CHAPTER 2

MATERIALS AND METHODS

A total of 37 virgin female rats were used in this project. These non-pregnant rats were bred at the Animal House, Flinders Medical Centre. Non-pregnant rats for titration studies and for determining the optimum protocol for processing into resin were sacrificed without reference to their stage in the estrous cycle. Non-pregnant rats in which uterine innervation was mapped were sacrificed in estrous or late estrous/early diestrous.

Timed-pregnant rats were mated at Adelaide University or Flinders Medical Centre. Four rats at day 20 of a 21 to 22 day pregnancy were used in this project.

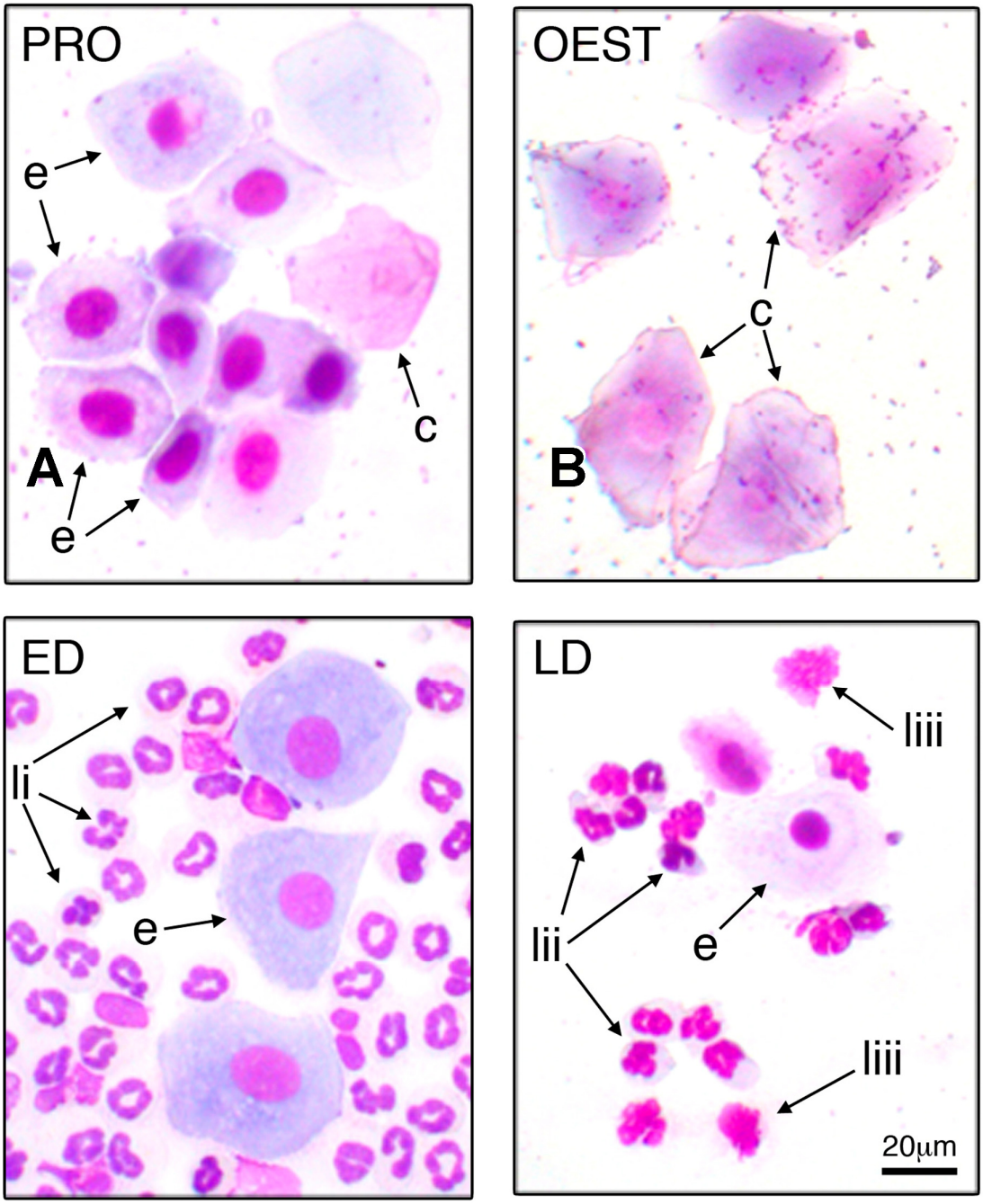
Experimental protocols were approved by the Animal Welfare Committee of Flinders University.

