3.1 **Chemicals**

Standard OT was purchased from Sigma Chemicals Co. (St. Louis, MO). EIA kit for OT quantitation was procured from Assay Design (Ann Arbor, MI) and Sep-Pak column was a product of Waters (Milford, MA). $[^{3}H]$ OT (oxytocin, tyrosyl-2,6-$^{3}$H) (30–60 Ci/mmol) was a product of PerkinElmer (Waltham, MA). Primary antibodies [phosphorylated epidermal growth factor receptor (pEGFR), epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2), phosphorylated extracellular signal regulated kinase 1/2 (pERK1/2), extracellular signal regulated kinase 1/2 (ERK1/2), growth differentiation factor-9 (GDF-9), hyaluronic acid synthase-2 (HAS-2), TNF stimulating gene-6 (TSG-6) and β-actin] and HRP-conjugated secondary antibodies (anti rabbit, anti mouse and anti goat) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). $[^{3}H]$ thymidine (6.7 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). PGE-2, pERK inhibitor (PD98059) and PGE-2 inhibitor (Arachidonoyl trifluoro methyl ketone) were procured from Cayman Chemicals (Ann Arbor, MI). OT antagonist [d (CH)$_{2}$ 51Tyr(Me)$_{2}$Arg8 vasopressin] (OTA) was purchased from Tocoris Bioscience (Bristol, UK). All other chemicals and reagents used were of highest purity commercially available. Antibodies for ERK1/2, p-ERK1/2, AKT, p-AKT, GDF-9, p-P38, p-NFkB, COX-2, HAS-2, TSG-6, BCL-2, PARP and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pregnant mare serum gonadotropin (PMSG) and human choriono gonadotropin (HCG) were purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals used were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise specified.

3.2 **Procurement of milk samples**

Milk samples from the local market of Lucknow, India were procured and after drying were kept at -20 °C till further analysis.

3.3 **Extraction of OT from milk**

OT from milk samples was extracted as described in figure 3.1. Milk sample (5mL) was brought to a final concentration of 10 percent trichloroacetic acid (TCA) by adding 500 μL of 100 per cent...
TCA (grams per volume) and stirred for 10 minutes. The precipitate was removed by centrifugation at 10000 rpm for 15 minutes and supernatant was collected. To enhance the recovery of OT, the precipitate was re-suspended in 1 ml of 0.25 per cent acetic acid and stirred for 15 minutes and again 100μL TCA (100 percent) was added, stirred for 5 min and centrifuged. After centrifugation, the previous step (the addition of 0.25 percent acetic acid to the precipitate and treatment with TCA), was repeated and the three supernatants were pooled. The second step of washing of precipitate with acetic acid can be optional step. Further, the pooled supernatants were passed through the Sep-pak vac (100 mg) solid phase extraction (SPE) column, which was pre-conditioned with methanol (1 mL) and water (1mL). The loaded cartridge was washed with water (1mL) and 30% methanol (1mL) to get rid of impurities, if any. The OT was eluted with 2ml mixture of ethanol; 6N hydrochloric acid (1000:1; v/v) and evaporated to dryness under a gentle stream of nitrogen.

3.4 Limit of detection and limit of quantitation measurement

The LOD and LOQ were calculated by calibration curve procedure (Eurachem Guide, 1998). Each of a series of blank samples, spiked with different concentrations of the OT, is analyzed. The threshold or ‘cut-off’ concentration is determined based on a response curve, plotting the % positive results versus the concentration. In this respect, the LOD is the concentration at which 95% of the values give a positive signal (Eurachem Guide, 1998). For LOQ determination, sample blanks are spiked with various OT concentrations close to the LOD. Independent replicates of each concentration are measured and the standard deviation of the measured was calculated. These standard deviations (or the relative standard deviations %RSD) are then plotted against the concentration. LOQ value was the concentration of OT corresponding to % RSD values of 10 (Eurachem Guide, 1998; Codex, 2002).

