Chapter 2

Materials and Methods

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2.1 General methods

2.1.1 Animals

All experiments were conducted using adult male Sprague Dawley rats (250-500g). Animals were sourced from the Gore Hill Research Laboratories (Sydney, NSW). Animals were housed in small groups (up to 8 rats per cage), in temperature controlled conditions with a fixed 12hr light/dark cycle. Food pellets and water were available *ad libitum*.

2.1.2 Ethics

All experimental protocols were approved by the Animal Care and Ethics Committee of the Royal North Shore Hospital. All procedures were conducted in accordance with the requirements of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.1.3 Anaesthesia

Anaesthetics used were Halothane (Zeneca), pentobarbitone sodium (Rhone Merieux), or ethyl carbamate (Urethane, Sigma). At induction, atropine sulphate (60μg, i.p.) was given to reduce airway secretions. Temperature was maintained at 36-37˚C via a combination of homeothermic blanket control (Harvard apparatus) and infrared heating lamp. Regular doses of 0.9% saline were given to maintain adequate hydration (approximately 0.5ml / hr).

2.1.3.1 Halothane

Anaesthesia was induced initially in all animals by placing the animal in a sealed box and exposing the animal to a mixture of Halothane (0.5-2%) in 100% oxygen via a Fluotec 3 (Cyprane Ltd, UK) vaporiser for 1-2 minutes. Depth of anaesthesia was determined by noting withdrawal following paw pinch. Once adequate anaesthesia was obtained, the animal was removed from the sealed chamber and other anaesthetic medications, such as sodium pentobarbitone, or Urethane, were administered via intra-peritoneal injection.

2.1.3.2 Pentobarbitone

In experiments where animal recovery was necessary, an initial dose of sodium pentobarbitone (60mg/ kg) was given intraperitoneally (i.p). If anaesthesia was inadequate, or additional doses were required, 6-12mg / kg sodium pentobarbital was administered i.p. as required.

In experiments where animal recovery was not necessary, the initial dose of sodium pentobarbital was given as above. Additional doses of 3-6mg sodium pentobarbital were given i.v. when required. In these experiments, depth of anaesthesia was determined by the stability of blood pressure, absence of paw withdrawal following toe pinch and the characteristics of the phrenic nerve discharge.

2.1.3.3 Urethane (ethyl carbamate)

Urethane (10% solution in 0.9% saline, Sigma) was injected intraperitoneally (1.3g/kg) in non-recovery experiments. Occasionally additional doses of anaesthetic were

required, in which case Urethane (0.2g/kg i.v) was given. Adequacy of anaesthesia was determined as described in section 2.1.3.2.

2.1.3.4 Ventilation

In non-recovery experiments, the trachea was cannulated so as to permit mechanical ventilation. The animals were paralysed with the muscle relaxant pancuronium dibromide (0.8mg i.v. initially, then 0.2mg /hr i.v., Astra). The tracheal cannula was connected to a Harvard rodent ventilator and the animals were artificially ventilated with O_2 enriched air with a tidal volume of approximately 3.5ml and a ventilation rate approximately 80 /min. End tidal CO_2 was maintained between 4.5 - 5% by varying the ventilator frequency.

2.2 General surgical procedures

Different experiments required different surgical procedures. However, many procedures were common to several experiments. These procedures are described below.

2.2.1 Arterial and venous cannulation

The right femoral vein and artery were cannulated using PVC tubing (0.96mm OD x 0.58mm ID, Critchley Electrical Products). In some animals the right carotid artery and jugular vein were used following tracheal cannulation (see below). The arterial

catheter contained heparinized saline (approx. 3 IU/ml) and the venous catheter 0.9% saline. The arterial catheter was connected to a pressure transducer (Sorenson Transpac, Abbot Laboratories). The signal was amplified, digitised (CED 1401 analogue to digital converter, CED, UK) and sent to the computer for display using Spike 2 software (version 4.1, Cambridge, UK.). Periodic checking of the accuracy of ABP recording was performed with a manual sphygmomanometer.

