSH). In Sc, FSH-R coupling with Gαs causes increase in the level of the intracellular cAMP which further activates classical protein kinase A (PKA) pathway or additionally results in calcium influx or activation of the phosphatidylinositol 3-kinase (PI-3K) or p38 MAPK or ERK MAPK pathways (Reviewed by Walker and Cheng, 2005).

FSH β-knock out (generated by Kumar et al. 1997) and FSH-R knock out (generated by Dierich et al. 1998 and Abel et al. 2000) male mice are fertile indicating that FSH is not essential for male fertility. Expression of either FSH or FSH-R (constitutively active) in hpg mice background also show lack of fertility (Allan et al. 2001 and Haywood et al. 2002). However, FSH determines the testicular size as well as the limit of sperm output in adult males (Orth et al. 1988 and Sharp, 1994). The number of FSH-R increases by about 3-fold from fetal stage to after birth and peaks at 15 days of postnatal age. Sc proliferate under the direct influence of FSH during fetal, and neonatal stage of
development and the proliferation ceases after around 15 days of age in rats (Reviewed by Walker and Cheng, 2005). FSH induced cAMP production by 5-days-old Sc is less compared to 19-days-old Sc (Crépieux et al. 2001). This change in the level of cAMP generated within Sc decides the fate of downstream signaling cascade. In 5-days-old Sc, FSH mediated low cAMP evokes a PKA dependent ERK activation that ultimately leads to Sc proliferation. However, elevated level of cAMP in 19-days-old Sc deactivates ERK and classical PKA dependent pathway becomes dominant (Crépieux et al. 2001). So it is evident that mode of FSH action changes in Sc as Sc undergo differentiation. This may be a probable reason for lack of spermatogenesis during infancy in spite of sufficient FSH. In this section, we have evaluated the expression level of FSH-R mRNA and protein in Sc obtained from different postnatal ages of rats. We also evaluated the ability of Sc to generate cAMP upon FSH treatment followed by the expression of various genes important for spermatogenesis.

**Methods**

**Isolation of Sc**

Described in Chapter I.

**Culture Conditions**

Described in Chapter I.

**Hormonal Treatment**

Different hormone treatments were given to Sc culture depending upon the objective and read out of experiment.

1. **Evaluation of the effect of continuous hormone supplementation** (from the first day of culture) in Sc culture.

Described in Chapter I. See Chapter I, **Cartoon: 15**.
2. Evaluation of cAMP produced by Sc.

Sc isolated from testes of different age groups and were cultured for 4 days without any hormonal supplements. 24hr after the hypotonic shock, Sc were treated with

i) gf media{ with IBMX (10^-4M)} alone,

ii) gf media containing o-FSH (50 ng/ml),

iii) gf media containing Cholera toxin (CT 100ng/ml) and

iv) gf media containing forskolin (10μM)

for either ¼ hr or 24hr. At the end of treatment, Sc exposed media were boiled for 5min and then were stored in -80°C. Cells present in each well were counted and generated cAMP (data) were represented in terms of per ml of media (per well) per million Sc.

A schematic diagram describing the experiment is given in **Cartoon: 16. A.**


One portion of Sc culture were preincubated with gf media alone and the other portion of Sc were preincubated with gf containing Pertussis toxin (PT, 100ng/ml) for 1hr. After that Sc were treated with

i) gf media{ with or without IBMX (10^-4M)} alone,

ii) gf media containing o-FSH (50 ng/ml),

iii) gf media containing Cholera toxin (CT 100ng/ml) and

iv) gf media containing forskolin (10μM)

for 24hr. At the end of treatment, Sc exposed media were boiled for 5min and then were stored in -80°C. Cells present in each well were counted and generated cAMP (data) were represented in terms of per ml of media (per well) per million Sc.

A schematic diagram describing the experiment is given in **Cartoon: 16. B.**
Cartoon 16: Experimental Protocol for evaluating cAMP production by rat Sc

- **C** → gf media alone
- **F** → FSH 50 ng/ml
- **CT** → Cholera Toxin 100 ng/ml
- **Forsk** → Forskolin 10 μM

\( \frac{1}{2} \) hr and 24 hr cAMP (+IBMX 10\(^{-4}\) M)

24 hr cAMP

Sc isolated from testes of different age groups and were cultured for 4 days without any hormonal supplements. 24hr after the hypotonic shock on day 3 of culture, Sc were treated with

i) gf media{ without IBMX (10^{-4} M)} alone,

ii) gf media containing o-FSH (50 ng/ml),

iii) gf media containing forskolin (10\mu M) and

iv) gf media containing 8Br-cAMP (0.5mM)

for either constant 2 hr or constant 24hr. At the end of treatment, cells were treated with Trizol and stored in -80°C for mRNA analysis.

\textbf{125I o-FSH Binding Assay}

FSH binding assay was done as described by Dahia and Rao (2006). Briefly, iodination of 5 \mu g of o-FSH was carried out with 50 \mu Ci of \textsuperscript{125}I-Na by the iodogen method. Cultured Sc from different age groups of rats were dislodged from the wells by scraper (without using any enzymatic treatment e.g. Trypsin EDTA) and washed twice in ice cold DMEM. 1-2 million Sc were incubated with 100,000 cpm of \textsuperscript{125}I-oFSH for 2hr at 34°C. Non-specific binding was evaluated by incubating splenocytes (which lack FSH-Rs) with 100,000 cpm of \textsuperscript{125}I-oFSH (radioactive FSH). Specific binding of FSH was evaluated by incubating radioactive FSH in presence of increasing concentration of excessive (minimal 100 fold to 1000 fold) cold FSH for 2hr at 34°C. Approximate FSH-R present per Sc was calculated using GraphPad radioactivity calculator (http://www.graphpad.com/quickcalcs/radcalcform.cfm).

For determining the intracellular binding sites of FSH, cultured Sc obtained from different age groups of rats were dislodged from the wells by scraper and washed twice in ice cold DMEM. Sc pellets was fixed by 2% PFA for 15min at 4°C, and were washed twice with cold PBS containing 0.1% saponine. Fixed Sc were incubated with radioactive FSH (100,000 cpm of \textsuperscript{125}I-oFSH) in presence of 0.1% saponine for 2hr at 34°C. Specific
binding of the ligand was detected in the presence of 1000 fold more cold FSH. Data were represented as total bound cpm per mg of protein of Sc pellet or total bound cpm per million Sc.

**cAMP assay**

Described in Chapter I.

**Flow Cytometry Analysis**

Experiment was done as described by Gong *et al.* (2008). Sc obtained from different age groups were cultured for 4 days without any hormones. Cultured Sc from different age groups of rats were dislodged from the wells by scraper and washed twice in ice cold HBSS. One fraction of Sc were directly used (without fixation) by washing with B-PBS (1X PBS containing 1% BSA only). Rest of the fraction of Sc was fixed by 2% PFA for 15min at 4°C and washed once with CPB (cell permeabilizing buffer i.e. 1X PBS containing 0.1% saponine and 1% BSA) before incubating with antibodies. For detection of FSH-R expression on the cell surface and inside the cell, we used N-terminal specific anti rabbit polyclonal antibody (Santa cruz, USA, sc-13935) and C-terminal specific anti rabbit polyclonal antibody (Abcam, USA, ab 65975-100) respectively. Unfixed Sc (live) in presence of B-PBS and fixed cells in presence of CPB were incubated with the specific primary antibody in 1:100 dilution for 1hr at 4°C. Thereafter, cells were washed twice with either B-PBS or CPB for unfixed and fixed Sc respectively. They were incubated further with anti rabbit IgG secondary antibody (goat raised) labeled with FITC (Alexa 488, Molecular Probes, Invitrogen) for 30min at 4°C. The cells were subsequently washed in cold HBSS and analyzed by a flow cytometer (FACS caliber, BD Biosciences). Cells were gated using forward scatter versus side scatter to exclude dead cells and debris. Fluorescence, detected in FL-1 filter of 25,000 cells/samples (i.e. ages) were acquired in logarithmic mode for visual inspection of the distributions. Acquisition of signal in linear mode was used to quantify the expression of the relevant molecules by calculating the mean fluorescence intensity. Data were analyzed by the software, winmdi version 9.0.
**Isolation of RNA from samples in Trizol**

Described in Chapter I.

**Analyses of the mRNA Expression of the Hormones Regulated Genes by Semi quantitative RT-PCR**

As described in Chapter I.

The list of primer sequences, annealing temperature ($T_m$), number of PCR cycles and PCR product sizes are given in Chapter I Table-2 for SCF, GDNF, AR, FSH-R, Cyp-19, cyclophilin A.

**Quantitative RT-PCR Analysis of mRNA Expression**

As described in chapter II.

Primers of FSH-R used are listed in Table 1. GAPDH primer sequence was given in chapter II, Table-2.

**Data Representation and Statistical Analysis**

As described in Chapter II.
Results

Expression of FSH-R mRNA in Various Postnatal Age Groups

a) Hormonal dependence of FSH-R mRNA expression in Sc culture obtained from various postnatal ages of rats

Expression of FSH-R mRNA in freshly isolated Sc and in cultured Sc with or without FT supplementation was evaluated by semi-quantitative RT-PCR analyses. FSH-R mRNA transcription continued in culture (without hormones) irrespective of ages but the levels of transcripts were lesser compared to that of the freshly isolated Sc for all the ages. In other words, transcript levels of FSH-R mRNA were less in cultured Sc without FT supplementation compared to that of the freshly isolated Sc (Direct Assay) irrespective of age groups. Continuous presence of FT in culture induced FSH-R mRNA expression up to 3 days of culture. However, on day 4 of culture, the basal transcription of FSH-R mRNA was recovered up to the level of the freshly isolated Sc, even in absence of hormones (Fig: 1. A, B, C and D). Expression of cyclophilin A mRNA was considered as internal control (Fig: 2. A, B, C and D).

b) Evaluation of the basal level FSH-R mRNA expression by q-RT-PCR in various postnatal ages of rats

Sc obtained from 5-days, 9-days, 12-days and 18-days-old rats were isolated and one fraction was used for Direct Assay. Rest of the cells were cultured for 4 days without any hormonal supplements. On day 4 of culture, Sc (of different age groups) were treated with Trizol and stored in -80°C for future mRNA extraction. Level of FSH-R mRNA expressions both in freshly isolated Sc and in cultured Sc (of various age groups) were evaluated by q-RT-PCR and data were represented by relative quantity of FSH-R mRNA normalized against GAPDH (endogenous control).