3.5 Stability measurement of OT at different conditions:

3.5.1 Temperature stability to OT
5 mL milk
Vortex for 10 min
Precipitate Supernatant (1) (~ 5mL)

1 mL of 0.25%
acetic acid
Vortex for 15 min
Vortex for 5 min
Precipitate Supernatant (2) (~ 1mL)

Process was repeated
and Supernatant (3)
was taken

SPE Process

Supernatants (1&2) were pooled (~ 6mL)
Passed through sep-pak column (100mg)
pre-conditioned with 1mL of methanol (~ under gravity)
1 mL of water was passed
ml of 30% methanol was passed
Elute with 2 mL of ethanol:6N HCl (1000:1)
Eluate (~ 2mL) was evaporated under
stream of Nitrogen gas
Residue dissolved in
solvent (0.1mL)
or buffer (1mL)

Figure 3.1 Flow sheet of steps involved in extraction of OT from milk

~ 31 ~
OT spiked milk samples (100 mg/L) were incubated for 1 hour at 25, 37, 55, 75 and 100°C. For all the temperature used for incubation, '0' min time point was taken as control. All the samples were analyzed by UV-HPLC.

3.5.2 pH resistance to OT

OT spiked (100 mg/L) milk samples were prepared in phosphate buffer (80 mM) having pH of 2, 4, 6, 8 and 10 and incubated for 1 hour. All the samples had their '0' min time point as controls and were analyzed by UV-HPLC.

3.5.3 Resistance of OT in Simulated Gastric Fluid (SGF)

3.5.3.1 Preparation of SGF

SGF was prepared as described by Roesler et.al. (Roesler & Rao, 2001). Pepsin, 4 mg (approximately 3460 unit activity/mg) was dissolved in 1 ml of 34 mM NaCl and 0.7 percent HCl, pH 1.2. Activity of freshly prepared SGF solution was defined as the increase of OD of 0.001 per min at 280 nm and a pH of 1.2 at 37°C following trichloroacetic acid (TCA) precipitation. SGF solution was freshly prepared and used on the same day.

3.5.3.2 Digestibility of OT and BSA (reference protein)

OT solution (100 mg/L) in water and BSA (1 g/L water solution) as positive control were taken for analysis. SGF was incubated at 37°C prior to the addition of test protein solution in the ratio of 1:1 (v/v) (Mishra et al., 2010). The contents were mixed by mild vortexing and the tube was immediately placed in a 37°C water bath. The reaction was stopped at different time intervals (0.083, 0.25, 0.5, 1, 5, 15 and 60 minutes) by the addition of 50 μL of stopping solution (2x Tris-tricine SDS sample buffer (containing 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 100 mM Tris, pH 8.8) to 50 μL of the incubation mixture in the case of BSA while for OT only 50 μL of 100 mM Tris of pH 8.8 was used. SGF and protein were added directly to the stopping solution prior to the incubation for '0' min time point.

3.5.3.3 Analysis to study degradation of OT by SGF
OT digestibility was analyzed by reverse phase HPLC. Undigested OT was analyzed at different time point by calculating peak area and comparing with that of OT. However, BSA digestibility was analyzed by SDS PAGE. Boiled samples (20μL/well) were subjected to SDS-PAGE at 22 milliampere for 4:30 h. Standard molecular weight markers were run with the gels to calculate the molecular weight of the proteins. Coommasie brilliant blue (R-250) stain was used to stain the gels and images were captured using Syngene Bio Imaging System (Syngene, Cambridge, UK). The undigested bands were quantified using the Gene tools software (Syngene, Cambridge, UK). Each sample protein was digested at least 4 times and was subjected to SDS-PAGE. Densitometry of each protein band was performed thrice and average values were used for preparing graphs.

3.6 Surveillance for consumption pattern of milk

A limited household survey on milk consumption in 255 subjects from 150 families was conducted within 15 year of age. The subjects were subdivided into three age groups; 0.5-1 year (infant), 2-12 year (children) and 13-15 year (adults) as per Indian Council of Medical Research (ICMR) guidelines (ICMR, 2000).