Vaginal Smearing

The stages of the estrous cycle in non-pregnant rats were assessed by vaginal smearing. A few drops of sterile water (Astra Zeneca Pty Ltd, Australia) were flushed into and out of the vagina of the rat 2-3 times to dislodge and suspend vaginal cells. The water with suspended cells was placed on a glass slide and allowed to dry. The slide was then fixed and stained using Diff Quik Stain (Lab Aids Pty Ltd, Australia), which consists of a fixative solution, stain 1 (red) and stain 2 (blue). The slides were dipped for 15 seconds each in fixative, stain 1 and stain 2 and allowed to dry. After this procedure, the cytoplasm of the vaginal cells was stained blue/violet; and the nuclei were stained reddish pink. Over 80% of non-nucleated cells indicated that rats were in the estrous stage of the estrous cycle

FIGURE 2.1. Vaginal smear cytology at different stages of the estrous cycle.

PRO = proestrous, OEST = Oestrous, ED = Early diestrous, LD = Late diestrous. Figure kindly provided by Dr Thelma Lovick, Department of Physiology, University of Birmingham.


PRO
e
c
A
e
OEST
c
B
ED
li
e
LD
liii
lii
e
liii
20µm

(Figure 2.1). The rats were staged for at least two complete estrous cycles before being sacrificed as close as possible to the estrous stage.

Perfusion

The rats were injected with pentobarbitone sodium (60 mg/100 g; Virbac Australia Pty. Limited). After anesthesia had been achieved, a mid-line incision was made to expose the abdominal organs. The intestines and kidneys were clamped off to discontinue blood supply. The sternum was clamped and the diaphragm was snipped to expose the heart. A needle was then inserted into the aorta through the left ventricle and heparin (1000 IU in 1 ml; Mayne Pharma Pty Ltd, Australia) was injected to prevent clotting of blood. The left atrium was snipped and 500 ml of phosphate buffered saline (PBS; Appendix 1) was flushed through the vasculature to remove blood. The uteri were harvested into single strength calcium-free Kreb's solution (Appendix 1).

Tissue Preparation

Non pregnant Uteri

The uteri were bubbled in calcium-free Kreb's solution (Appendix 1) for 30 to 60 minutes to relax the smooth muscle. The uteri were slit along the mesentric or anti-mesentric border, stretched out and pinned flat in a Petri dish coated with Sylgard (Dow Corning Corporation, USA). The tissue was fixed in 4% formaldehyde in 0.1M phosphate buffer, pH 7.4, (Appendix 1) for 3 to 4 days at room temperature.

The fixed uterine horns were rinsed three or four times with 0.1M phosphate buffer (Appendix 1) and then weighed and scanned in order to measure areas, lengths and widths. The uteri were placed on a gauze pad on the scale to absorb as much phosphate buffer as

possible. The uteri were removed and the scale was tared. The uteri were returned to the scale and weighed. Uteri placed flat in a Petri dish, covered with dark piece of cardboard and scanned with a CanonScan LiDE 25 scanner and saved as .tif files.

The areas, lengths and widths of the non-pregnant uterine horns were measured from the files of the scans. Each uterine horn was carefully traced in Scion Image and the option for area measurement was selected on the software. The lengths and widths of each uterine horn were measured in Adobe Photoshop 5.0. After measurement, the uteri were stored in 0.1M phosphate buffer containing 0.05% sodium azide (Appendix 1) until they were stained for immunohistochemistry.