2.2.2 Cannulation of the trachea

A midline incision was made in the ventral surface of the neck. Limited diathermy was used to clear the subcutaneous fat. The strap muscles of the neck overlying the trachea were parted and a small incision was made in the trachea with the diathermy. A plastic cannula (14 gauge i.v. catheter, Johnson & Johnson) was cut to approximately 1.5cm length and inserted into the trachea. This was tied in place with silk thread. Following cutting of the right vagus the neck wound was sutured.

2.2.3 Nerve dissection

2.2.3.1 Right vagus nerve

Following cannulation of the trachea, the right vagus nerve was dissected clear of the carotid sheath and cut. The neck wound was then sutured (see above).

2.2.3.2 Left phrenic nerve

A longitudinal postero-lateral incision was made at the cervico-thoracic junction. Following blunt dissection through the subcutaneous fat and diathermy through the

parascapular muscles, the scapula was retracted laterally and the phrenic nerve was located posterior to the carotid sheath at the root of the neck. The phrenic nerve was dissected free, cut and tied distally. Cotton wool soaked in saline was used to keep the nerves moist until the rat was placed on the stereotaxic frame.

2.2.3.3 Left aortic depressor and vagus nerves

Following dissection of the phrenic nerve the aortic depressor nerve was dissected free from the carotid sheath. Prior to cutting and tying the nerve, a handheld bipolar silver wire electrode was used to record nerve activity to ensure a rhythmic discharge pattern in time with the cardiac cycle. Cotton wool soaked in saline was used to keep the nerve moist. The left vagus nerve was also dissected from the carotid sheath. This was cut following commencement of mechanical ventilation to avoid respiratory failure.

2.2.3.4 Splanchnic sympathetic nerve

The splanchnic nerve was isolated following a longitudinal para-lumbar incision and supero-medially directed blunt dissection in the retroperitoneal plane following retraction of the paraspinal muscles and diathermy through the oblique and transverse muscle layers. The splanchnic sympathetic nerve was cut and tied distally and kept moist with saline soaked cotton wool.

2.2.3.5 Tibial nerve

An incision was made in the right leg and the tibial nerve dissected free but not cut. The nerve was kept moist with saline soaked cotton wool and the skin edges held together with a small metal clip.

2.2.4 Retrograde tracer injection into spinal cord

After securing the rat in a stereotaxic frame and flexing the head to keep the surface of the dorsal medulla horizontal, a clamp was placed on the proximal tail and light traction applied. Local anaesthetic (1% lignocaine gel) was applied to the shaved skin surface at the T2 level in the midline. A midline incision was made at the T2 level. The midline connective tissue and muscles were removed, along with the spinous process and lamina of the T2 vertebra. The dura was incised and the spinal cord kept moist with cotton wool soaked with 0.9% saline. A small incision was made over the lower lumbar spine and a clamp applied to the lower lumbar spinous processes to maintain the spine in good alignment.

Using a single barrel glass micropipette, 100nl of cholera toxin B subunit (1% CTB; Sapphire Bioscience) was microinjected bilaterally into the IML (co-ordinates 0.4mm lateral from midline and 0.7 ventral to dorsal surface). Following microinjections, a piece of Gelfoam was placed over the laminectomy defect and the wound closed with 4/0 silk sutures.

The animals were allowed to recover and perfused 48hrs later (see section 2.4.1).

2.2.4.1 Exposure of the dorsal medulla

An occipital incision, followed by removal of the occipital musculature and connective tissue, was performed. The atlanto-occipital membrane was removed, followed by removal of the inferior occipital bone with bone rongeurs. Any bleeding from the exposed bony surfaces was sealed with bone wax. Using the bevelled edge of a 25 gauge needle the dura was incised in a cruciform pattern and the edges retracted, to expose the dorsal surface of the medulla at the level of the obex and 4^{th} ventricle.