3.7 Intake assessment of OT through milk

The intake of OT through milk was assessed through the Food Frequency Recall (FFR) method (Burdock, 1996) employing a Food Frequency Questionnaire (FFQ), which searched for information of respondent's name, age, gender and followed by the queries on the consumption and frequencies of consumption of specified milk. Quantities of milk consumed were recorded based on the standard stainless steel measuring vessels. On the basis of milk consumption and the values of OT in milk, the actual intake of OT was calculated.

3.8 Cell culture

3.8.1 IEC-6 Cell culture

IEC-6 cells (NCCS, Pune, India) were routinely cultured in 25-cm² flasks at 37°C under the influence of 5% CO2 and 95% humidified atmosphere in DMEM medium having 100 IU/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% FBS and 0.25% insulin. When a sub confluent state was reached, cells were detached from the flasks with trypsin/EDTA and cell
viability was checked. The cell viability of cells was performed by 1:1 (V/V) dilution of the cell suspension with 0.4% trypan blue solution. This mixture was loaded on to the chambers of a haemocytometer. After 1-2 minutes, number of stained cells and total number of cells were counted in haemocytometer. The numbers of unstained cells represent viable cells and calculated as percentage of total cells. The cells were plated in 12 well plates for each experiment.

3.8.2 Granulosa cell culture

Granulosa cells were harvested from estradiol primed intact immature (day 25) rats as described previously (Carlone et al. 1997). Briefly, cells were harvested by aspiration and cultured at a density of 5 x 10^4 cells per 0.5 ml of medium (1% FBS containing DMEM F-12 medium with penicillin and streptomycin) in each well of 48 well plates and kept overnight. After 24 h the medium was removed and attached cells were washed with serum free medium and kept overnight followed by addition of OT (10 nM), OTA (19 μM), pERK1/2 inhibitor (10 μM), PGE-2 (1 μM) and PGE-2 inhibitor (25 μM) in 0.5 ml of serum free DMEM F-12 medium as indicated in the figures legends. The concentrations of these inhibitors were similar to those uses by other investigators (Parent et al. 2007; Seto-Young et al. 2003; Markosyan et al. 2006). After 24 h of treatment cells were analyzed for proliferation and pERK1/2 expression by thymidine incorporation and western blot analysis respectively.

3.9 OT Internalization assay in IEC-6 cells

For the OT internalization assay, cells were seeded in 12 well plates at a density of 1X 10^5 cells/2ml. The IEC-6 cells after 24 h of plating were treated with 2 μci of [3H] OT for 15 min and 24 h, respectively. After completion of incubation, cells were gently scraped and harvested in glass fiber filters. These filters were transferred to scintillation cocktail W (Sisco Research Laboratories Pvt. Ltd. Mumbai, India) and counts were recorded on Hewlett-Packard β counter (Palo Alto, CA). To study the localization of internalized OT, cells were homogenized and cell fractionation was carried out by differential centrifugation (Das et al. 1982). The radioactivity was counted in sub cellular fractions in β counter as described above.

3.10 Measurement of [3H] thymidine incorporation in granulosa cells
To measure cell proliferative rate, $5 \times 10^4$ granulosa cells were seeded in each well of 48 well plates in 0.5 ml of complete medium in triplicate with OT, OTA, pERK1/2 inhibitor, PGE-2 and PGE-2 inhibitor as mentioned above in section 2-10. [$^3H$] thymidine (2 μCi) was added to the each well, 18 h prior to the completion of 24 h of incubation time (Mishra et al. 2010; Yadav et al. 2011). The cells were collected with the help of harvester and incorporated radioactivity was measured in a liquid scintillation counter (Packard Bioscience Company, Meriden, CT).

3.11 Animals and treatment protocol for oral administration

Healthy female Wistar rat pups (8-10 day old) along with their mother were obtained from the animal breeding colony of Indian Institute of Toxicology Research (IITR) Lucknow, India. The animals were acclimatized under standard laboratory conditions for 3 days prior to the experiment. Animals were housed in air-conditioned room in plastic cages and maintained at 22 ± 2 °C under standard laboratory conditions of light/dark cycle (12-12 h) and have free access to food and water ad libitum.