Pregnant Uteri

The pregnant uteri were also bubbled in calcium-free Kreb's solution for 30 to 60 minutes. Each pregnant uterus was cut approximately in half, slit along the mesentric border, stretched to 3cm in width and pinned flat in a Petri dish coated with Sylgard. Two pups randomly selected from the left uterine horn and two from the right uterine horn were weighed and the total number of pups in each horn was noted. The ovary remained connected to the uterus to mark its ovarian end and the cervical end was marked with a piece of thread. The tissue was fixed in 4% formaldehyde in 0.1M phosphate buffer, pH 7.4, for 3 to 4 days at room temperature. At the end of the fixation period, uteri were rinsed three or four times with 0.1M phosphate buffer, weighed and scanned in the same way as the non-pregnant uteri. Each pregnant uterus was then divided into ovarian, middle and cervical regions and each region was cut into horizontal strips about 1.5 cm wide. The strips were then stored in 0.1M phosphate buffer containing 0.05% sodium azide (Appendix 1) until they were stained for immunohistochemistry.

Immunohistochemistry

Antibody titrations

Details of the primary and biotinylated secondary antibodies used in this project are provided in Tables 1 and 2, respectively.

All primary antibodies were titrated in order to determine the optimal dilution for use in whole mounts of uterine tissue. Pieces of non-pregnant uterine whole mounts about 1 cm × 1 cm that had been separated into muscle and mucosal layers were used for all titrations. In all subsequent experiments, the dilution showing minimal non-specific staining and the maximum number and best-stained immunoreactive axons was used (Table 3).

Absorption controls have been carried out on the anti-NPY (Llewellyn-Smith et al., 1990), anti-SP (Llewellyn-Smith & Minson, 1992) and anti-NOS (Llewellyn-Smith & Hinrichs, 2009). The anti-TH labels neurons with the expected distribution in the medulla and the anti-VAChT labels cholinergic neurons in the expected locations in the brain and spinal cord.

Immunohistochemical Protocol

Peroxidase immunohistochemistry (Figure 2.2) was used to reveal nerve fibers in the full-thickness whole mount preparations of intact uterine horns.

Whole mounts were incubated for 30 minutes in distilled water containing 30% methanol and 1% hydrogen peroxide solution (Appendix 1) to block endogenous peroxidase activity, followed by 3 washes of 20 minutes each in Immunobuffer (IB, Appendix 1) to remove cell membranes and 30 minutes in 10% normal horse serum in immunobuffer (NHS-IB, Appendix I) to prevent non-specific antibody binding. The whole mounts were incubated

Table 1. Primary Antibodies Used

Antigen	Immunogen	Manufacturer, Species antibody was raised in, Mono- vs. polyclonal, Catalogue & Lot number	Dilution used
Tyrosine Hydroxylase (TH)	Tyrosine hydroxylase from rat pheochromocytoma (denatured with sodium dodecyl sulfate)	Chemicon International , rabbit, polyclonal, Catalogue # AB152, Lot # LV1375881, LV1382810	1:2,000
Neuropeptide Y (NPY)	Synthetic Neuropeptide Y	Kind Gift of Dr Bevan Jarrott, Monash University	1:10,000
Substance P (SP)	Synthetic Substance P	Kind gift of Dr Roger Murphy, Ludwig Institute	1:10,000
Calcitonin gene related peptide (CGRP)	Synthetic rat Tyr-CGRP (23-37)	Biogenesis (UK), goat, polyclonal, Catalogue # 1720-9007, Lot # 22110851	1:5,000
Neuronal nitric oxide synthase (NOS)	C-terminal synthetic peptide sequence corresponding to amino acids 1419-1433 of human nNOS couple to keyhole limpet haemocyanin	Diasorin (USA), rabbit, polyclonal, Catalogue # 24287, Lot # 043005	1:20,000
Vesicular acetylcholine transporter (VAChT)	Synthetic peptide (CSPPGPFDGCEDDYNYYSRS, corresponding to amino acids 511-530 predicted from the cloned rat VAChT)	Chemicon International, goat, polyclonal, Catalogue # AB1578, Lot # 196157	1:7,500

