CHAPTER II

MATERIALS AND METHODS
ANIMALS

All the experiments were performed in colony bred rats, derived from Wistar strain, raised in our animal facility. Adult, ovariectomized (OVX), OVX-estrogen, progesterone primed and immature female rats of age 21, 30, 40 days old, pubertal rats (42 days old) and postpubertal rats (43 days old) were used. They were fed standard rat pellet (Lipton India Ltd., Bangalore, India) ad libitum with free access to drinking water. Rats were housed in an air conditioned room (25 ± 2°C) and were maintained under controlled conditions of light (12 hrs light : 12 hrs dark).

SURGICAL PROCEDURES

a. Ovariectomy

Adult female rats weighing 180 - 200 g were bilaterally overiectomized under light ether anaesthesia using semi-sterile conditions. The animals were used 3 - 4 weeks after ovariectomy.

b. Implanation of third ventricular (IVT) cannula

Cannulae were prepared from 23 guage stainless steel tubing (Small Parts Inc. Miami, USA). Each cannula was 17 mm in length and had a flat tip with a beveled edge and was provided with a mandril to prevent its obstruction. Cannulation was performed following the procedure described by Antunes-Rodrigues and McCann (1970) and Vijayan and McCann (1978a & b). The animals were anaesthetized with nembutal (Abbot Labs, USA 40 mg/kg, ip) and the rat's head was fixed on a David-Kopf rat stereotaxic instrument. The following coordinates were used for the third ventricle...
anterior-posterior = 1.3 mm behind bregma, lateral = just on the midline (above the superior longitudinal sinus) and vertical = 0.3 mm above the base of the skull. The cannula was mounted in the stereotaxic instrument with the aid of a stainless steel wire fitting exactly its inside diameter which served as the cannula guide. Two small holes were drilled in the adjacent parietal and frontal bones. Brass microscrews were screwed firmly into the holes to serve as an anchor for the dental cement. The cannula was introduced through a hole over the superior longitudinal sinus after the sinus was pulled gently to the left side with a hypodermic needle. This procedure prevented rupture of the sinus and consequent hemorrhage. The cannula was lowered to the skull base and then raised 0.3 mm. Its location in the 3rd ventricle was confirmed when cerebrospinal fluid (CSF) flowed continuously from the cannula. A small amount of dental cement was placed around the cannula and screws. Finally, after removing the guider mounted in the stereotaxic instrument an additional amount of cement was added around the cannula to cover the screws completely. A small amount of neosporin ointment was mixed along with the dental cement to prevent bacterial infection. The cannula was now firmly fixed into position. The skin was left unsutured and the animals were returned to their own individual cages until the day of the experiment. The mandril was removed every other day rinsed in isotonic saline and returned to its original position so as to maintain the cannula clean and to get the animal acquainted with the experimental procedure to be followed (Mistry and Vijayan, 1987).

The intraventricular cannulated rats were primed with estradiol benzoate (50 µg SC) and progesterone (25 mg SC) 72 hours before intraventri-
Fig. 1. Diagram showing intraventricular injection cannula. A 28 gauge cannula was placed inside the chronically implanted 23 gauge guide tube at time of injection.
cular injection (Vijayan and McCann, 1978b).

c. **Intraventricular Injection**

Five to seven days after the cannulation, third ventricular injection of the peptide and or control medium was performed according to the following procedure. The mandril was removed and the inner cannula of the same length was introduced. The cannula was connected by a polyethylene tube to a 10 μl Hamilton microsyringe filled with freshly prepared test material to be injected. After 60 seconds with the inner cannula in its position in the animal's brain the test solution was injected slowly in a volume of 2 μl while the animal was freely moving around. The inner cannula was removed approximately 60 seconds later so that the entire test substance reached the third ventricle.