2.2.4.2 Exposure of cerebrum for rCBF studies

The connective tissue and musculature overlying the parietal lobe were removed using diathermy. A dental drill was used to make a small burr-hole over the left parietal lobe. The bone surface was irrigated with 0.9% normal saline to avoid bone heating during drilling. The dura was incised by the bevelled end of a 25-gauge needle to expose the pial surface of the left parietal lobe. The burr hole and exposed parietal lobe pial surface were kept moist with 0.9% saline.

2.2.5 Nerve recording and stimulation

2.2.5.1 Phrenic nerve

Custom made bipolar silver wire electrodes were used to record the phrenic nerve. The left scapula was retracted laterally using sutures and tied to the stereotaxic frame. This created a ‘well’ with the dissected phrenic nerve at the bottom. The well was filled with paraffin oil and sealed with agar soaked cotton wool.

The recorded phrenic nerve output was pre-amplified 10X (series Z bioamplifier, CWE), and then further amplified 2000-5000X using a scaling amplifier (BMA-400 AC/DC/ Bioamplifier, CWE). The signal was filtered (100-3000 Hz band pass), full wave rectified, and integrated using a Paynter filter with a 50-ms time constant. The resulting analogue signal was digitised using an analogue to digital converter (CED 1401, Cambridge Electronic Designs, UK) and displayed in real time on a computer using Spike 2 software (CED).

2.2.5.2 Splanchnic sympathetic nerve (sSNA)

Bipolar silver wire electrodes were used to record sSNA. As with the phrenic nerve, a 'well' was formed by lateral retraction of the left flank cut skin edge and medial retraction of the paraspinal muscles with sutures. This well was filled with paraffin oil and sealed with agar soaked cotton wool. The signals from the silver wire electrodes were amplified in a similar fashion to the phrenic nerve, filtered (100-3000 Hz band pass), full wave rectified, and integrated using a Paynter filter with a 50-ms time constant. The signal was digitized with a CED 1401 analogue to digital converter (Cambridge Electronic Designs, UK) and displayed in real time on a computer using Spike 2 software (CED, UK)

The zero level of sSNA was determined using supramaximal electrical stimulation of the aortic depressor nerve (0.2 ms stimulation, 50 Hz for 5 seconds).

2.2.5.3 Aortic depressor nerve

The aortic depressor nerve was stimulated using custom made bipolar silver wire electrodes. As previously mentioned, the zero level of sSNA was determined using supramaximal electrical stimulation of the aortic depressor nerve (0.2ms, 50 Hz for 5 seconds, 0.5-4.0 volts). This was achieved using electrical stimulators (AMPI, Israel) driven by a Master 8 eight channel pulse generator (AMPI, Israel).

2.2.5.4 Tibial nerve

Stimulation of the cutaneous afferent fibres was achieved by a custom made bipolar silver wire cuff electrode placed on the right tibial nerve. The nerve was stimulated at 0.5 Hz (1ms duration, 20-30 volts). To prevent nerve dehydration, the nerve and cuff electrode were surrounded by cotton wool soaked in paraffin oil.

2.2.6 Data analysis

2.2.6.1 Sympathetic baroreflex

To assess the sympathetic baroreflex, the average SNA inhibition in response to intermittent ADN stimulation was determined. The ADN was stimulated (0.2 ms duration, 2 pulses at 2.5 ms interval, 0.5 Hz) and the SNA response was averaged at least 50 times. The SNA responses to intermittent ADN stimulation was analyzed using peristimulus waveform averaging. The amplitude of the SNA from –200 to 0 ms prior to stimulation was taken as the baseline. The maximum response to stimulation was then expressed as a percentage change from the baseline (see Fig. 2.1*A*).