Table 2. Biotinylated Secondary Antibodies Used

Immunogen	**Manufacturer, Species antibody was raised in, Mono- vs. polyclonal, Catalogue & Lot number**	**Dilution used**
Rabbit Immunoglobulin G	Jackson ImmunoResearch Laboratories Inc.	1:500
Goat Immunoglobulin G	Jackson ImmunoResearch Laboratories Inc.	1:500

Table 3. Antibody Titrations

Antibody	**Dilutions tested**	**Dilution used**
rabbit anti-TH	1:1000, 1:2000, 1:5000, 1:10,000	1:2,000
rabbit anti-NPY	1:1000, 1:2000, 1:5000, 1:10,000	1:10,000
rabbit anti-SP	1:2000, 1:5000, 1:10,000, 1:20,000	1:10,000
goat anti-CGRP	1:2000, 1:5000, 1:10,000, 1:20,000	1:5,000
rabbit anti-NOS	1:5,000, 1:10,000, 1:20,000, 1:50,000	1:20,000
goat anti-VAChT	1:5,000, 1:10,000, 1:20,000, 1:50,000	1:7,500

for 3 days each in primary antibody (Table 1) diluted in 10% NHS-IB (Appendix 1), 1:500 biotinylated secondary antibody (Table 2) in 1% NHS-IB (Appendix 1) and 1:1500 ExtrAvidin-horseradish peroxidase (Catalogue No: E2886, Sigma Aldrich Inc., 3050 Spruce Street, St. Louis, MO 63103 USA). Between each incubation, the whole mounts were given 3 × 30 min washes in Tris-phosphate buffered saline (TPBS; Appendix 1). Because of the large size of the whole mounts, large containers (Figure 2.3) and big volumes of immunoreagents were used for staining. Twelve ml of each immunoreagents was used when intact non-pregnant uteri were stained in glass crystallizing dishes (Sigma Aldrich, USA) or 20 ml when plastic Petri dishes were used. The 1.5 cm wide strips of pregnant uteri were stained in 70 ml plastic specimen containers with 8 ml of each immunoreagent.

Immunoreactive structures in the whole mounts were then visualized using a nickel-intensified diaminobenzidine (DAB) reaction with peroxide being generated with glucose oxidase (Llewellyn-Smith et al., 2005). Whole mounts were agitated on a shaker for 10 mins in freshly prepared pre-incubation solution (Appendix 1). The reaction mix (Appendix 1) was then added to the pre-incubation solution. The volume of pre-incubation solution varied with the type of container used for the reaction. In crystallizing dishes 11ml of pre-incubation solution, in 70 ml specimen containers 7 ml of pre-incubation solution and in Petri dishes, 19 ml of pre-incubation solution were used. One ml of reaction mix was added to the pre-incubation solution in all cases. The quantity of glucose oxidase in the reaction mix was adjusted so that the final concentration of glucose oxidase in each container was 1µl/ml. The whole mounts from non-pregnant rats were reacted for 7 minutes and the whole mounts from pregnant rats were reacted for 8 minutes. These reaction times were found to be the most favourable for good staining of immunoreactive axons with minimal background staining. The reaction was stopped by quickly washing

FIGURE 2.2 Avidin-biotin-peroxidase Immunohistochemistry

Diagram detailing the steps involved in immunohistochemical detection of antigens with peroxidase-based detection systems. Step 1: Incubation in primary antibody; Step 2: Incubation in biotinylated secondary antibody; Step 3: Incubation in ExtrAvidin Peroxidase; Step 4: Nickel intensified peroxidase reaction.