FSH-R mRNA(s) were found to be uniformly expressed in freshly isolated Sc obtained from different postnatal ages of rats. However, the basal level of FSH-R transcripts were less in cultured Sc compared to that of the Direct assays irrespective of age groups. No significant change in the basal level of FSH-R mRNA was observed in the cultured Sc of the different age groups (Fig: 3.).
Expression of basal level of FSH-R protein in Sc culture obtained from various postnatal age groups of rats

a) Detection of FSH-R using N-terminal specific antibody
Total FSH-Rs present on Sc surface were measured by flow cytometry analysis using N-terminal specific anti rabbit FSH-R primary antibody and fluorescent (FITC) tagged specific secondary antibody. Total fluorescence was measured by FL-1 filter per 25,000 Sc obtained from 18-days-old rat. No binding was detected in live cells (without fixation and permeabilization). Binding was detected in fixed cells followed by permeabilization (Fig: 4). However, this binding was non-specific as the antibody was found to be cross reactive with B-16 F-10 melanoma cell line that does not express FSH-R (Fig: 5).

b) Detection of total intra cellular FSH-R using C-terminal specific antibody
Total FSH-R protein present inside Sc was measured by flow cytometry analysis using C-terminal specific anti-FSH-R primary antibody and fluorescent (FITC) tagged specific secondary antibody. Total fluorescence was measured by FL-1 filter per equal number of Sc (e.g. 25,000 cells) obtained from various postnatal age groups. This data indirectly indicated the total FSH-R protein present in Sc. Anti FSH-R antibody bound with Sc obtained from various ages of rats gave similar fluorescence intensity detected in FL-1 filter (Fig: 6.A and B). Binding of both primary and secondary antibody were found to be specific as very low fluorescence was detected in splenocytes (-ve control) and no fluorescence was detected with secondary antibody alone in 18-days-old Sc (+ve control) respectively (Fig: 6.C).

Binding ability of FSH to Sc culture obtained from various postnatal ages

a) Extra cellular Binding
Approximate FSH-R number were detected by incubating radioactive FSH in presence of increasing concentration of excess (minimal 100 fold to 4000 fold) cold FSH for 2hr at 34°C. Total FSH bound to Sc cultured from 5-days-old and 9-days-old rats was found to
be profoundly less when compared to that of Sc cultured from 12-days-old and 18-days-old rats. Non-specific binding of radioactive FSH with rat splenocytes (which lack FSH-R) was remarkably low (Fig: 7. and 8.A).

FSH-R per Sc was calculated by GraphPad radioactivity calculator. About 40-60 detectable binding sites for FSH (FSH-R / each Sc) were discernible in Sc cultured from 5-days-old and 9-days-old rats. In contrast, about 200-220 detectable binding sites for FSH were found per each Sc cultured from 12-days-old and 18-days-old rats (Fig: 8.A-B).

b) Intra cellular Binding

Fixed and permeabilized Sc and live Sc (non fixed and un permeabilized) were incubated for 2hr with radioactive FSH in presence of 1000 fold more cold FSH.

Intracellular binding were found to be more in all the age groups compared to that of the extracellular binding. However, total intra cellular binding of radioactive FSH to Sc obtained from 5-days and 9-days-old rats were less compared to that of the 12-days and 18-days-old rat Sc (Fig: 9.A). However, fold increase in FSH binding upon membrane permeabilization was more during infancy compared to that of the 12-days and 18-days of age (Fig:9.B).
### Table -1

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<th>Tₘ</th>
<th>Product Size (bp)</th>
<th>Melting Temperature</th>
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Ability of Sc obtained from various age groups of rats to produce of cAMP upon treatment of FSH and other GPCR modulators -

a) 1/2hr data
FSH mediated augmentation in cAMP production were not detected in 5-days and 9-days-old rats. CT also failed to produce cAMP in these two age groups by 1/2hr of treatment. However, forskolin mediated cAMP generated by Sc were detected in both the age groups (Fig: 10. A-B). FSH induced cAMP production were detected in 12-days and 18-days-old rats. However, CT could not produce cAMP even in these two age groups for 1/2hr of treatment. Forskolin mediated cAMP generated by Sc were detected in both the age groups (Fig: 10. C-D).

b) 24hr data
CT or forskolin mediated cAMP production was detected at 24hr in all the age groups. We observed that significant amount (P<0.05%) of cAMP was induced by FSH only in 12-days and 18-days-old rat Sc. FSH mediated cAMP response was found highest in 18-days of age (Fig: 11).

Evaluation of the endogenous PDEs activity in postnatal rat Sc
Accumulation of cAMP (from 1/2hr to 24hr) was detected in 9-days-old rat Sc only in presence of IBMX. However, cAMP was detectable in 12-days-old rat Sc after 24hr even in absence of IBMX (Fig: 12. A-B).

Recruitment of G\(_{\alpha_i}\) and G\(_{\alpha_s}\) by FSH-R in Sc obtained from various postnatal age of rats followed by FSH treatment

a) 5-days-old Sc
In absence of IBMX, cAMP was elevated by FSH or CT or forskolin after 24hr. However, the level of cAMP produced by FSH was less compared to that of the CT or forskolin. Preincubation with PT increased the basal level of cAMP significantly (P<0.05%) and no further rise in cAMP production was observed when cells were further
treated with FSH. CT or forskolin mediated cAMP generation remained uniform with that of the PT untreated group (Fig: 13.A).

However, in presence of IBMX, the basal level of cAMP was found to be increased significantly (P<0.05%) compared to that of the IBMX and PT untreated cells. This elevated basal level left no further scope for FSH to show a rise in cAMP production. However, CT or forskolin induced cAMP production was discernible. In PT preincubated group, the basal level of cAMP was found to be elevated further. No further augmentation of cAMP production was observed with FSH or CT or forskolin treatments (Fig: 13.A).

b) 9-days-old Sc
In absence of IBMX, cAMP was elevated by FSH or CT or forskolin treatment after 24hr of exposure. However, the level of cAMP produced by FSH was less compared to that of the CT or forskolin. Preincubation with PT increased the basal level of cAMP and no further rise in cAMP production was observed when cells were further treated with FSH or CT or forskolin (Fig: 13.B).

However, in presence of IBMX, the basal level of cAMP was significantly (P<0.05%) increased compared to IBMX and PT untreated cells. This left no scope for FSH or CT or forskolin to show a rise in cAMP further. PT preincubation elevated the basal level of cAMP even more and the pattern remained the same (Fig: 13.B).

c) 12-days-old Sc
In absence of IBMX, cAMP was progressively increased with FSH or CT or forskolin treatment for 24hr. Preincubation with PT failed to increase the basal level of cAMP but an elevated cAMP production was observed when cells were further treated with FSH compared to that of the PT untreated cells. However, PT preincubation could not increase cAMP production with CT or forskolin treatments.

In presence of IBMX, no significant (P<0.05%) rise in the basal level of cAMP was observed when compared with that of the IBMX and PT untreated group. cAMP levels were uniformly elevated with FSH or CT or forskolin treatments. PT pre incubation, failed to elevate the basal level of cAMP further. No further rise in cAMP production was
observed with FSH or CT or forskolin treatments compared to that of the PT untreated group (Fig: 13.C).

d) 18-days-old Sc
In absence of IBMX, cAMP was increased with FSH or CT or forskolin treatment for 24hr. However, the level of cAMP produced by forskolin was more compared to that of the FSH or CT. Preincubation with PT failed to increase the basal level of cAMP. An elevated cAMP production was observed when Sc were further treated with FSH or CT compared to that of the PT untreated cells. However, PT preincubation could not increase forskolin induced cAMP production.

In presence of IBMX, no further increase in the basal level of cAMP was observed compared to that of the IBMX and PT untreated cells. Same level of cAMP was induced by FSH or CT or forskolin treatment. No significant rise in the basal level of cAMP was observed with PT preincubation in these cells. No further rise in cAMP production was also observed with FSH or CT or forskolin treatments (Fig: 13.D).

Evaluation of gene expression in various postnatal ages
Sc obtained from 5-days, 9-days, 12-days and 18-days-old rats were cultured for 4 days without any hormonal supplements. On day 4 of culture, Sc were treated with i) gf media alone ii) gf Media containing o-FSH (50 ng/ml) iii) gf media containing forskolin (10μM) iv) gf Media containing 8-Br-cAMP (0.5mM) for 2hr and 24hr. Semi-quantitative RT-PCR analyses were performed to evaluate the expression of SCF, GDNF, and Cyp-19 and the expression level were normalized against house keeping gene cyclophilin A.

a) SCF
2hr data
Low levels of soluble isoform of SCF mRNA was detected in Sc cultured from 5-days-old rats upon 2hr of constant exposure with various treatments (Fig: 14.A). SCF mRNA expression was found to be more in 9-days-old rats. Augmentation of soluble SCF mRNA was observed upon treatment with either FSH or agents (forskolin or 8 Br-cAMP) that increased intracellular cAMP concentration by bypassing FSH-R in this age group.
Basal level of SCF transcripts were found to be higher in 12-days of age compared to that of the 9-days of age. More membrane bound isoform was expressed basally in this age. A significant rise (P<0.05%) in the expression of both the isoforms of SCF mRNA was observed with FSH or forskolin or 8Br-cAMP treatment (Fig: 14.C). No significant rise (P<0.05%) in SCF mRNA was observed in 18-days of age with various treatments (Fig: 14.D). Expression of membrane bound isoform was dominant over the soluble isoform in 18-days-old Sc in all the treatments (Fig: 14.D). Internal control cyclophilin A were uniformly expressed in all the ages irrespective of different treatments (Fig: 14.E-H).