3.11.1 Experiment 1

Pups (15 ± 3 g) were randomly divided into four groups, of 15 each. Oral intubation of freshly prepared OT (0.1, 1 and 10 ng) was given daily for 25 days. For this treatment schedule pups were kept with respective dams for the first 7 days after which they were separated for the next 18 days treatment with OT. The doses of OT were given through soft plastic tube faced cannula to avoid damage in buccal cavity of pups. The animals of control group received daily oral intubation of vehicle. During the treatment schedule, the animals were observed for body weight twice weekly. After 25 days of treatment, rats were monitored daily for estrus stage through vaginal smear and sacrificed in the morning of estrus by cervical dislocation according to the guidelines for the care and use of laboratory animals of IITR. All the major organs including liver, lungs, kidney, heart, brain and ovaries from each animal were dissected out and weighed. Three animals from each group were sacrificed after 20 days of OT exposure (before puberty) and ovaries were dissected out to check the effects of oral OT on immature ovaries.

3.11.2 Experiment 2: Ovulation Induction

~35~
To examine in vivo follicle maturation status in OT treated female Wistar rat pups, a standard super ovulation regimen using a combination of PMSG and HCG protocol as described earlier (21, 22). In this protocol 25-day-old immature female rats were taken to study ovarian responses to exogenous gonadotropins to avoid the complexity of ovarian functions associated with estrous cycles and endogenous surges of gonadotropins. We utilized this protocol in our experiments by taking 80 immature female rat pups in each group and treated them with a daily oral intubation of 1ng or 10 ng of OT from d 10–25 of age. Eighty immature female rat pups receiving an oral intubation of saline vehicle from d 10–25 of age served as control. On d 25, all pups received i.p injection of 5 IU PMSG for 48 hrs followed by 5 IU HCG to stimulate pre-ovulatory follicular development, ovulation and luteinization. Ovaries and blood samples were collected at d 25, PMSG 48 and 0, 2, 4, 8, 24 and 48 hour after HCG injection from 10 animals at each time points.

3.12 Locomotive behavior study in rats

Neurobehavioral studies in rats were undertaken prior to the termination of experimental schedule, using the fully computerized Actimot Monitor (TSE, Tubingen, Germany) housed in a quiet, temperature-controlled (21 ± 2 °C) room. In brief, rats were placed individually in the measuring chamber and after 1min of acclimatization, different activity scores were recorded. Each animal was subjected to one activity session of 5 min. At the end of every session, the chamber was cleaned and wiped thoroughly with 70% ethanol to remove any odour and then used after 10 min. Locomotive markers such as distance travelled, resting time and movement time were monitored (Ali et al., 2000).

3.13 Specimen collection and estimation of serum markers

Blood was allowed to clot at room temperature for 5 min followed by keeping it on an ice bath for 30 min. Serum was separated by centrifugation at 3000g for 10 min and stored at -80°C until further analysis. Activities of alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST), creatine, creatine kinase and glucose were estimated in serum by using commercially available kits (Accurex Biomedical Pvt. Ltd., Mumbai, India) following the instructions given by the manufacturer.

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3.14 Serum protein levels and profile

Total protein and albumin (A) contents in serum was assayed by kit (Accurex Biomedical Pvt Ltd, Mumbai, India). Globulins (G) and A/G ratio were calculated from these values and expressed as g/dl. Serum profiling of serum was carried out by electrophoresis using a pre-formed SDS-PAGE gel from Sebia (Gwinnett, GA) and densitometric scanning was performed on Phoresis software from Sebia (Gwinnett, GA).

3.15 Histopathological processing

All the organs were washed in cold saline, soak dried on filter paper and weighed. A portion of organ was fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin for microscopic examination (Bogovski, 1978).

3.16 Prostaglandin E2 (PGE2) assay

The level of PGE2 in the ovaries and brain of animals of experiment 1 were determined by enzyme-linked immunoassay according to the instructions provided by the manufacturer (Cayman Chemical Co, Ann Arbor, Michigan). Total protein concentration of the tissue was determined with the BCA kit (Thermo Fisher Scientific, Rockford, IL). PGE2 values were expressed as pico-gram of PGE2 per milligram of tissue protein.