Peroxidase Immunohistochemistry

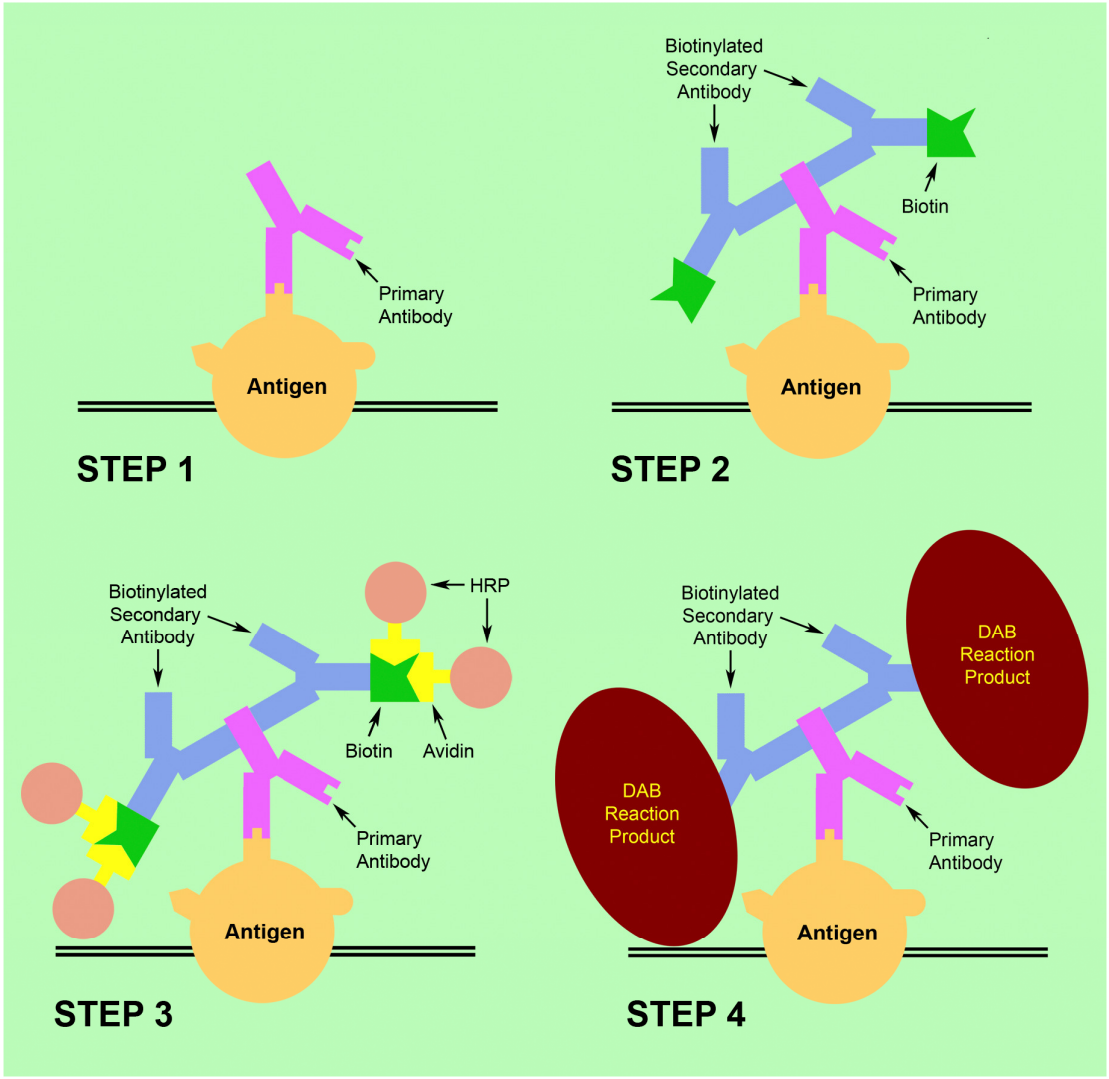


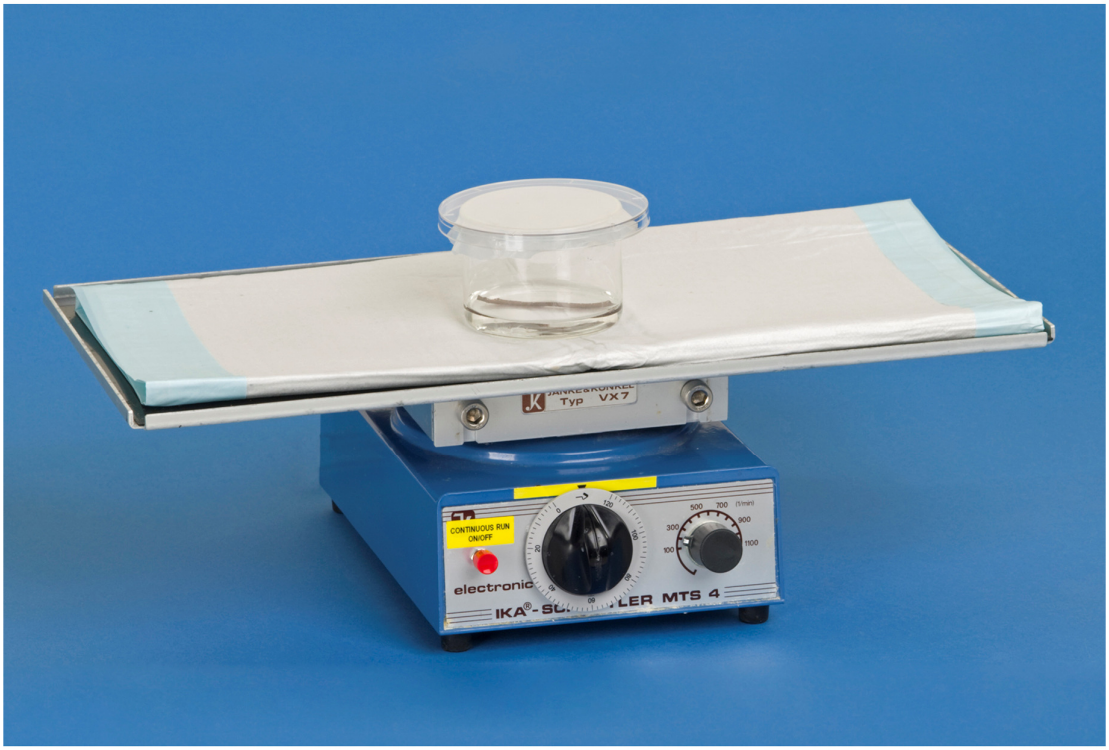
FIGURE 2.3. Containers for Immunostaining of Uterine Whole Mounts

Petri dishes, 70 ml specimen containers and crystallizing dishes were used to stain uterine whole mounts.


SPECIMEN CONTAINER
TIME
No.
TECHNO-PLAS
PYREX®
Made in Germany