24hr data
Expression of soluble isoform of SCF mRNA was significantly (P<0.05%) discernible in Sc cultured from 5-days-old rats when Sc were over stimulated for 24hr with forskolin or 8Br-cAMP. Very less amount of transcripts was detected in control cells or FSH treated cells in this age group (Fig: 15.A). Sc cultured from 9-days-old rats showed a rise in the basal level of soluble isoform but the level remained uniform upon FSH treatment. Forskolin or 8Br-cAMP mediated augmentation of the soluble isoform was observed in this age group (Fig: 15.B). Treatment of FSH or forskolin or 8Br-cAMP to 12-days-old Sc augmented both the isoforms. However, more membrane bound isoform was detected in this age group (Fig: 15.C). In 18-days of age, SCF mRNA were uniformly expressed in all the treatment groups with a higher level of membrane bound isoform. No augmentation of SCF mRNA was detected in this age group with any of the treatments (Fig: 15.D). Expression of membrane bound isoform was maintained from 12-days to 18-days-of age. However, soluble isoform was found to be significantly (P<0.05%) downregulated in 18-days of age compared to that of the 12-days-old Sc (Fig: 15.C and D). Internal control cyclophilin A were uniformly expressed in all the ages irrespective of different treatments (Fig: 15.E-H).

b) GDNF

2hr data
GDNF mRNA was barely detected in 5-days-old Sc at 2hr in different treatment groups (Fig: 16.A). GDNF mRNA was found to be elevated basally in 9-days-old Sc at 2hr and
FSH or forskolin mediated augmentation of GDNF mRNA were observed (Fig: 16.B). A significant (P<0.05%) increase in GDNF transcripts was detected at 2hr in 12-days of age with FSH treatment. Forskolin also augmented GDNF mRNA in this age but the level of expression was less compared to that of the FSH treatment. 8-Br-cAMP however failed to induce GDNF mRNA in this age (Fig: 16.C). Rise in GDNF mRNA were observed with treatment of FSH or forskolin in 18-days-old Sc. 8-Br-cAMP failed to induce GDNF mRNA in this age also (Fig: 16.D). Internal control cyclophilin A were uniformly expressed in all the ages irrespective of different treatments (Fig: 16.E-H).

24hr data

GDNF mRNA was barely detected in 5-days-old Sc at 24hr in different treatment groups. (Fig: 17.A). Unlike FSH, only forskolin or 8-Br-cAMP augmented GDNF mRNA expression in 9-days-old Sc at 24hr (Fig: 17.B). Elevated basal level of GDNF mRNA was observed in 12days-old Sc compared to that of the previous age groups. FSH or forskolin or 8Br-cAMP could not augment GDNF mRNA at 24hr in this age (Fig: 17.C). In 18-days-old Sc the basal level of GDNF expression was also high like that of the 12-days of age. No FSH or forskolin or 8Br-cAMP mediated up regulation in GDNF mRNA expression was observed in this age group (Fig: 17.D). Internal control cyclophilin A were uniformly expressed in all the ages irrespective of different treatments (Fig: 17.E-H).

c) Cyp-19

2hr data

Basal level of Cyp-19 transcripts were not detected in all the age groups. However, Cyp-19 mRNA was augmented by FSH or forskolin at 2hr in all the age groups. FSH mediated augmentation of Cyp-19 expression was more prominent. 8Br-cAMP mediated augmentation of Cyp-19 mRNA expression was less compared to that of the FSH or forskolin (Fig: 18.A-D). Internal control cyclophilin A were uniformly expressed in all the ages irrespective of different treatments (Fig: 18.E-H).
Fig: 1. RT-PCR analyses of FSH-R mRNA expression in freshly isolated and cultured Sc.

A. Sc from 5-days-old rats
B. Sc from 9-days-old rats
C. Sc from 12-days-old rats
D. Sc from 18-days-old rats

For each panel (A to D)
Lane 1 = Day 1 (Direct Assay DA)
Lane 2 = day 2 of culture, FT untreated
Lane 3 = day 2 of culture, FT treated
Lane 4 = day 3 of culture, FT untreated
Lane 5 = day 3 of culture, FT treated
Lane 6 = day 4 of culture, FT untreated
Lane 7 = day 4 of culture, FT treated

F = 50ng/ml o-FSH, T = \(10^{-7}\) M Testosterone

Note: Provided gel picture (for each age group of rats) is a representative of three sets of independent experiments.
Fig: 2. RT-PCR analyses of cyclophilin A mRNA expression in freshly isolated and cultured Sc.

A. Sc from 5-days-old rats
B. Sc from 9-days-old rats
C. Sc from 12-days-old rats
D. Sc from 18-days-old rats

For each panel (A to D)

Lane 1 = day 1 (Direct Assay, DA)
lane 2 = day 2 of culture, FT untreated
lane 3 = day 2 of culture, FT treated
lane 4 = day 3 of culture, FT untreated
lane 5 = day 3 of culture, FT treated
lane 6 = day 4 of culture, FT untreated
lane 7 = day 4 of culture, FT treated

F = 50ng/ml o-FSH, T = 10^-7 M Testosterone

Note: Provided gel picture (for each age group of rats) is a representative of three sets of independent experiments.
Fig 3. Evaluation of the relative quantity of FSH-R mRNA expression by q-RT-PCR in various postnatal ages of rats both in freshly isolated (day 1) and in cultured Sc (day 4).
Fig: 4. Flow Cytometry dot plot analysis using N-terminal specific FSH-R antibody for detecting FSH binding with Sc obtained from 18-days-old rats. A. Live cells without fixation and permeabilization. B. Fixed and permeabilized cells.

Fig: 5. Flow Cytometry dot plot analysis using N-terminal specific FSH-R antibody for detecting FSH binding with melanoma cells (B-16 F-10) as negative control that do not express FSH-R. A. Live cells without fixation and permeabilization. B. Fixed and permeabilized cells.
Results Chapter III (v)

A

5 day Sc 0.01% FL-1 FSH-R intensity / 25,000 cells

9 day Sc 0.04% FL-1 FSH-R intensity / 25,000 cells

12 day Sc FL-1 FSH-R intensity / 25,000 cells

18 day Sc FL-1 FSH-R intensity / 25,000 cells

B
Fig: 6. Histogram analyses of flow cytometry data obtained from Sc of various ages of rats using C-terminal specific FSH-R antibody.

A. Percentage of binding of anti FSH-R antibody with Sc obtained from 5-days and 9-days-old rats.

B. Percentage of binding of anti FSH-R antibody with Sc obtained from 12-days and 18-days-old rats.

C. Control of the primary antibody using splenocytes (that do not express FSH-R, negative control) and control of the secondary antibody using only secondary antibody in 18-days-old rat Sc (that express FSH-R, positive control).
Fig: 7. Binding ability of FSH to Sc obtained from various postnatal ages of rats. (* = P<0.05%)

Fig: 8. Detection of FSH Binding sites on Sc obtained from various postnatal ages of rats.
A. Radioactive FSH binding ability to Sc obtained from various postnatal ages of rats in presence or absence of excessive (in increasing concentration) cold FSH.
B. Detection of approximate FSH-R number on Sc surface. Three different bars are three different experiments indicating different range of the number of FSH-R in each age group.
Fig: 9.A. Comparisons of external and internal FSH binding sites in Sc obtained from various postnatal ages of rats.
1, 2 = Unfixed cells, 3, 4 = fixed cells permeabilized with saponine.

Fig: 9.B. Fold change in radioactive FSH binding sites in after permeabilization of Sc obtained from various postnatal ages of rats.
Fig: 10. Production of cAMP by Sc obtained from various postnatal ages of rats in presence of IBMX at 1/2hr.
A. cAMP produced by 5-days-old rats,
B. cAMP produced by 9-days-old rats,
C. cAMP produced by 12-days-old rats,
D. cAMP produced by 18-days-old rats. (* P<0.05% as compared to control).
Fig: 11. Production of cAMP by Sc obtained from various postnatal ages of rats in presence of IBMX at 24hr.
A. cAMP produced by 5-days-old rats, B. cAMP produced by 9-days-old rats, C. cAMP produced by 12-days-old rats, D. cAMP produced by 18-days-old rats.
(* P<0.05%).

Fig: 12. Accumulation of basal level cAMP produced by Sc obtained from various ages of rats in presence or absence of IBMX over 24hr.
A. cAMP generated by 9-days-old Sc, B. cAMP generated by 12-days-old Sc
1. cAMP generated in 1/2hr in absence of IBMX. 2. cAMP generated in 24hr in absence of IBMX. 3. cAMP generated in 1/2hr in presence of IBMX. 4. cAMP generated in 24hr in presence of IBMX.
Fig: 13. Change in the production of cAMP due to dual coupling of FSH-R with $G_{as}$ and $G_{ai}$ in Sc obtained from various postnatal ages of rats.

A. cAMP produced by 5-days-old rat Sc by FSH or CT or forskolin with or without IBMX, either preincubated with gf media alone or gf media containing for PT for 1hr.