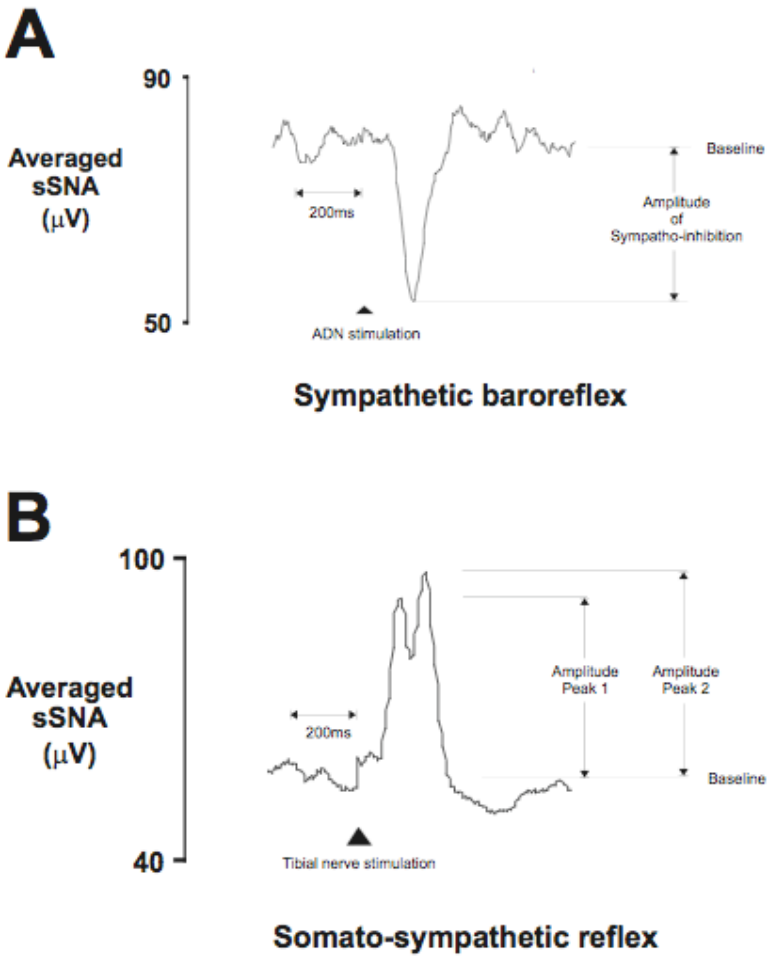

Figure 2.1. Sympathetic baroreflex and somato-sympathetic reflex. **A. Sympathetic baroreflex.** Peri-stimulus waveform average following electrical ADN stimulation. The amplitude of sympatho-inhibition is expressed as percentage change from baseline (average sSNA for 200ms prior to stimulation-see text). **B. Somato-sympathetic reflex.** Peri -stimulus waveform average following electrical tibial nerve stimulation. The amplitudes of Peak 1 and Peak 2 sympatho-excitatory responses are expressed as percentage change from baseline (average sSNA for 200ms prior to stimulation-see text).

2.2.6.2 Somato-sympathetic reflex

To assess the somato-sympathetic reflex, the sSNA response to electrical stimulation of the tibial nerve (0.5 Hz, 1ms duration, 20-30 volts) was averaged at least 50 times, and analyzed using peristimulus waveform averaging. The amplitude of the sSNA from –200 to 0 ms prior to stimulation was taken as the baseline. The maximum response to stimulation was then expressed as a percentage change from the baseline (see Fig. 2.1*B*).

2.2.6.3 Sympathetic chemoreflex

Activation of the sympathetic chemoreflex was achieved using a brief period of hypoxia. The animals were ventilated with 100 N_2 (BOC Gases, Australia) for 15 seconds. . The response to hypoxia was quantified by comparing the average sSNA for 10 seconds following the onset of excitation of phrenic nerve discharge caused by the inhaled 100% N_2 as a percentage change from a control period of 10 seconds average sSNA immediately prior to 100% N_2 inhalation.

2.2.6.4 Phrenic amplitude, frequency, and inspiratory time

Phrenic discharge frequency was determined using Spike 2 software (CED, UK) using a 'data rising through a threshold trigger'. Phrenic amplitude and inspiratory time were determined using phrenic discharge triggered phrenic nerve waveform average over a 100 second period (see Fig. 2.2). Phrenic waveform amplitude, inspiratory time and frequency were expressed as a percentage of pre-stimulus control period (averaged over 100 seconds).