Rats were killed by instant decapitation at different time intervals following injection of test substance and/or control medium. Trunk blood was collected into heparinized tubes and plasma was separated by centrifugation at 4°C and stored frozen. The frozen plasma samples were later assayed for hormones by radioimmunoassay. Brains were rapidly removed, cerebral cortex, cerebellum and brain stem were quickly dissected out as per the procedure of Sadasivudu and Lajtha (1970). Hypothalami were dissected out as a single block which included the preoptic area and was limited laterally by the hypothalamic fissures and posteriorly by the mamillary bodies. The upper section of the block was cut 3-4 mm from the basal surface of the hypothalamus. Thus the hypothalamus included medial basal hypothalamus (MBH), preoptic area (POA) and median eminence (Vijayan, 1974).
CHEMICALS

Luteinizing hormone releasing hormone (LHRH), somatostatin (SRIF), Gamma-glutamyl para-nitroanilide, glycylglycine, 5-5'-dithiobis-2-nitrobenzoic acid (DTNB), glutamic acid, gamma aminobutyric acid (GABA), scphadex G-75, sodium azide, chloramine-T, heparin, bovine serum albumin were purchased from Sigma Chemical Company, St. Louis, USA. Kits for RIA of LH, FSH and Prl were obtained from the National Pituitary Agency, National Institute of Arthritis Metabolism and Digestive diseases (NIAMDD), Bethesda, USA. Ninhydrin was purchased from Fluka, Switzerland. All other chemical used were of analytical grade.

ESTIMATION OF GAMMA GLUTAMYL TRANSPEPTIDASE ACTIVITY

The activity of gamma glutamyl transpeptidase was estimated according to the method of Tate and Meister (1974) as described by Vali Pasha and Sadasivudu (1984). The assay mixture (2.0 ml) consisted of 80 micromoles of Tris-HCl buffer (pH 9.0), 150 micromoles of NaCl, 40 micromoles of glycylglycine, 5 micromoles of gamma-glutamyl para-nitroanilide and 0.4 ml 2% homogenate (in 0.25 M sucrose). After 30 min of incubation at 37°C the reaction was terminated by the addition of 2 ml of 10% acetic acid. A control was set up having all the above ingredients except that the homogenate was added after the addition of 10% acetic acid. After centrifugation at 5,000 rpm for 20 min the absorbance of clear supernatant was read at 410 nm. The enzyme activity was expressed as micromoles of paranitroaniline liberated per gram weight of tissue per hour with reference to a standard graph plotted using different concentrations of paranitroaniline and treating it with acetic acid.
ESTIMATION OF SULFHYDRIL GROUPS IN THE TISSUE

The total sulfhydryl groups and non-protein sulfhydryl groups were estimated according to the procedure of Sedlak and Lindsay (1968). A known weight of the tissue was homogenized in 5.5 ml of cold EDTA (0.02 M) using a potter-Elvehjem homogenizer with a teflon pestle.

ESTIMATION OF TOTAL SULFHYDRIL GROUPS

0.5 ml of the above homogenate was mixed with 1.5 ml of 0.2 M tris buffer (pH 8.2). The mixture was then treated with 0.1 ml 5-5'-dithiobis-2-nitrobenzoic acid (DTNB, 0.01 M). 7.9 ml of absolute methanol was then added to make up the volume to 10 ml and centrifuged for 15 minutes at 3,000 rpm. The absorbance in the supernatant was read at 412 nm against a reagent blank having 0.5 ml of EDTA (0.02 M) in place of homogenate and treated as in the case of homogenate described above.

DETERMINATION OF REDUCED GLUTATHIONE (Non-protein sulfhydryl groups)

5 ml of the above EDTA homogenate was mixed with 4 ml of distilled water followed by the addition of 1 ml of 50% TCA after shaking intermittently for 10 min using a vortex mixer. The solution was centrifuged at 3,000 rpm for 15 min. 2 ml of the supernatant was mixed with 4 ml of 0.4 M tris buffer (pH 8.9) followed by the addition of 0.1 ml DTNB (0.01 M). The absorbance was measured at 412 nm within 5 min against a blank containing 2 ml of EDTA (0.02 M) in place of TCA supernatant and mixed with 4 ml of 0.4 M tris buffer (pH 8.9) and 0.1 ml of DTNB (0.01 M). The
concentration of sulfhydryl compound was determined using the molar extinction coefficient of 13,100.

**ESTIMATION OF GLUTamate AND GABA**

Glutamate and GABA contents were estimated by paper chromatography as described by Chandrakala *et al.*, (1987). Immediately after decapitation, the tissues were separated and weighed. The tissue was homogenized in 0.5 ml of ice cold 80% ethyl alcohol and centrifuged at 8,000 rpm at 0°C for 20 min. The clear supernatant was evaporated to dryness at 70°C-80°C and the residue was dissolved in 200 μl of distilled water. The amino acid content was determined by paper chromatography using butanol : acetic acid : Water (65 : 15 : 25) as the solvent and multiple developments as given Sadasivudu and Lajtha (1970). Color was developed by ninhydrin (0.25% in acetone with pyridine). The amino acids were eluted in 3 ml of 75% alcohol (with 0.005% CuSO₄) and the color was measured at 515 nm. Amino acid content was expressed as μmoles of amino acid/gram wet weight of tissue.