B. cAMP produced by 9-days-old rat Sc by FSH or CT or forskolin with or without IBMX, either preincubated with gf media alone or gf media containing for PT for 1hr.

C. cAMP produced by 12-days-old rat Sc by FSH or CT or forskolin with or without IBMX, either preincubated with gf media alone or gf media containing for PT for 1hr.

D. cAMP produced by 18-days-old rat Sc by FSH or CT or forskolin with or without IBMX, either preincubated with gf media alone or gf media containing for PT for 1hr.
Fig: 14. RT-PCR analyses of SCF mRNA obtained from 5-days, 9-days, 12-days and 18-days-old rat Sc after 2hr of treatments.


The gel picture is a representative of three sets of independent experiments. Bar diagrams represent calculated mean with standard error of the three experiments.

Aa and Ab are significantly different from each other. (* = P < 0.05%).
Results Chapter III (xvi)

SCF Soluble Isoform mRNA Expression at 24 hr

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Relative Expression of SCF Soluble Isoform mRNA

SCF Membrane Bound Isoform mRNA Expression at 24 hr

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Relative Expression of SCF Membrane Bound Isoform mRNA

A  B  C  D

E  F  G  H
Fig: 15. RT-PCR analysis of SCF mRNA obtained from 5-days, 9-days, 12-days and 18-days-old rat Sc after 24hr of treatment.


For all the 8 panels (A to H), lane 1, = gf media alone (control), lane 2, = gf media containing o-FSH (50 ng/ml) lane 3 = gf media containing forskolin (10μM) lane 4 = gf media containing 8Br-cAMP (0.5mM).

The gel picture is a representative of three sets of independent experiments. Bar diagrams represent calculated mean with standard error of the three experiments.

Aa and Ab are significantly different from each other. (* = P < 0.05%).
Fig: 16. RT-PCR analysis of GDNF mRNA obtained from 5-days, 9-days, 12-days and 18-days-old rat Sc after 2hr of treatment.

A. Expression of the GDNF mRNA in 5-days-old rat Sc. B. Expression of the GDNF mRNA in 9-days-old rat Sc. C. Expression of the GDNF mRNA in 12-days-old rat Sc D. Expression of the GDNF mRNA in 18-days-old rat Sc. E. Expression of the cyclophilin A mRNA in 5-days-old rat Sc. F. Expression of the cyclophilin A mRNA in 9-days-old rat Sc. G. Expression of the cyclophilin A mRNA in 12-days-old rat Sc. H. Expression of the cyclophilin A mRNA in 18-days-old rat Sc. For all the 8 panels (A to H), lane 1 = gf media alone (control), lane 2, = gf media containing α-FSH (50 ng/ml) lane 3 = gf media containing forskolin (10µM) lane 4 = gf media containing 8Br-cAMP (0.5mM). (* = P < 0.05%). The gel picture is a representative of three sets of independent experiments. Bar diagrams represent calculated mean with standard error of the three experiments.
Fig. 17. RT-PCR analysis of GDNF mRNA obtained from 5-days, 9-days, 12-days and 18-days-old rat Sc after 24hr of treatment.


For all the 8 panels (A to H), lane 1 = gf media alone (control), lane 2 = gf media containing o-FSH (50 ng/ml) lane 3 = gf media containing forskolin (10μM) lane 4 = gf media containing 8Br-cAMP (0.5mM). Aa and Ab are significantly different from each other.

The gel picture is a representative of three sets of independent experiments. Bar diagrams represent calculated mean with standard error of the three experiments.
Fig: 18. RT-PCR analysis of Cyp-19 aromatase mRNA obtained from 5-days, 9-days, 12-days and 18-days-old rat Sc after 2hr of treatment.


For all the 8 panels (A to H), lane 1, = gf media alone (control), lane 2, = gf media containing o-FSH (50 ng/ml) lane 3 = gf media containing forskolin (10μM) lane 4 = gf media containing 8Br-cAMP (0.5mM). (* = P < 0.05%).

The gel picture is a representative of three sets of independent experiments. Bar diagrams represent calculated mean with standard error of the three experiments.
Cyp-19 mRNA Expression at 24 hr

Fig: 19. RT-PCR analysis of Cyp-19 aromatase mRNA obtained from 5-days, 9-days, 12-days and 18-days-old rat Sc after 24 hr of treatment.


For all the 8 panels (A to H), lane 1 = gf media alone (control), lane 2 = gf media containing o-FSH (50 ng/ml) lane 3 = gf media containing forskolin (10μM) lane 4 = gf media containing 8Br-cAMP (0.5mM). (* = P < 0.05%).

The gel picture is a representative of three sets of independent experiments. Bar diagrams represent calculated mean with standard error of the three experiments.
24hr data

Cyp-19 mRNA was barely detected in 5-days-old Sc at 24hr in different treatment groups. (Fig: 19.A). A significant (P<0.05%) rise in the transcripts were detected with FSH or forskolin or 8-Br-cAMP treatments in 9-days-old Sc at 24hr, although the level of augmentation was most prominent in FSH treated group (Fig: 19.B). FSH or forskolin or 8Br-cAMP mediated augmentation of Cyp-19 mRNA was detected in 12-days of age (Fig: 19.C). In 18-days-old Sc, FSH or forskolin augmented Cyp-19 mRNA expression. Augmentation of Cy-19 mRNA by 8Br-cAMP treatment at 24hr was less compared to that of the FSH or forskolin in this age (Fig: 19.D). Internal control cyclophilin A were uniformly expressed in all the ages irrespective of different treatments (Fig: 19.E-H).

Discussion and Inferences

Although FSH is not crucial for male fertility, it still plays a major role in testicular physiology. The critical influence of FSH on testes relates both to its impact on Sc proliferation in perinatal life and to its influence on the synthesis of Sc-derived products essential for Gc survival and differentiation in the developing adult testis. FSH induces Sc proliferation in fetal and neonatal testes to establish the final number of Sc in the seminiferous tubule. In rats, Sc proliferate until around 15 days after birth (Clermont and Perey, 1957). The final number of Sc directly regulates the upper limit of sperm count (Orth et al. 1988 and Sharp, 1994). FSH acts via FSH-R present only in Sc in the testes. FSH binds to FSH-R and subsequent receptor coupling with Gs causes increase in the level of the intracellular cAMP which further activates classical PKA pathway or additionally results in calcium influx or activation of the PI 3-K, p38 MAPK and ERK MAPK pathways (Reviewed by Walker and Cheng, 2005).

Two studies have been done so far to evaluate the mode of action of FSH in rat testes during the first wave of spermatogenesis.

Using primary rat Sc culture, Crépieux et al. (2001) have demonstrated that mode of FSH action changes with Sc maturation. FSH induced cAMP production in 5-days-old rat Sc culture is found to be less compared to that of the 19-days-old rat Sc. This change in

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the level of cAMP generated within Sc decides the fate of downstream signaling cascade. In 5-days-old Sc, FSH mediated low cAMP evokes a PKA dependent ERK activation that ultimately leads to Sc proliferation. However, this elevated level of cAMP in 19-days-old Sc deactivates ERK and stimulates classical PKA dependent pathway (Crépieux et al. 2001).

Meachem et al. (2005) have taken an in vivo approach where endogenous FSH has been selectively suppressed by passive immunization for 2 days or 4 days prior to testis collection at day 3, 9 and 18 of postnatal ages of rats. 3-days-old rat testes after 2 days of FSH suppression display no measurable changes in terms of Sc and Gc proliferation and survival. 4 days of FSH suppression decreases Sc proliferation and numbers only in 9-days-old rats, but not in 18-days-old rats. In contrast, Gc numbers are unaffected at 9 days of age but decrease at 18 days of age following FSH suppression, with a corresponding increase in Gc apoptosis noticed in 18-days-old rats. Therefore, from these two studies it is obvious that, the dominant function of FSH shifts from driving Sc proliferation to support Gc differentiation in between 9 and 18 days of postnatal age which coincides with the first wave of spermatogenesis.

We measured plasma FSH level in rats of various postnatal ages (See Chapter I). We found that plasma FSH level was high in 5-days and 9-days-old rats compared to that of 12-days and 18-days-old rats. However, robust initiation of Gc differentiation starts at around 12-days of age. A differential action of FSH during the perinatal stage (in rats upto 9-days of age) may be responsible for this.

**Evaluation of FSH-R mRNA expression in various postnatal ages of rats**

FSH-R is present in the testis before significant concentrations of FSH appear in the fetal circulation. In rats, ligand-binding experiments reveal the presence of the FSH-R from fetal day 17.5 onwards. Concentration of FSH-R increases between fetal day 20.5 and birth (Warren et al. 1984). Transcripts encoding the ECD (N-terminal) have been detected from fetal day 14.5 onwards, and full-length FSH-R mRNA appears around fetal day 16.5 by northern blot and RT-PCR analysis. The extracellular domain is detected first. This may be due to differences in the onset of transcription of two mRNA varients
or alternatively, the two transcripts may have different half-lives. A C-terminally truncated ovine-FSH-R splice variant (alternative splicing in exon 10) has been described by Sairam et al. (1996). This protein exhibits membrane localization, binds to FSH but is unable to transduce the signal. Co-expression of this kind of FSH-R with the native receptor can create a dominant negative effect on the FSH signaling. Kraaij et al. (1998) have cloned two alternatively spliced mRNA species from rat Sc directional cDNA library. However, these two splice variants are not detected by RNase protection assay either in total testicular extracts (obtained from 21-days-old rat fetus and/or 21-days-old rats and/or 56-days-old rats) or in Sc primary culture (prepared from 21-days-old rats). Northern hybridization reveals presence of FSH-R mRNA in the rat testis on day 7 (youngest postnatal age so far studied). The expression decreases substantially by day 14, and remains at the same level until day 35 (Ito et al. 1993). However, FSH-R binding is highest in the postnatal testis at 15 days of age (Ketelslegers et al. 1978; Tsai-Morris et al. 1985).