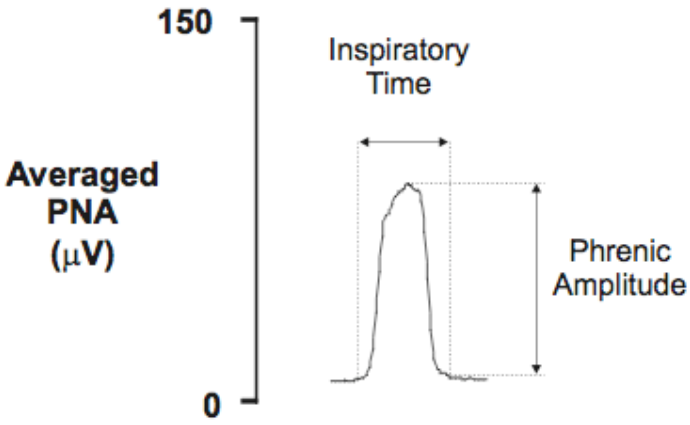

Figure 2.2 Peri-phrenic waveform averaging. Inspiratory time, phrenic discharge frequency and phrenic amplitude were calculated using peri-phrenic waveform averaging (see section 2.2.6.4).

2.2.7 Pipettes

Single barrel glass micropipettes for microinjection of drugs or retrograde neuronal tracers were prepared using Clay Adams graduated micropipette glass on a moving coil microelectrode puller (model 753, Camden Instruments).

Triple barrel microinjection pipettes were prepared using borosilicate glass capillaries (1.0mm OD x 0.50mm ID, Harvard apparatus) on a PMP-100 multibarrel puller (Micro Data Instruments, NJ, USA).

Microinjection pipettes were filled via suction applied by attached air filled tubing and air filled syringe.

Microinjections were performed via pressure injection using an air filled 5ml syringe and attached tubing. The volume of injection was determined by direct visualization of the fluid meniscus within the glass micropipette against an adjacent calibrated grid (25nl per division) using the operating microscope.

2.2.8 Microinjection drugs

Drugs used in microinjection experiments are shown in Table 2.1

Drug	**Source**	**Concentration**	**Injection Volume**	**Vehicle**
L-Glutamate	Sigma	50mM	50nl	10mM phosphate buffered saline (0.9%)
($pGlu^5$, $MePhe^8$, Sar^9)-SP(5-11) DiMe-SP	Sigma	600pmol in 50nl	50nl	10mM phosphate buffered saline (0.9%)
[Sar^9, $Met(O_2)^{11}$]-substance P	Sigma	600pmol in 50nl	50nl	De-ionised water
WIN 51,708	Sigma	5nmol in 100nl	100nl	1% DMSO
Albumin-colloidal Gold	Sigma	20%	25nl	10mM phosphate buffered saline (0.9%)

Table 2.1 Microinjection drugs. The pH of all injections was adjusted to between 7.3-7.5. DMSO, dimethyl sulfoxide.

In chapter 5, the dose of ($pGlu^5$, $MePhe^8$, Sar^9)-SP(5-11) for microinjection into the RVLM was 600pmol in 50nl. This has previously been demonstrated to result in a maximal increase in MABP following bilateral RVLM microinjection (Urbanski *et al.,* 1989). The dose of [Sar^9, $Met(O_2)^{11}$]-substance P for brainstem microinjection (600pmol in 50nl) is slightly higher than previously microinjected into the rat ventral tegmental area (100pmol, Deschamps and Couture, 2005) or rat striatum (75nl of 1mM, Tang *et al.*, 1998), and less than microinjected in rat nucleus basalis magnocellularis (25ng, Ciccocioppo *et al.*, 1997). As demonstrated in chapters 5 and 6, this dose resulted in significant alterations in multiple physiological parameters when microinjected into various brainstem regions, and those effects were abolished by the

highly specific neurokinin-1 receptor antagonist WIN 51708. Dose response curves were not performed for either (pGlu^5, MePhe^8, Sar^9)-SP(5-11) or [Sar^9, Met(O$_2$)^{11}]-substance P. These curves could be incorporated into future studies.