**ASSAY OF HORMONES**

Plasma levels of LH, FSH and Prl were measured by radioimmunoassay (RIA) using a double antibody procedure as standardised in our laboratory (Babu, 1982). Radioimmunoassay kits for rat LH, FSH and Prl were obtained from the NIAMD-NIH pituitary hormone distribution programme. Radioimmunoassay was performed according to the guidelines provided with the kit for each hormone.
RIA OF LH

The LH kit consisted of

1. Rat luteinizing hormone antigen, highly purified for iodination. NIAMDD - rLH - 15.

2. Rat luteinizing hormone antiserum (rabbit) NIAMDD-antiserum (rabbit) NIAMDD-anti-r-LH-S-6.


PREPARATION OF THE GEL

Five grams of sephadex G-75, was added to 100 ml of phosphosaline buffer (PBS) (0.01 M PO₄, 0.05 M NaCl, 0.1% sodium azide, pH 7.6) and stirred for 30 min using a magnetic stirrer. It was (a) kept in a boiling water bath for .5 h (b) allowed to stand 72 h at room temperature (c) stored in a refrigerator upto 4 weeks (d) placed at room temperature for 2k h before use.

PREPARATION OF THE COLUMN

1. Ten ml of pipettes were used. The mouthpiece was cut off.

2. Tubes were scrupulously cleaned with chromic acid, hot water, tap water and double distilled water and dried.

3. A three way stopcock (Pharmaseal, Puerto Rico, USA) was attached to the tube by a 4 cm long latex tubing. Glass wool was placed in the tip of the tube.
4. The tube was washed twice with phosphosaline buffer and filled up to the 7 ml mark.

5. The gel was continuously stirred using a magnetic stirrer to keep the suspension homogenous.

6. The gel was pipetted from the bottom of the flask as a well mixed slurry. When settling was under way, the outlet was opened and allowed to run freely. The slurry was continuously added as needed. The top was never allowed to settle before adding more slurry.

The column was filled to a height of 15-20 cm. About 2 ml of buffer (PBS) was left at the top of the column. On the day of iodination (maximum 4 h before use) the column was equilibrated with 1 ml of 2% bovine serum albumin (BSA) in PBS and then washed with PBS. After a single use the column was discarded.

IODINATION OF RAT LH

Reagents

1. Iodine, carrier free, as sodium iodide with specific activity of 400 mCi/ml suitable for iodination of protein.

2. 0.5 M sodium phosphate buffer, pH 7.6.

3. Chloramine-T (5 mg/10 ml of 0.05 M PO₄, pH 7.6 buffer).

4. Sodium metabisulfite (Na₂S₂O₅) 25 mg/10 ml of 0.05 M PO₄, pH 7.6 buffer.

Chloramine-T and sodium metabisulfite were prepared freshly just prior to use.
1 mCi of I was added to a small disposable glass vial used as the reaction vessel. 25 µl of 0.5 M PO buffer pH 7.6 was added. 2 µg of NIAMDD-r-LH-1-5 in 20 µl of PO buffer was added next. 10 µl of chloramine T was then added. The vial was then agitated for 50 seconds after which 25 µl of sodium metabisulfite was added. The entire reaction mixture was applied to the sephadex G-75 column. The column was then eluted with phosphosaline (0.01 PO, 0.15 M NaCl buffer, pH 7.6). Fractions of 0.5 ml were collected in test tubes containing 50 µl of 2% BSA in PBS buffer. These fractions were counted in a Packard autogamma scintillation spectrometer. Two peaks of radioactivity were detected. The first peak began at tubes 3-4 and trailed off by tube 6. A second peak containing free I began at about tube 7. The iodinated rat LH was contained in the first peak (tubes 4-5). The fraction high on the trailing shoulder of this peak (tubes 4 and 5) contained the most immunoreactive and least damaged rat LH. This fraction was added to buffer in order to give 10,000 cpm per 100 µl, and stored at -20°C until use.