The initial increase in FSH-R mRNA expression is related both to the increase of receptor numbers per Sc and to the proliferation of Sc up to day 10 (Almiron et al. 1988). However, Heckert and Griswold have found that FSH-R mRNA expression seems to be comparable in immature (10-days-old rats) and adult (60-days-old rats) Sc (Heckert and Griswold, 1991).

Most of these studies have been conducted in the past by taking total testicular extracts. No data is available which quantify the exact number of FSH-R in primary Sc culture using q-RT-PCR techniques.

We have studied the expression of FSH-R mRNA in Sc culture obtained from 5-days, 9-days, 12-days and 18-days-old rats. We first evaluated the status of FSH-R mRNA expression in culture with or without hormonal (FSH and T) supplements. The expression level was found to higher in freshly isolated Sc compared to that of the cultured Sc (irrespective of the age of rats, upto 3 days of culture in FT untreated groups). However continuous FT supplementation augmented FSH-R mRNA in cultured Sc obtained from all the age groups of rats. This finding indicated that transcription of FSH-R mRNA could carry on even without hormones in vitro and hormones only augmented the expression. In the hypogonadal (hpg) mouse which lacks circulating gonadotrophins,
levels of FSH-R mRNA remain normal up to 15 days. This suggests that gonadotrophins are not necessary for the expression of FSH-R mRNA levels (O'Shaughnessy et al. 1996) and further supported our findings. Sadate-Ngatchou et al. (2004) and Abel et al. (2009) have demonstrated an abundance of a large number of mRNAs affected by the FSH treatment in 21-days-old and 10-weeks-old hpg mice respectively. These observations directly support that the expression of FSH-R is independent of the presence or influence of endogenous FSH. 21-days-old rat Sc in culture shows substantial expression of FSH-R without FSH supplementation for 4 days (Kraaij et al. 1998).

Therefore, in our study, we evaluated the basal level of expression of FSH-R mRNA (in both freshly isolated Sc and in Sc culture without any hormonal supplements upto 4 days) by q-RT-PCR in various postnatal ages of rats. FSH-R mRNA was found to be uniformly expressed in freshly isolated and cultured Sc irrespective of the ages of rats. However, the expression levels were less in cultured Sc compared to that of the freshly isolated Sc. This uniform expression of FSH-R mRNA from 5-days to 18-days of postnatal age matched with that of the previous finding of Heckert and Griswold (1991).

**Evaluation of FSH-R binding sites on Sc surface obtained from various postnatal ages of rats**

Our finding of uniform expression of FSH-R mRNA in different age groups did not affirm the previous reports of increase in FSH-R binding with the advancement towards puberty. Low levels of FSH-R activity in rat gonads is detected after 14.5 days of gestation but FSH binding activity is increased about four-fold over fetal days 19.5 to 21.5 (Warren et al. 1984). This increase in FSH-R is concurrent with increased Sc proliferation. Theoretically, proliferation of Sc is dependent upon the increase in number of FSH-R levels on Sc itself. However, it is an endless debate till date, whether Sc proliferation during perinatal age is dependent upon the increasing FSH-R concentration per Sc or the increased number of Sc actually accounts for the detection of the elevated FSH-R numbers (FSH binding sites). Ketelslegers and colleagues have evaluated FSH binding activity in testis extracts after birth and have shown that at 2 days of life FSH-R are approximately 40 fmol/testis (Ketelslegers et al. 1978). FSH-R numbers per testis then continually increase up to a plateau of 1000 fmol/testis 60 days
after birth. Studies by Bortolussi and colleagues have demonstrated similar trends but receptor concentrations are found to be 3 to 10-fold lower (Bortolussi et al. 1990). Both groups have observed that after birth the concentration of receptors in the testis increases about 3-fold, peaking at 15 days after birth, thereafter receptor levels fall in the adult to levels approximately that of the 2 days-old Sc. These changes in concentration reflect the increase in FSH-R due to Sc proliferation, 2 to 15 days after birth and the subsequent dilution of Sc due to the expansion of Gc (Orth, 1984, Meachem et al. 1996). The number of FSH-R per Sc actually remain relatively constant from 2 to 21 days after birth (1500–1900 receptors/cell) but increase by another 2-fold each by 40 days and again by 60 days after birth (Bortolussi et al. 1990).

In order to find out the approximate FSH-Rs expressed on Sc surface in various ages of rats we did radio receptor assay for FSH-R with iodinated (I—125 ) o-FSH. Most of the FSH-R binding studies in Sc have been down on membrane fraction. As the expression of FSH-R is extremely low in Sc, we incubated radioactive hormone (I—125 -o-FSH) directly with the cells in presence of increasing order of excessive cold hormone. We were eventually able to detect approximately 50-60 and 200-220 FSH biding sites (FSH-R) on Sc surface obtained from 5-days, 9-days and 12-days and 18-days-old rats respectively. This observation was similar with that of the pervious work (Ketelslegers et al. 1978 and Tsai-Morris et al. 1985).

**Evaluation of FSH-R protein in various postnatal ages of rats**

The FSH binding data and the level of FSH-R mRNA expression did not match in different age groups. Infant Sc (upto 9-days of age) express similar amount of FSH-R mRNA compared to that of the 12-days and 18-days-old rats. However, binding of FSH to Sc increased sufficiently from 12-days of age and was maintained upto 18-days of age. Therefore, it was essential to measure the levels of FSH-R protein in various ages of rats. Traditionally, people use western blot analysis to detect the protein level. However, the number of FSH-Rs per Sc are too low to be detected by western blot. Therefore, we took an alternative approach described by Gong et al. (2008).

We tried to measure the total number of FSH-Rs present on Sc surface by flow cytometry analysis using N-terminal specific FSH-R antibody and fluorescent (FITC) tagged...
secondary antibody. However, we failed to detect any binding in live Sc obtained from 18-days-old rats using this antibody. Moreover, the antibody was found to be cross reactive with melanoma cell line (B-16 F-10) which lacks FSH-R. Therefore, we searched for new antibodies specific to FSH-R.

FSH-R is a GPCR and has a large ECD, a TMD and a short intracellular/cytosolic tail (C-terminal tail). The ECD has a LRR is essential for the ligand binding, while TMD is essential for transducing the signal. C terminal end is essential for the regulation of the basolateral localization (membrane targeting) of the receptor (Beau et al. 1998), the stability on the membrane and for desensitization. The C-terminal segment of FSH-R has one or more conserved cysteine residues that are potential sites for palmitoylation. This post translational modification contributes to membrane association, internalization, and membrane targeting of proteins (Uribe et al. 2008). Recently, Thomas et al. (2007) have identified an unique post translational cleavage site in the C-terminal domain and mature FSH-R and FSH-R present on the membrane of the cell lack this particular C-terminal domain. We chose a C-terminal specific antibody to evaluate the level of mature FSH-R protein present inside the cells. For this purpose, cultured Sc obtained from various postnatal ages of rats were fixed and were permeabilized by saponine treatment followed by incubation with primary anti-FSH-R antibody and fluorescent labeled secondary antibody. Total fluorescence detected in FL-1 filter per 25,000 cells obtained from various postnatal ages of rats indicated amount of FSH-R protein present in Sc. Splenocytes (that do not express FSH-R) were used as negative controls. Like equal expression of FSH-R mRNA, we found similar binding of FSH-R antibody to FSH-R protein inside the cells obtained from all the different ages. This observation indicated that FSH-R mRNA translated up to the protein level uniformly in all the ages. The possibility exists the proteins (mature FSH-R) either were not transported to the membrane appropriately or were not bioactive in terms of binding.

Evaluation of FSH binding sites inside the cells (Sc obtained from various postnatal ages of rats)

The expression of FSH-R mRNA and protein were uniform from 5-days to 18-days of age. However, the ligand binding ability was found to be increased remarkably only after
12-days of age. We hypothesized that FSH-R expressed as mature protein inside infant Sc (upto 9-days of age) might not be transported to the cell membrane, thus leading to poor ligand binding at this age. However, we failed to detect approximate FSH-R numbers present on Sc surface using unfixed Sc with N-terminal specific antibody. Therefore, we evaluated the ligand binding sites present inside the cells. More FSH binding sites (radioactive FSH specifically bound to Sc after replacing excessive cold FSH) were detected inside the cells compared to that present on Sc surface irrespective of the ages of rats. However, the fold increase in radioactive FSH binding to FSH-R upon Sc permeabilization was more in infant Sc compared to that of the 12-days and 18-days-old Sc. Total binding sites (both extra and intra cellular) were found to be more in 12-days and 18-days of age when compared against that of the 5-days and 9-days-old Sc. These data indicated that although FSH-R proteins were present in equal amount inside Sc from 5-days to 18-days-old Sc, the distribution of FSH-R from cytoplasm to membrane was impaired during infancy and biologically active receptors were more prevalent in 12-days of age onwards. It seems that majority of the FSH-Rs present during infancy (upto 9-days of age) were not able to bind with the ligand.