2.2.9 Marking injection sites

Injection sites were marked by the microinjection of 25nl of 20% albumin colloidal gold. The animals were killed with an i.v. injection of 1M KCl. The brainstem was removed immediately and fixed overnight in 4% formaldehyde in de-ionised water. The next morning the brainstem was washed 3x30 mins in de-ionised water. The pia was carefully dissected from the medulla, which was then cut into serial 100μm sections on a vibrating microtome and the sections washed a further 3x30 mins in de-ionised water.

The injection sites were revealed using a silver enhancer kit (SE-100 Silver Enhancer Kit, Sigma, USA) according to the manufacturers instructions. Briefly, silver ions (in the form of a solution of silver bromide) are catalyzed by the gold particles in the presence of a reducing agent to precipitate metallic silver, which is visible as a dark spot. The sections were washed 3x30 mins in de-ionised water, mounted on gelatenised glass slides and air-dried. After dehydration with serial dilutions of alcohols, the sections were counterstained with cresyl violet and then delipidated (Histoclear, National diagnostics, Australia). Ultramount (Fronine, Australia) mounting media was applied and the slides coverslipped and viewed using a brightfield microscope (Leica, Germany). An example is provided in Figure 2.3.

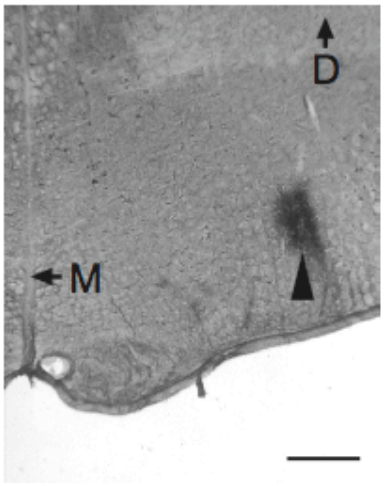

Figure 2.3. Microinjection sites. Microinjection sites were marked with a 25nl microinjection of albumin-colloidal gold and revealed using a silver enhancer kit, precipitating metallic silver at the injection site, which was visualised as a dark spot (see section 2.2.9). This example is in the RVLM. M, midline. D, dorsal. Scale bar = 500 μm.

2.3 Laser Doppler blood flow probe

2.3.1 Theory

Laser Doppler flowmetry provides a continuous measurement of relative blood cell perfusion of the microcirculatory beds of tissues without influencing the blood perfusion. The output signal from the laser Doppler flowmeter is proportional to the blood cell perfusion in the tissue illuminated by the laser, defined as:

Microvascular Perfusion= number of red blood cells X mean velocity

To achieve this, the tissue is illuminated with a coherent beam of laser light (in this case laser wavelength 830 ± 10 nm). This laser light is scattered by both moving and static structures within the microcirculatory beds. Photons that are scattered by moving red blood cells have their wavelength spectrally broadened according to the Doppler effect. The maximum Doppler shift occurs when the blood cells are moving in a direction parallel to the incident light beam and the scattered light from the cells is in the opposite direction from its origin. However, the Doppler frequency shift is very small. If laser light with a wavelength of 830 nm is reflected by a red blood cell moving 1mm / second parallel to the incident beam, the Doppler shift is only about 4 kHz. The static tissues illuminated by the laser undergo no Doppler shift.