70 X 50
No. 3140

FIGURE 2.4. Uterine whole mounts undergoing peroxidase immunohistochemistry

Entire non-pregnant uterine horns were stained in a crystallizing dish covered with Parafilm and a Petri dish lid on a shaker.


Typ VX7
CONTINUOUS RUN
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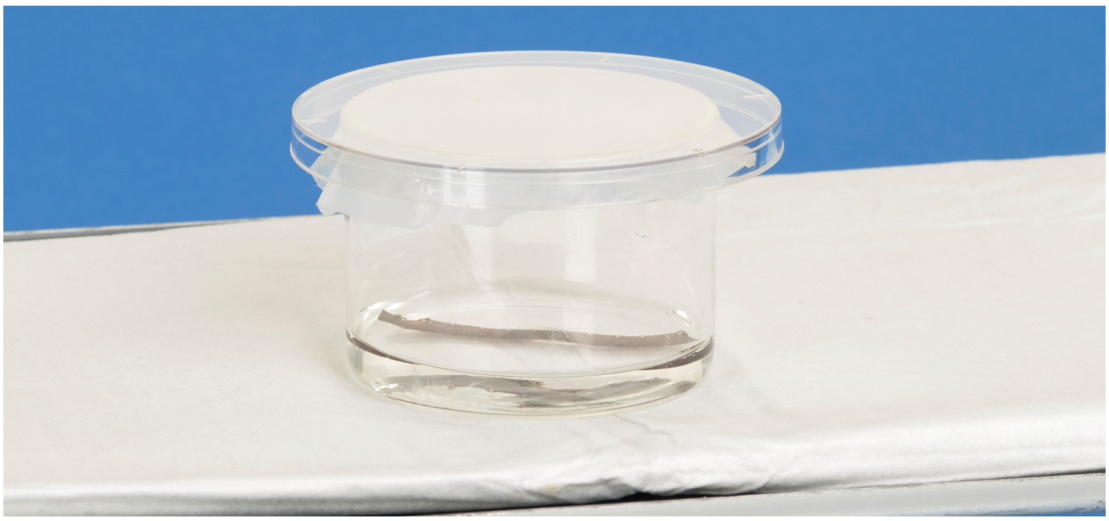