**DOUBLE ANTIBODY RIA PROCEDURE**

The following steps were performed in sequence for the assay of plasma LH.

1. 10 X 75 mm disposable test tubes were used.

2. Buffer (1% BSA in 0.01 M PO, 0.15 M NaCl, 0.1% sodium azide, pH 7.6) was added to each tube in sufficient quantity to produce a final volume of 0.7 ml.
3. (a) 25. μl plasma to be assayed was added or

(b) The reference preparation (NIAMDD-r-LH-RP-1) was dissolved in 1% BSA in phosphosaline and added in doses ranging from 1,000 ng to 1 ng per tube, in sufficient detail (1,000, 500, 250, 100, 50, 25, 10, 5, 2.5 and 1 ng) so that the entire curve can be constructed graphically.

4. Iodinated rat LH was added such that approximately 10,000 cpm were contained in 100 μl of 0.1% BSA-phosphosaline buffer.

5. 200 μl of the antiserum (NIAMDD-r-LH-S-6) in a final dilution of 1 : 40,000 in 3% normal rabbit serum (NRS) - 0.05 M EDTA-PBS was added (at these dilutions, the antiserum was observed to bind 25% of the labelled rat LH (B X 100, see below).

6. In some tubes 200 μl buffer and 200 μl 3% NRS-EDTA-PBS and 100 μl label were added to serve as background.

7. In a few tubes 200 μl buffer and 100 μl label and 200 μl antiserum were added to serve as zero (100% binding Z).

8. In 2 or 3 tubes 100 μl label was taken to get the total counts (TC).

9. Tubes were agitated on vortex mixer.

10. Tubes were incubated for 24 h at room temperature.

11. At the end of this period, 200 μl of goat anti rabbit gamma globulin (ARGG) was added to precipitate maximally the antibody bound labelled rat LH.
12. Tubes were agitated on a vortex mixer.

13. Tubes were again incubated for 24 h at room temperature.

14. At the end of this incubation period all tubes were centrifuged at 1,000 g for 20 min in a refrigerated centrifuge. The supernatant was discarded and the precipitate was counted in a gamma spectrometer.

15. The unknown samples were compared to the percentage of counts precipitated with the rat LH reference preparation, NIAMDD-r-LH-RP-1. A curve was constructed on semilogarithmic paper, and the unknown was read directly from the curve obtained with LH-RP-1. Results are expressed as nanograms (ng) of rat LH-S-1 per ml of plasma.

**RIA OF FSH**

The following were provided with the FSH kit.

1. Rat FSH antigen NIAMDD-r-FSH-1-5, highly purified for iodination.

2. Rat FSH antiserum (Rabbit) NIAMDD-Anti-r-FSH-S-1 I.


**IODINATION OF RAT FSH**

Iodination was performed as for LH except that 10 mg/ml of chloramine T was used. Double antibody RIA procedures: Procedures was same as LH except reference preparation (FSH-RP-1) was dissolved in 1%
BSA phosphosaline in doses ranging from 2,000 ng to 10 ng (2,000, 1,000, 500, 250, 100, 50, 25, 10 ng). FSH antiserum was used at a dilution of 1 : 2,500.

**RIA OF Prl**

The RIA kit for Prl consisted of:

1. Rat prolactin antigen NIAMDD-r-Prl |I-1-5, highly purified for iodi-

2. Rat prolactin antiserum (rabbit) NIAMDD-anti-r-Prl-S-8.

3. Rat Prl reference preparation NIAMDD-r-Prl-RP-2. (Biological

**IODINATION**

As for LH.

**DOUBLE ANTIBODY PROCEDURE**

As per LH and FSH except the reference preparation was diluted in PBS in a range of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 ng and antiserum was diluted so as to get a final dilution of 1 : 12,500.

As far as possible samples from a particular experiment were run in one assay, each in duplicate, to avoid interassay variation. In our laboratory the sensitivities of the assay were 5 ng LH, 10 ng FSH and 0.25 ng Prl. The inter and interassay co-efficients of variation were 10 and 6% for LH, 9 and 5% for FSH and 10.4 and 5.5% for Prl respectively.
Samples for GH were assayed at the Department of Physiology, University of Texas Health Science Centre at Dallas, Dallas, Texas.

**STATISTICAL EVALUATION**

Statistical evaluation of the data was done using the Student's *t* test.