3.17 Brain γ-aminobutyric acid (GABA) and glutamate quantitation

Brain tissues from experiments 1 were taken for analysis of GABA and glutamate according to the procedure of Pearson et al (2). Briefly brain tissues were homogenized in glass homogenizer in 0.5 ml 0.1 M perchloric acid containing 100μM ascorbic acid. The homogenate was centrifuged for 3 minute at 12000 g. One part of supernatant was used for analysis of GABA while other part was diluted 1:10 (v/v) times with 0.1 M perchloric acid containing 100μM ascorbic acid for glutamate estimation. The supernatants (diluted and undiluted) were used for pre-column derivatization with o-phthaldialdehyde (OPA) and 2-mercaptoethanol reagent as described by Loscher et al., (1993) and GABA and glutamate levels were estimated on HPLC using florescent detector as described by Loscher et al. (1993). Reverse phase C18 bonded column (Symmetry) was utilized on HPLC conditions according to Loscher et al. (1993). The chromatograms were processed by water’s
Empower software. The peaks of GABA and glutamate were identified by comparing the retention time with that of standard and quantified by comparing the integrated peak area with that of reference standard.

3.18 Progesterone and Estradiol measurement

Animals from experiment 2 at d25, P48, H-24 and H-48 time period were anesthetized and blood was collected by cardiac puncture. Progesterone and estradiol levels were analyzed in serum by the micro plate EIA kit (Abbott Diagnostics, Abbott Park, IL) and read on system Abbott AxSYM System according to the protocol mentioned there in.

3.19 Immunofluorescence analysis of pERK and pAKT proteins

Paraffin sections (5 μm) of ovaries from animals of experiment 2 were dewaxed with xylene and dehydrated in graded alcohols. Endogenous peroxidase was blocked with 1.5% hydrogen peroxide diluted in methanol for 10 min. Sections were then washed with Tris-buffered saline (TBS). For improved antigen retrieval for p-AKT and p-ERK staining, tissues were heated at 70 °C in 0.01 M citrate buffer, pH 6, for 10 min. Sections were washed again in TBS and sequentially probed with primary antibodies of pAKT (1:100), and pERK (1:100) and secondary FITC-conjugated goat anti-mouse IgG antibodies (Sigma, St, Louis, MO) as previously described. Slides were mounted using DAPI/antifade solution from Invitrogen (Paisley, Scotland). Digital images were captured using Nikon Eclipse Ti-S System software NIS-Elements D (Melville, NY). For all the experimental slides, exposure time was kept constant for control and treated samples.

3.20 Quantification of ovarian follicles

Quantification of ovarian follicle in animals of experiment 2 was performed according to the procedure described earlier (Pedersen and Peters, ). Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. To count the numbers of follicles, paraffin-embedded ovaries were serially sectioned at 5-μm thickness and stained with hematoxylin for morphological observation. Ovarian follicles at different developmental stages, including primordial, type 3b, type 4, type 5, and type 6 were counted in all sections of an ovary, based on the well-accepted standards established by Pedersen and Peters (26).
3.21 RNA Isolation and RT-PCR