The physiological relevance of the existence of such non-functional receptors is not well understood. Not all the FSH-Rs expressed on the surface of Sc are available for binding. Enzymatic deglycosylation of plasma membranes unmasks cryptic binding sites of FSH in testicular preparations from calves (Nishimori et al. 1989) and monkeys (Berman and Sairam, 1982). One interesting factor regarding the ligand-receptor interaction is the functional role of glycosylation for gonadotropin receptors. The FSH-R possesses three (rat) or four (human) potential glycosylation sites in the N-terminal ECD. The concept that local enzymatic activities in the gonads could potentially modulate hormone action by unmasking receptors remains highly speculative. Experiments employing site-directed mutagenesis have shown that at least two of the three potential glycosylation sites of the rat FSH-R are actually N-linked glycosylated. Removal of N-linked glycosylation sites does not alter the binding affinity of the mutated receptor, but binding is lost when both glycosylation sites are mutagenized or when glycosylation of the nascent receptor is totally prevented by tunicamycin (Davis et al. 1995). In contrast, exhaustive deglycosylation of mature receptors does not impair binding properties, suggesting that
carbohydrates are essential for receptor folding and transportation to the cell surface, but not for hormone binding (Davis et al. 1995). Moreover, deglycosylation of the calf testis does not affect functional coupling of FSH-R to Gs protein (Dattatreyamurty and Reichert, 1992).

Here, for the first time, we showed that although, total FSH-R protein present inside Sc remain constant from infancy (upto 9-days of age in rats) to prepuberty (18-days of age in rats) only few of the FSH-Rs were functionally active to bind efficiently with FSH, during infancy.

The number of FSH-Rs capable of binding to FSH increases significantly (P<0.05%) in 12-days and 18-days of age. The significance of the presence of FSH-Rs that do not bind with FSH is not clear. The reason for which the proportion of FSH-R remains nonfunctional during infancy is beyond the scope of the present study. A detail structure-function analysis of age specific native FSH-R can answer this question.

However, a probable justification of this phenomenon from a physiological view point could be "intermolecular cooperation", observed in GPCRs due to di/oligomerization during their biosynthesis before membrane delivery and ligand binding. Di/oligomerization observed in GPCR explains both the negative or positive receptor cooperativity, reconstitution of activation by mutant receptors, and ligand promiscuity. The interactions of some GPCR dimers are covalent, noncovalent (interactions involving the transmembrane domains or coiled-coil interactions, or both). In the later case, a ligand bound receptor interacts sequentially with another or multiple nonligand bound receptors in its vicinity, pushing the equilibrium from an "active" to an "active-active" state. This trans conformations of GPCRs result in G protein activation known as "intermolecular noncovalent positive cooperation," and has a broad implication in biological systems where a specific signal could be rapidly amplified, with minimal ligand binding, yet maintaining the specificity of action (Reviewed by Bulenger et al. 2005). There is currently no biochemical data that demonstrate that FSH-R exists as a dimer or higher-order oligomer in cell membranes. A fluorescence resonance energy transfer assay has been used by Thomas and colleagues to determine whether full-length native FSH-R is an oligomers (Thomas et al. 2007). FSH-R-specific monoclonal antibody or Fab fragments, labeled with two different fluorophores, have allowed the
study of nontagged receptor *in situ*. Unoccupied FSH-R exhibits strong fluorescence resonance energy transfer profiles *in situ*. Complementary coimmunoprecipitation experiments by myc- or FLAG-tagged FSH-R indicate that FSH-R forms oligomers early in receptor biosynthesis. No effect of FSH on this process is observed (Thomas *et al.* 2007). Recently, Rivero-Müllera and colleagues have demonstrated that transgenic mice coexpressing binding-deficient and signaling defective forms of LH-R can reestablish normal LH actions through intermolecular functional complementation of the mutant receptors in the absence of functional wild-type receptors. This provides the *in vivo* evidence for the physiological relevance of intermolecular cooperation in GPCR signaling (Rivero-Müllera *et al.* 2010).

However, it is not clear whether the binding of a ligand to a receptor could influence the binding of a second ligand to a second receptor linked with the first receptor (as receptors are in oligomers) by this intermolecular noncovalent cooperation. *If it is so then we can hypothesize that oligomerization of FSH-R proteins inside Sc either starts or increases between 9 to 12-days of postnatal age in rats which results into a intermolecular noncovalent positive cooperation leading to increase in FSH binding sites.*

**Ability of FSH to produce cAMP from Sc culture obtained from various postnatal ages of rats**

FSH binding to its receptor is known to activate at least five different pathways but the *cAMP pathway is thought to be the major pathway triggered by FSH in Sc*. In the fetal testis, despite FSH binding, it is difficult to demonstrate FSH-stimulated cAMP production (Ketelslegers *et al.* 1978; Huhtaniemi *et al.*, 1987; Picon and Gangnerau, 1980) which is the hallmark of the FSH action postnatally. Eskola *et al.* (1993) using testicular homogenates have showed that the cAMP response is low at birth and at days 30 of postnatal age; and the maximal cAMP response is observed at around 10-days of age. However, Crépieux *et al.* (2001) have described that, in 5-days-old Sc, FSH mediated low cAMP evokes a PKA dependent ERK activation that ultimately leads to Sc proliferation. However, elevated level of cAMP in 19-days-old Sc negatively regulates ERK activation and stimulates classical PKA dependent pathway.
We evaluated the cAMP produced by Sc under FSH or agents that selectively bind with cell membrane components and eventually generate cAMP inside the cells. Cholera toxin (CT) or forskolin directly activates the G_s subunit and adenyl cyclase (AC) enzyme respectively without interacting with FSH-R and increase cAMP concentration inside the cell. *Accumulated cAMP diffuses out from the cell and cell-exposed media can thus be used to measure cAMP.* The level of cAMP secreted out in the media is comparable with the cAMP generated inside the cell. Hence, measuring cAMP from Sc exposed media is a routine practice rather than measuring intracellular cAMP. However, **Galdieri et al. (1994)** have measured more cAMP in the extracellular media compared to that of the intracellular part. This is probably because of the activity of PDE present inside the cell. As we have discussed in chapter I, non-specific PDE inhibitor IBMX was used in our system (even though we have done our cAMP assay in Sc exposed media) for generating better read out.

Unlike 5-days and 9-days-old Sc, FSH mediated rise in cAMP production was observed within ½ hr in 12-days and 18-days-old rats. CT treatment for ½ hr failed to stimulate any detectable rise in cAMP in all the age groups. However, CT treatment for 24hr generated sufficient amount of cAMP in the Sc exposed media of all the age groups. This suggested that the *G_s subunit was expressed and active from the neonatal stage of development in rats.* Forskolin induced cAMP generation was detected in all the age groups indicating that the AC action was also not responsible for getting no rise in cAMP production after FSH treatment in 5-days and 9-days-old rats. Sc. *Although the binding of FSH to FSH-R in 12-days-old Sc was similar to that of the 18-days-old rats, FSH mediated cAMP production was most prominent in 18-days of age.* This observation was supported by previous findings by **Crépieux et al. (2001)** and **Levallet et al. (2007).** The reason of this discrepancy between FSH binding and cAMP generation might be either due to a change in the action of PDEs or change in action of Regulators of G-protein Signaling (RGS) class of proteins in different age groups.

**Evaluation of the endogenous PDEs activity in postnatal rat Sc**

The regulation of intracellular cAMP levels is controlled through the modulation of both AC and cAMP-catabolizing PDEs. PDEs constitute a family of more than 50 members of
cyclic nucleotide-catabolizing enzymes, which has been subdivided in 11 subfamilies (from PDE1 to PDE11) according to substrate selectivity, inhibitor sensitivity, and sequence. Each member of the different PDE subfamilies is encoded by different genes, which can give rise to multiple variants by alternative splicing of mRNA or by the use of alternate promoters. PDE4 is the main, if not the sole, PDE subfamily that is subjected to regulation in the testis (Reviewed Mehats et al. 2002).

In our experiments using forskolin showed earlier that AC activity was conserved in rats from 5-days to 18-days of age in terms of cAMP production. Hence, it was essential to estimate the action of PDEs in these age groups. In our study, using undifferentiated Sc from 9-days-old rats (that failed to induce cAMP production upon FSH treatment) and differentiating Sc from 12-days-old Sc (that produced appreciable cAMP upon FSH treatment) we have measured the activity of PDEs indirectly. Sc from these rats were cultured and on day 4 of culture, Sc was treated with gf media with or without presence of IBMX ($10^{-4}$ M) for 1/2hr and 24hr. The basal level of cAMP accumulated over 24hr of time in the Sc exposed media would indirectly indicate the basal level of PDEs activity. We considered that due to the presence of IBMX all the endogenous PDEs would be inactivated properly. The dose of IBMX was determined from previously published work (Steinberger et al. 1978). Accumulation of cAMP (from 1/2hr to 24hr) was detected in 9-days-old rat Sc only in presence of IBMX. cAMP was detectable in 12-days-old rat Sc after 24hr even in absence of IBMX. This indirectly suggested that the endogenous PDEs activity might be more in 9-days-old rat Sc, as in absence of IBMX, cAMP was not detectable even after 24hr. Levallet et al. (2007) have measured intracellular level of cAMP (generated inside Sc basally) in Sc cultured from 10-days, 20-days, and 30-days-old rats without IBMX. Level of cAMP is less in 10-days-old Sc compared to that of the 20-days-old rats Sc. This observation also suggests high level of PDEs activity (as less cAMP) in 10-days-old rats. However, Monn et al. (1972) have described low activity of PDEs in 5-days-old rats. Crépieux et al. (2001) have found higher level of basal cAMP in 5-days compared to that of the 19-days-old rat Sc culture. Therefore, the existing reports are very conflicting. A five-fold increase in PDE activity has been reported from 20 to 60 days postpartum in the whole testis (Epplen et al. 1980), while in partially purified cultured Sc from 18- to 24-days-old rats, a decline in total PDE
activity occurs concomitantly with a drastic decrease in the cAMP response to FSH (Steinberger et al. 1978). We have found that FSH induced cAMP production was most prominent in 18-days-old Sc. The differences in the FSH responsiveness of immature and mature rats are still misunderstood due to the lack of information about the evolution of PDE activities in purified Sc during postnatal development. Levallet et al. (2007) using Sc cultures isolated from 10-, 20-, and 30-days-old rats, have recorded a specific increase in PDE4 activity in both the soluble and particulate subcellular fractions of 20-days-old Sc, which also displayed the highest cAMP response to FSH and the highest FSH-induced increase in PDE4 activity in both subcellular compartments, although the subcellular distribution and expression of PDE4D proteins were unaffected by the developmental status of the Sc.