To measure this small Doppler shift, the optical heterodyne technique is used (Pike *et al.*, 1968). When two signals of similar frequency are added together (such as scattered light from red blood cells with a small Doppler shift and the original output light from the laser), a 'beat' signal is formed due to the slightly out of phase signals alternately cancelling out and reinforcing (Pike *et al.*, 1968). This beat frequency is the difference

between the two added frequencies, and is equal to the Doppler shift frequency (Pike *et al.*, 1968). In the laser Doppler flowmeter, the original source laser light is added to the reflected light and sampled by a photodiode. The frequency and amplitude of the alternating current from the photodiode (i.e. the power spectral density) is related to the mean velocity and concentration of red blood cells present in the measured volume. The signal is digitised and spectral analysis performed with a digital signal processor, producing the Doppler flow signal. The resulting signal is proportional to blood flow rather than an absolute quantitative measure of tissue perfusion, and is therefore termed *relative* blood flow (e.g. rCBF).

2.3.2 Laser Doppler blood flow probe placement

The pial surface of the left parietal lobe was exposed via a left parietal burr hole (see section 2.2.4.2). Following this a needle regional blood flow laser Doppler probe (1mm diameter probe, ML191 Blood FlowMeter, ADInstruments) was placed in a micromanipulator mounted to the stereotaxic frame and the tip placed approximately 1mm above the cortical surface. Care was taken to avoid any large vessels overlying the cortical surface that might interfere with accurate blood flow readings. The probe was left in position for the remainder of the experiment.

2.4 Immunohistochemistry

2.4.1 Perfusion

Animals were deeply anaesthetised with sodium pentobarbitone (100mg/kg i.p.), given 5000 units heparin and 1%(w/v) sodium nitrite transcardially, and then perfused transcardially with 400ml of phosphate buffered saline (PBS, 100mM sodium phosphate buffer plus 0.9% sodium chloride: pH 7.4) to wash out the blood, followed by 700ml 4% formaldehyde in 0.1M PB (sodium phosphate buffer).

The brainstem was then removed and post-fixed with gentle agitation at room temperature for 4 hours in 4% formaldehyde in 0.1M PB. Following this the brainstem was washed 3x30 mins in 0.1M phosphate buffered saline, and the dura and pia mater carefully removed.

2.4.2 Sectioning of brainstem

The brainstem was mounted on a metal block and surrounded by agar to prevent distortion during cutting. Serial 50μm coronal sections were cut on a vibrating microtome into 0.1M PB. The sections were then washed for 30min in 50% ethanol (in de-ionised water) solution to increase antibody penetration, followed by 3x30min washes in Tris-phosphate buffered saline (TPBS; Tris-HCl 10mM, sodium phosphate buffer 10mM, 0.9% NaCl, pH 7.4).

2.4.3 Antibodies

2.4.3.1 Primary antibodies

Sections were incubated for 48hrs in primary antibody solutions containing primary antibodies (see Table 2.2), 5% normal horse serum (NHS) to prevent non-specific primary antibody binding, and TPBS with 0.05% merthiolate (TPBS-M). The addition of merthiolate to the TPBS prevents bacterial growth during incubation.

Primary	**Source**	**Species**	**Antigen**	**Dilution**
Neurokinin-1 receptor	Sigma	Rabbit	Amino acids 393-407 rat NK1-receptor C-terminal	1:5,000
PNMT	Dr Peter Howe	Sheep		1:10,000
DBH	Chemicon	Mouse	Bovine dopamine beta hydroxylase	1:500
CTB	List Biological Laboratories	Goat	Cholera toxin B subunit	1:1,000

Table 2.2. Primary antibodies used in immunohistochemical experiments (Chapter 4)

2.4.3.2 Secondary antibodies

Following the 48hr incubation with primary antibodies, the sections were washed 3x30 mins with TPBS and then incubated overnight in a secondary antibody solution. This solution consisted of secondary antibodies (see Table 2.3), 2% normal horse serum to prevent non-specific secondary antibody binding, and TPBS-M.