A semi-quantitative RT-PCR analysis of PR m-RNA in ovarian homogenates of experiment 2, GAD-1 & GAD-2 m-RNA in brain homogenates and COX-2 mRNA in brain MBH homogenates of experiment 2 were performed taking gene specific primers. Gene specific primers for RT-PCR were designed using Primer 3 software (http://seqtool.sdsc.edu/CGI/BW.cgi). Primer sequence for PR (PCR amplicon size: 616 bp): forward primer 5' GATCTTGTTAATCTGGG 3'; reverse primer & 5' ATGCTTGTACGACCTCCACC 3'; primer sequence for GAD-1 (PCR amplicon size: 230 bp) forward primer 5' AAAACAAAAGGCATGGCG 3'; reverse primer 5' CCTGGATCGGATCAAACG 3'; primer sequence for GAD-2 (PCR amplicon size: 220 bp) forward primer 5' CTGAGAAGGCACAGACAGGC 3'; reverse primer 5' CAGAAATGCAGACGTGGGC 3'; primer sequence for COX-2 (PCR amplicon size: 320 bp) forward primer 5' TGAGGAGAGATGTCATCCC 3'; reverse primer 5' GCACCAGACAAAGACTCC 3'; primer sequence for β-actin (PCR amplicon size: 120 bp) forward primer 5'-GCTACAGCTTCACCACA-3'; reverse primer 5'-TCTCCAGGGAGAAGAGGAT-3'. Total RNA was extracted from rat brain and ovaries at different time points (H-0, H-2 & H-4 h) using TRI reagents (Sigma Chemical Company, St. Louis, USA) according to the manufacturer's instructions. Total RNA was treated with RNase free DNase (Fermentas, Glen Burnie, Maryland, USA) and the integrity of RNA was determined by running on 1.2% agarose gel prior to further use for RT-PCR. The RT-PCR was carried out using One Step RT-PCR kit (Qiagen, Valencia, CA, Netherland) and 2 μg total RNA was taken as the template for each reaction. β-Actin gene was selected as an endogenous internal standard. Thermal cycler (G-Storm, Rayne, Brentree, Essex, United Kingdom) was programmed as follows; reverse transcription at 50 °C for 30 min followed by 35 cycles at 94 °C denaturation for 20 s, annealing (56 °C for PR, and 58 °C for GAD-1 & GAD-2) for 30 s and extension at 72 °C for 30 s, with final extension at 72 °C for 7 min. After completion of PCR cycles, 10 μl of PCR product was resolved on 2% agarose gel electrophoresis and stained with ethidium bromide. The density of each band was estimated by the Gene tools software (Syngene, Nuffield road, Cambridge, UK).
3.22 Cumulus oocyte complex (COC) expansion and germinal vesicles breakdown (GVBD)

The expansion status of the cumulus oocyte complex (COC) in animals of experiment 2 was checked by microscopic examination in histopathological slides of ovary (method reference). To assess cumulus expansion, images of COCs were captured under an inverted microscope and the projected cumulus areas of COCs were calculated using the Nikon Eclipse Ti-S System software NIS-Elements D (Melville, NY). Germinal vesicles (GV) status was checked by fluorescence microscopic examination of slides.

3.23 Western Blot Analysis

For western blot analysis, whole cell lysates were prepared from 6 pooled ovaries collected from control and treated groups using lysis buffer [10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1mM EDTA, 50 mM NaF, 1 mM DTT, 20 mM sodium pyrophosphate (Na2P2O7), 2 mM sodium orthovanadate (Na3VO4), 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor cocktail tablets]. Ovarian cell lysates were electrophoresed on 6% SDS-polyacrylamide gel for pEGFR and EGFR protein, while rest of the proteins like COX-2, pERK1/2, ERK1/2, GDF-9, HAS-2, TSG-6, and β-actin were electrophoresed on 10% SDS-polyacrylamide gels. Separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking the membranes with 1 % BSA in TBS-T [20 mmol/L Tris, 150 mmol/L NaCl (pH 7.5), 0.02% Tween 20] for 1 h at room temperature and further incubated with different antibodies as of EGFR (1:1000), pEGFR (1:500), ERK (1:1000), p-ERK1/2 (1:1000), AKT (1:1000), p-AKT (1:1000), p-P38 (1:1000), p-NFkB (1:1000), BCL-2 (1:1000), PARP (1:1000), COX-2 (1:1000), HAS-2 (1:500), TSG-6 (1:500), GDF-9 (1:500) and β-actin (1:1000). After probing with primary antibodies the membranes were incubated with horseradish peroxidase-linked anti-rabbit/mouse/goat antibodies (Cell Signaling Technologies, Danvers, MA), washed and the bound antibodies were visualized using the ECL Substrate (Thermo Fisher Scientific, Rockford, IL).
3.24 Statistical analyses

The data for RT-PCR assays, hormone levels and ovulation tests are represented as means ± SE.