*PDEs activity increases only with an increase in the substrate (cAMP) concentration and found to be highest in postmitotic differentiated Sc (Levallet et al. 2007).*

Therefore, we inferred that the generation of lesser cAMP during infancy (upto 9-days of age) compared to that of the 12-days and 18-days of age upon FSH treatment is directly linked with the lesser binding of FSH to FSH-R at younger age and not due to change in PDEs expression or activity.

*Most prominent cAMP response in 18-days as found by us was probably because of some role of RGS class of proteins that might be more dominant in 12-days of age. RGS3 and RGS22 are studied in testes (Crépieux et al 2001 and Hu et al. 2008 respectively). However, RGS are not reported to deactivate Gαs subtype (Dolhman and Thorner, 1997). A detail investigation of this question has not been pursued further since it was beyond scope of the present study.*

**Recruitment of G\(_{αi}\) versus G\(_{αs}\) by FSH-R followed by FSH treatment**

FSH is a glycoprotein hormone, the degree of glycosylation directs the preferential recruitment of G\(_{αi}\) versus G\(_{αs}\) upon FSH binding with FSH-R (Arey et al. 1997). Pertussis toxin (PT) is a protein-based AB\(_5\)-type exotoxin produced by the bacterium *Bordetella pertussis*, which causes whooping cough. PT catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric G proteins G\(_{αi}\), G\(_{αo}\) and G\(_{αt}\). This predominantly
prevents the G proteins from interacting with GPCR on the cell membrane, thus interfering with intracellular communication. The G\textsubscript{ai} subunits remain locked in their GDP-bound, inactive state, thus being unable to inhibit AC activity, leading to increased cellular concentrations of cAMP. **Therefore, FSH treatment to Sc pre-incubated with PT will only recruit the G\textsubscript{as} subunit due to suppression of G\textsubscript{ai} activation.**

We investigated the age dependent nature of coupling of these two G proteins upon FSH binding to FSH-R in Sc. Crépieux et al. (2001) have described that FSH induced ERK pathway is dependent upon cAMP and involvement of G\textsubscript{ai} is essential for neonatal Sc proliferation. We evaluated FSH or CT or forskolin mediated cAMP production by Sc obtained from different ages of rats after either preincubating Sc with PT or no prior PT incubation. CT was the positive control to detect the participation of G\textsubscript{as} only and forskolin was used as the negative control as it directly activates AC.

A rise in cAMP was detected in 5-days and 9-days-old rat Sc upon FSH treatment in absence of IBMX for 24hr. PT preincubation induced significantly (P<0.05%) high rise in basal cAMP in these cells. This might be because of the removal of G\textsubscript{ai} mediated inhibition of AC. However, further FSH treatment could not increase cAMP in PT preincubated cells. This was probably due to less number of functionally active FSH-R available for FSH binding in these cells. An appreciable rise in cAMP level was detected in Sc from both of the age groups after CT or forskolin treatment. This observation indicated that the G\textsubscript{as} subunit and AC were active during infancy. CT or forskolin treatment did not elevate cAMP production further when Sc were preincubated with PT. Presence of IBMX elevated the basal level of cAMP in all the treatment groups in 5-days and 9-days-old Sc. FSH or CT or forskolin in presence of IBMX, failed to augment cAMP significantly in these age groups. However, in presence of IBMX, the basal level of cAMP was found to be increased further with PT preincubation in 5-days of age. 12-days and 18-days-old rat Sc showed an appreciable rise in cAMP production upon FSH treatment for 24hr in presence or absence of IBMX. This rise in cAMP upon FSH treatment was significantly (P<0.05%) higher than that of the 5-days and 9-days of rats. PT preincubation had no effect in 12-days and 18-days-old Sc in terms of induction of the
basal rise in cAMP. *This observation suggested that G\textsubscript{a,i} mediated inhibition of AC was compromised in 12-days and 18-days-old Sc and action of G\textsubscript{a,i} was more dominant during infancy (upto 9-days of age) without the involvement of FSH-R.* However, unlike 5-days and 9-days-old rat Sc, FSH treatment to PT preincubated Sc further induced cAMP production in 12-days and 18-days-old rat Sc. This probably was due to availability of more bioactive FSH-R in 12-days and 18-days of age.

The reason behind the FSH-R independent G\textsubscript{a,i} mediated inhibition of AC can be explained by the age dependent change in adenosine receptor present in Sc. Adenosine receptor is known to be coupled with G\textsubscript{a,i} thereby inhibits the AC (*Monaco et al. 1988* and *Conti et al. 1989*). PT incubation deactivates G\textsubscript{a,i} and hence removes the inhibition upon AC. Expression of adenosine receptor is also developmentally regulated. However, the physiological role of these receptors in the testis is not clear. The rat testis expresses high levels of A\textsubscript{1} adenosine receptors that are coupled to the inhibition of AC activity. To date, four subtypes of adenosine receptor have been identified, these being the A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B}, and A\textsubscript{3} receptors. Both the A\textsubscript{1} and A\textsubscript{3} adenosine receptor couple with G\textsubscript{a,i} proteins and thereby promote inhibition of AC. In contrast, the A\textsubscript{2A} and A\textsubscript{2B} adenosine receptor positively couple to AC through the G\textsubscript{o,s} proteins. Other effectors shown to be regulated following activation of adenosine receptor include K\textsuperscript{+} channels, Ca\textsuperscript{2+} channels, and phospholipase C. In Sc culture, activation of A\textsubscript{1} adenosine receptor leads to inhibition of FSH-mediated cAMP production and the aromatization of androgen to estrogen (*Conti et al. 1989*). Study by *Bhat et al. (1998)* indicates substantial reductions in A\textsubscript{1} adenosine receptor in the testes with age, which might reflect by our finding of increase in cAMP production only in infant Sc (upto 9-days of age) after PT treatment compared to that of the 12-days and 18-days-old Sc. *Therefore, inhibition of AC during infancy via G\textsubscript{a,i} as shown by us was probably due to an age related change in A1 adenosine receptor expression in rat testes. This could be another cause of getting less amount of cAMP generated by FSH during infancy.*
**Evaluation of SCF expression in various postnatal ages**

In testis, Kit ligand (KL, also named stem cell factor, SCF) is produced by the Sc, whereas its receptor (the transmembrane tyrosine kinase encoded by the c-kit proto-oncogene) is expressed predominantly in differentiating type A spermatogonia, in Lc and, at a lower level, in type B spermatogonia and in early spermatocytes but not in undifferentiated spermatogonia i.e., spermatogonial stem cells (Ssc) or in postmeiotic Gc. The interaction of SCF with its receptor causes the activation of a signal transduction pathway required for the proliferation and the survival of mitotic Gc both in the embryonal gonad and in the postnatal testis. SCF might also be involved in the control of meiotic differentiation of male Gc. In Steel17H mutant mice, a specific alteration in the SCF gene causes defects selectively in spermatogenesis but not in other SCF-regulated processes, such as hematopoiesis, pigmentation, development of primordial germ cells, and oocyte maturation. Recent evidence supports an essential role of SCF activated signaling pathways for normal spermatogenesis, since homozygous mutations in the PI3K docking site of c-kit cause male sterility due to a block of spermatogonial proliferation in prepubertal mice, whereas no major defects are observed in other SCF-targeted cells. Expression of the mRNA for SCF is induced by the pituitary hormone FSH in prepubertal mouse and rat Sc through an increase of cAMP levels. Paracrine factors secreted in the microenvironment of the seminiferous tubules are also probably important for modulation of SCF expression in Sc, and at least one of these factors that has been identified in rats, growth hormone releasing hormone (GHRH), which works through an increase in cAMP levels (Reviewed by Bedell and Zama, 2004).

The existing information regarding the pattern of SCF expression throughout the early postnatal development is little controversial. Munsie et al. (1997) have showed that the level of SCF transcript is barely detectable at 1-days to 4-days of age whereas a striking elevation in the expression is evident in 5-days of age. The SCF transcripts remain detectable at all ages thereafter, although the signal intensity goes down. Blanchard et al. (1998) have reported that SCF mRNA increases progressively from 5-days to 16-days of age with a constant rise in the membrane bound form over the soluble form. However, in both of the studies total testicular extracts have been used for the preparation of the
mRNA. It is known that in addition to Sc, gonocytes and the interstitial Lc also express SCF during neonatal stage (Munsie et al. 1997). Therefore, Sc isolated and cultured from different ages of postnatal period should be used to validate the exact expression profile.

In chapter I, we have described that the expression of SCF mRNA dramatically increased in Sc cultured from 12-days-old rats. Here, we have evaluated the expression of SCF mRNA under the influence of either FSH or agents that directly induce intracellular cAMP (such as forskolin or 8-Br-cAMP) in Sc from various postnatal age groups by semi-quantitative RT-PCR. SCF mRNA was not detected in Sc cultured from 5-days-old rats upon 2hr of constant exposure with various treatments. Detectable SCF transcripts (soluble isoform) were observed from Sc cultured from 9-days-old rats. However, no augmentation by FSH or forskolin or 8Br-cAMP was observed in this age group. Sc cultured from 12-days and 18-days-old rats were found to have high basal level of SCF mRNA. Treatment of FSH and other compounds that elevate cAMP levels also augmented SCF production in 12-days-old Sc. Membrane bound isoform was expressed more compared to that of the soluble isoform in these two age groups.