twice with TPBS followed by another wash in TPBS for 5 minutes. After the reaction, the whole mounts were washed 3 x 10 min in 0.1M phosphate buffer, pH 7.4, and stored in this buffer at 4^{o}C until processed into resin. Immediately before processing into resin, the whole mounts were washed twice for 15 minutes in distilled water to remove salts.

Processing uterine whole mounts into resin

Optimizing the protocol

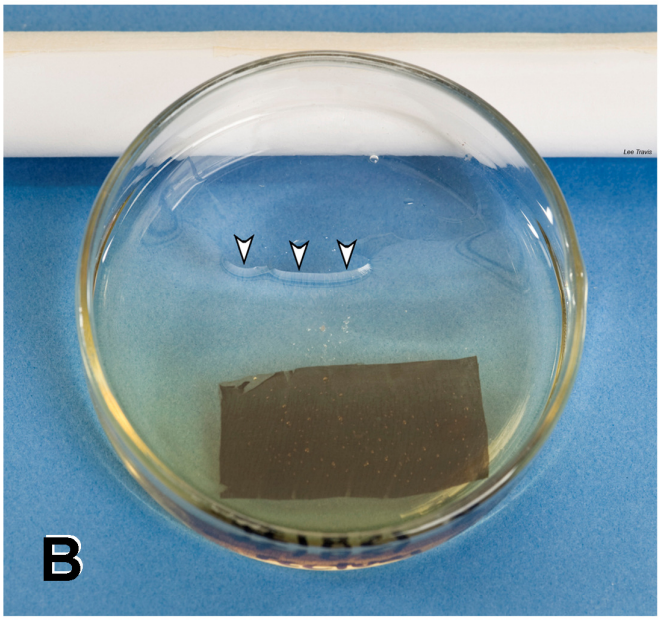
Whole mount preparations were initially processed into resin using a slight modification of the protocol that is used to dehydrate and infiltrate immunostained Vibratome sections for electron microscopy in the Llewellyn-Smith laboratory. The whole mounts are washed flat for 15 minutes and floating for 15 minutes in graded acetone solutions (Chem Supply, Australia; 70%, 90% and 95% in distilled water and undiluted 100%) in Petri dishes. 'Flat' indicates that the whole mounts were sandwiched between the lid and bottom of the Petri dish. 'Floating' indicates that the whole mounts were in the Petri dish without a lid so that they could float in the solution. After acetone treatment, the whole mounts were exposed to 2 x 5 minute washes in propylene oxide (Sigma Aldrich, USA) and then infiltrated and embedded in Durcupan resin (Sigma Aldrich, USA). The whole mounts curled after this procedure, indicating that they were not adequately dehydrated because they were so thick.

Several variations of this method were then tested to determine whether it was possible to produce whole mounts that were flat after dehydration. In these optimization experiments, the dehydration schedule was two incubations, each of 15 min duration, in 30%, 50%, 70%, 90%, 95% and 100% acetone solutions, followed by 2 x 10 min washes in propylene oxide, infiltration and embedding. Placing weights in the lid of the Petri dish during acetone treatment was not successful because the whole mounts tended to dry out.