Fluorophore	**Species**	**Anti-species IgG**	**Dilution**	**Source**
Dual Labelling Experiments				
FITC	Donkey	Anti-Sheep	1:500	Jackson Immunoresearch
Texas Red™	Donkey	Anti-Rabbit	1:500	Jackson Immunoresearch
Triple Labelling Experiments				
FITC	Donkey	Anti-Mouse	1:500	Jackson Immunoresearch
Texas Red™	Donkey	Anti-Rabbit	1:500	Jackson Immunoresearch
AMCA	Donkey	Anti-Goat	1:500	Jackson Immunoresearch

Table 2.3. Secondary antibodies conjugated to fluorophores for fluorescent immunohistochemistry. FITC, fluoroscein isothiocyanate; AMCA, 7-amino-4-methylcoumarin-3-acetic acid.

2.4.4 Mounting

The sections were mounted serially on glass slides. To prevent fading / photobleaching of the fluorophores, Prolong Antifade mounting media (Molecular Probes) was used according to the manufacturers instructions. The glass slides were then coverslipped.

2.4.5 Retrograde tracer

The cholera toxin B subunit (CTB) was introduced as a neuronal tract tracer by Luppi *et al* (Luppi *et al.*, 1987). The cholera toxin B subunit is taken up at neuronal terminals by binding to the ganglioside G_{M1} on the neuronal extracellular membrane (Lencer and Tsai, 2003). It is then internalised and undergoes active retrograde transport along the

axon to the cell body (Kobbert *et al.*, 2000). By labelling antibodies to CTB the cell bodies of neurons projecting to the site of CTB microinjection can be determined.

Cholera toxin B subunit is also taken up by fibres of passage (Llewellyn-Smith *et al.*, 1990; Chen and Aston-Jones, 1995). Neuronal gangliosides necessary for the binding of CTB were originally thought to be present only at synaptic terminals (Derry and Wolfe, 1967; Hansson *et al.*, 1977), however it was later demonstrated that they are also located along the axolemma, facilitating uptake by fibres of passage (Skrivanek *et al.*, 1982; Harry *et al.*, 1987).

2.4.6 Fluorescence microscopy

A Leica DML fluorescence microscope was used to view the brainstem sections. Each fluorophore was viewed separately using filter sets as described in Table 2.4. Images were obtained using a Spot2 digital camera (Diagnostic Instruments, MI, USA). The resulting images for each fluorophore were pseudocoloured and merged using Spot2 software (Diagnostic Instruments, MI, USA).

Fluorophore	**Excitation wavelength**	**Excitation wavelength**	**Filter set**	**Dichroic mirrors**	**Excitation filters**	**Suppression filter**
FITC	495 nm	520 nm	L4	RKP510	BP450-490	BP515-560
AMCA	350 nm	445 nm	A	RKP400	BP340-380	LP425
Texas Red™	590nm	615 nm	TX	RKP600	BP530-590	LP615

Table 2.4. Leica DML fluorescence microscope filter sets

2.4.7 Confocal microscopy

Some dual labelled sections were also examined using a confocal laser scanning TCS 4D system (Leica, Germany) equipped with a krypton-argon laser. Fluorochromes were viewed separately in single channel mode using 488nm excitation for FITC-labelled secondary antibodies and 568nm excitation for Texas red-labelled secondary antibodies. Images of varying thickness (1-3 μm) were obtained through the brainstem sections. To increase the signal to noise ratio, each image slice was averaged multiple times (8-16). The exported images were pseudocoloured and merged using Spot2 software (Diagnostic Instruments, MI, USA).

2.4.8 Immunohistochemical experiment counting and analysis

Sections were mounted in sequential rostro-caudal order. All sections were examined using fluorescence microscopy (Leica DML fluorescence microscope, Germany). For quantitative analysis every fourth section was counted. The ventral medulla was defined as the area ventral to the nucleus ambiguus, medial to the spinal trigeminal tract and lateral to the lateral edge of the inferior olive.

Double and triple labelling was determined using 40X magnification. Close appositions were defined only when the terminal and labelled cell were in focus in the same focal plane and there was no discernible gap between the two structures (Pilowsky *et al.*, 1992).