Expression of soluble SCF mRNA was discernible in Sc cultured from 5-days-old rats when Sc over stimulated for 24hr with forskolin or 8Br-cAMP, while no transcripts were detected in control or FSH treated cells in this age group again proving the failure of FSH action. Sc cultured from 9-days-old rats showed a rise in the basal level of SCF expression (soluble isoform) and the level were unaltered upon FSH treatment at 24hr. Forskolin or 8Br-cAMP mediated augmentation in the soluble isoform of SCF was also detected in 9-days at 24hr. All these observations together suggested that SCF is a developmentally regulated gene which starts expressing sufficiently from 12-days of age due to high FSH binding with FSH-R followed by cAMP production. Soluble and membrane bound isoforms were dominant during infancy and prepuberty respectively. This data was supported by a previous finding of Blanchard et al. (1998).

Treatment of forskolin or 8-Br-cAMP showed that the intracellular downstream signaling events were active from the time of early infancy (from 5-days of postnatal age). These data also provided the information that SCF expression was not developmentally regulated by some epigenetic control. In other words, unlike other developmentally
regulated genes, SCF promoter does not seem to be repressed against the activator transcription factors in an age dependent manner. Our observation is also supported by previous works. The proximal promoter regions of SCF genes in human (Taylor et al. 1996), rat (Jiang et al. 1997), and mouse (Grimaldi et al. 2002) contain elements that mediate cAMP induction of the genes in Sc. The lack of cAMP response element (CRE) sequences in the proximal 5'-flanking regions of all these genes suggests that cAMP-induced SCF expression in Sc is mediated by transcription factors different from the CRE binding protein in all these species. cAMP directs SCF expression via Sp-1 binding region (Grimaldi et al. 2003).

Our observation indicated that low cAMP levels during infancy (upto 9-days of age) fail to trigger robust SCF expression. Signaling components upstream of SCF expression were active and SCF promoter was accessible to them at this age.

**Evaluation of GDNF expression in various postnatal ages**

The regulation of Ssc maintenance, self-renewal, and differentiation is critical for normal spermatogenesis. GDNF, a distantly related member of the transforming growth factor-β superfamily, secreted by Sc regulates the fate of Ssc. Gene-targeted mice with one GDNF-null allele are fertile but show depletion of the reserve of Ssc, whereas mice overexpressing GDNF in the testis are infertile and accumulate a lot of undifferentiated spermatogonia (Meng et al. 2000). These results indicate that GDNF contribute to the paracrine regulation of spermatogonial self-renewal, differentiation, and survival in the mouse. *At a low GDNF level, spermatogonia favor differentiation, and at a high level, they favor self-renewal* (Meng et al. 2000). GDNF is expressed in Sc and the expression is known to be augmented by FSH (Han et al. 2008). The physiological response to GDNF requires the presence of the glycosyl-phosphatidylinositol-linked protein (GFR-α) that is expressed on Ssc. At the same time, GDNF promotes the formation of a physical complex between GFR-α and the orphan tyrosine kinase receptor Ret (GFR-β), thereby inducing its tyrosine phosphorylation (Reviewed by Hofmann, 2008).

In chapter I, we have discussed the expression pattern of GDNF mRNA in Sc culture with and without hormonal (FSH and T) supplements obtained from various postnatal age groups of rats. GDNF was not detected in 5-days-old Sc culture irrespective of presence
or absence of FSH and T. GDNF mRNA expression was continued in 9-days-old rat Sc culture only in presence of FSH and T. 12-days and 18-days-old Sc were able to express sufficient GDNF mRNA independent of hormonal supplements in the culture. Here, we evaluated whether the expression of GDNF mRNA under the influence of either FSH or agents that directly induce intracellular cAMP (such as forskolin or 8-Br-cAMP) in Sc from various postnatal age groups by semi-quantitative RT-PCR.

FSH mediated augmentation of GDNF mRNA was detected in 9-days-old rats Sc at 2hr. However, maximal augmentation of the GDNF mRNA by FSH was observed in 12-days-old Sc at 2hr. Forskolin or 8-Br-cAMP failed to augment GDNF mRNA expression at 2hr. This indicated that GDNF might be up regulated by FSH via an alternative pathway other than classical cAMP-PKA pathway. Treatment of FSH or forskolin or 8-Br-cAMP for 24hr failed to induce GDNF expression in 5-days and 9-days-old rat Sc. GDNF expression was not augmented by forskolin or 8-Br-cAMP upon 24hr treatment in 12-days and 18-days of age. This further suggested that GDNF expression was dependent upon an alternative pathway of FSH signaling (other than classical cAMP-PKA pathway) in all the age groups of rats.

A progressive elevation in the basal GDNF transcript was detected from 5-days to 9-days to 12-dasys-old rats. This elevated level was maintained in 18-days-old rats. In chapter II, we observed a similar expression pattern of GDNF mRNA when androgen mediated GDNF expression were studied. This indicated that Sc maturation is a graded process associated with a progressive elevation of GDNF expression. We assumed here that an epigenetic control might exist for the age dependent expression of GDNF. Action of GDNF to regulate the self renewal of Ssc is restricted to only the first wave of spermatogenesis. Transcription factor Ets variant gene 5 (ETV5; also known as Ets-related molecule, or ERM) is known to regulate Ssc self-renewal during the maintenance phase of spermatogenesis (Chen et al. 2005). Decrease in the expression of GDNF with age is also reported (Reviewed by Hofmann, 2008). Therefore, plausibility of having such epigenetic control over GDNF expression is not incomprehensible.

All these data together indicated that the expression of GDNF increases progressively with age of Sc (upto 18-days, studied here) under the control of FSH but it was
independent of cAMP. Expression of GDNF was found most elevated in 12-days, the age when robust initiation of Gc differentiation occurs.

Evaluation of Cyp-19 aromatase expression in various postnatal ages

Aromatase, the enzyme responsible for the transformation of androgens into estrogens, is encoded by the Cyp-19 gene expressed in the testis. An active PI3-K/AKT signaling pathway is required for the stimulatory actions of FSH, whereas an active ERK/MAPK pathway inhibits aromatase expression and further estradiol production in 10-days-old Sc (McDonald et al. 2006). A recent study has been performed by Bouraïma-Lelong et al. to analyze the evolution of aromatase gene expression under FSH control in rat Sc between 10 and 30 days of postnatal age, corresponding to the end of the proliferative period of Sc, establishment of the BTB and acquisition of the mature phenotype. The maximum stimulatory effect of FSH on aromatase gene expression is obtained in 20-days-old rat Sc, compared with cells from 10-days and 30-days-old rats, in parallel with the differentiation of Sc. Two activators of the PKA pathway (i.e. forskolin and dibutyryl-cAMP) reveal differential effects between cells from rats aged 20 and 30 days, implying the involvement of another signaling pathway. Experiments using the specific PI3-K inhibitor LY294002 indicate that PI3-K is strongly involved in FSH-induced aromatase expression in Sc from both 20- and 30-day-old rats (Bouraïma-Lelong et al. 2010).

In our experiments however, FSH mediated Cyp-19 augmentation was found in all the age groups at 2hr. Only forskolin (not 8-Br-cAMP) could augment Cyp-19 expression at 2hr, but the level of transcripts was less compared to that of the FSH. No basal level of the transcripts were detected in any of the age groups at 2hr. Low amount of transcripts were detected in 5-days-old Sc at 24hr. FSH mediated augmentation were observed in rest of the age groups at 24hr. Forskolin, unlike 8-Br-cAMP could augment Cyp-19 expression at 24hr, but the level of transcripts were less compared to that of the FSH. The differential expression patterns of this gene by forskolin and 8-Br-cAMP treatments observed in all the age groups at 2 or 24hr, was probably due to the different amount of
cAMP generated by these treatments (dose of forskolin 10µM/well, dose of 8-Br-cAMP 0.5mM/well) resulted into differential activation of PI3-K/AKT pathway. This further indicated that alternative pathways other than classical PKA were active during infancy (upto 9-days of age of rats) supporting the previous findings of Crépieux et al. (2001).

All these data together suggested that although FSH mediated cAMP production was less during infancy, alternative pathways of FSH (mediated by less cAMP itself) were functional to fulfill the physiological needs of the cells.

**Conclusion**

This study was initiated keeping in view of a situation in idiopathic male infertility where sufficient hormone fails to induce the onset of spermatogenesis, for which infant rats were used as a surrogate. Although infant rat Sc (5-days and 9-days of age) have a higher circulating level of FSH compared to that of the 12-days and 18-days of age, initiation of Gc differentiation was observed only at around 12 days of postnatal age in rats. In this study, we found that inefficient responsiveness of infant Sc to FSH in terms of cAMP production and SCF expression was responsible for this situation. Receptor binding (using radioactive FSH) and staining (followed by flow cytometric analysis) suggested that this lack of FSH mediated response was due to less number of functionally active FSH-Rs present either on the surface or inside the cell. High level of GDNF expression was also detected only during 12-days of age, presumably the period of the onset of robust spermatogenesis. This restricted the expression of SCF and GDNF genes upto 9-days of age and may be responsible for the inability of Ssc to differentiate. We termed the augmented appearance of SCF and GDNF expression in 12-days-old rat Sc as a “Developmental Switch” that came into effect from the age when the cells (Sc) mature to show hormone response coinciding with the time of the robust initiation of Gc differentiation.
The failure of such developmental switch in FSH mediated gene expression in Sc due to an inappropriate FSH-R, preventing the occurrence of its downstream cascades may be the principal cause of certain forms of idiopathic male infertility. An interesting finding is that, intracellular cAMP stimulation bypassing FSH-R with forskolin or 8Br-cAMP treatment, in infant Sc could generate necessary downstream signals to express relevant genes. This may be a basis of treatment of certain forms of idiopathic male infertility.