FIGURE 2.5 Dehydration and Embedding of full-thickness uterine whole mounts

A, Weighted wire meshes were designed for use in crystallizing dishes in order to keep whole mounts flat during dehydration. **B**, Uterine whole mount in resin on a Petri dish. The arrows indicate the meniscus of the resin. **C**, Uterine whole mount embedded on a glass slide under an Aclar coverslip. The edges of the Aclar coverslip are highlighted by dotted lines.


A


B


C

Wire meshes that fitted within the lids of the Petri dishes allowed the whole mounts to be in contact with the acetone solutions but were too light to keep the whole mounts flat. This problem was solved by placing small weights on the wire meshes. Finally, a design for a weighted wire mesh (Figure 2.5) was developed in conjunction with the Department of reaction times were found to be the most favourable for good staining of immunoreactive axons with minimal background staining. The reaction was stopped by quickly washing twice with TPBS followed by another wash in TPBS for 5 minutes. After the reaction, the whole mounts were washed 3 x 10 min in 0.1M phosphate buffer, pH 7.4, and stored in this buffer at 4^{o}C until processed further.

Most whole mounts were washed twice for 15 minutes in distilled water to remove salts before processing into resin (see below). However, small pieces of TH-immunostained uterine whole mount from 2 rats were paraffin embedded to precisely define the uterine layers in which sympathetic axons occurred. The paraffin-embedded tissue was serially sectioned either transversely or sagittally at 10 micrometers on a rotary microtome and alternate sections were stained with haematoxylin and eosin. This work was carried out by Michelle Norman, Department of Anatomy and Histology, School of Medicine Flinders University.

The optimum protocol

Once the optimum system for dehydration had been determined, the whole mounts were dehydrated through acetone under weighted wire mesh. Crystallizing dishes (Sigma Aldrich, USA) were used for incubations in graded acetone solutions since they accommodated the size of intact uterine horns. Dehydration in propylene oxide and infiltration with a 1:1 mixture of propylene oxide and Durcupan resin were done in 70 ml polypropylene specimen containers. The infiltrated whole mounts were then transferred to

Table 4. Schedule for Processing Immunostained Uterine Whole Mounts into Resin

Solution	Incubation Time	Incubation conditions
Distilled water	2×15 minutes	In crystallizing dish
30% acetone	2×15 minutes	Under weighted wine mesh
50% acetone	2×15 minutes	Under weighted wine mesh
70% acetone	2×15 minutes	Under weighted wine mesh
90% acetone	2×15 minutes	Under weighted wine mesh
95% acetone	2×15 minutes	Under weighted wine mesh
100% acetone	2×15 minutes	Under weighted wine mesh
100% acetone	2×15 minutes	In 70 ml specimen container
100% propylene oxide	2×10 minutes	In 70 ml specimen container
1:1 propylene oxide : resin	At least 30 min	In 70 ml specimen container
Durcupan resin	10 min 60^{o}C	In glass Petri dishes
Durcupan resin	Overnight	In glass Petri dishes

All steps were done at room temperature unless otherwise indicated.

glass Petri dishes for infiltration with undiluted Durcupan resin (Figure 2.6). The whole mounts were heated in the resin for 10 minutes at 60^{o}C to speed infiltration and then left overnight at room temperature. The schedule for processing the immunostained whole mounts into resin is shown in Table 4. After resin infiltration, the whole mounts were carefully embedded on 5 cm x 7.5 cm glass slides under Aclar coverslips (Figure 2.6) and finally polymerised for 24 to 48 hours at 60^{o}C.

Data Analysis

Means and standard deviations for lengths widths, areas and weights of uterine horns were calculated using Microsoft Excel. T tests were performed to compare the areas and weights between non-pregnant and pregnant uteri and to compare the areas and weights between right and left uterine horns from non-pregnant rats.

Innervation patterns in whole mounts were assessed using Olympus BH-2 microscope. Photographs were taken using a SPOT RT camera and SPOT software version 4.6. The photographs were sized, sharpened and cropped using Adobe Photoshop version 8, which was also used to construct plates.