

Stimulating Peripheral Afferents to Evoke Cardiorespiratory Reflex Responses in the In Situ Arterially Perfused Preparation

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Abstract

The in situ arterially perfused rodent preparation is an innovation that has allowed for significant progress in the study of cardiorespiratory reflex circuitry. This preparation provides a number of advantages over other preparations. The retention of peripheral cardiorespiratory afferents enables the study of reflex circuitry that is not possible in in vitro slice preparations. In addition, the in situ arterially perfused preparation provides unsurpassed mechanical stability of the brainstem compared with the in vivo preparation. This stability allows for better cellular recordings for prolonged periods.

Here, the basic technique for the in situ arterially perfused preparation including recording of a number of cardiovascular and respiratory parameters is described. In addition, some of the common techniques for stimulating peripheral afferent nerves that produce different cardiovascular and respiratory reflex responses are discussed.

Key words: Cardiorespiratory reflex, Baroreceptor, Chemoreceptor, Nociceptors, Somatic afferent, Locomotor-respiratory coupling, In situ arterially perfused preparation

1. Introduction

The study of neuronal mechanisms involved in cardiorespiratory control has been greatly aided by the development of reduced, in vitro preparations. In vitro brain slice and brainstem spinal cord preparations (1–9) have provided significant insights into the neurophysiological properties and synaptic mechanisms of cardiorespiratory networks. The major benefits of the reduced in vitro preparations include increased mechanical stability, visualisation of the neuronal network and the ability to precisely control the extracellular milieu. These advantages have allowed more direct

interrogation of the synaptic properties of neurones within the cardiorespiratory network. However, with *in vitro* brain slice preparations, only a small portion of the brain and its neuronal networks are maintained, severely limiting the ability to study integration of afferent inputs and outputs. This limitation was overcome to some extent, by the development of *in situ* perfused preparations, including the arterially perfused rodent preparation (10–12).

Since its first description in mouse in 1996 (10), the *in situ* arterially perfused preparation, also known as the ‘working heart-brain-stem preparation’, has been adapted for a variety of rodent species, including rats (12, 13), shrew (14, 15) and hamsters (16). Not only can the *in situ* arterially perfused preparation be adapted to many species, it also allows neurophysiological studies at ages that range from the day of birth (P0) through to adulthood (10, 16, 17), something that is not possible with most *in vitro* or *in vivo* preparations. However, the age range that can be utilised varies depending on the rodent species used. While adult mice can be used in this preparation, the age range for rats is typically no older than 6 weeks of age (equivalent to approximately ~150 g). In our experience, hamsters up to 7–8 weeks of age can also be used (16). Although the upper age range for use in this preparation is limited, it is generally considered that the sympathetic nervous system and the respiratory control systems are well developed before these time points.

The *in situ* arterially perfused preparation has a number of advantages compared with other approaches. First, autonomic reflex circuitry is intact, without the depressive and confounding effects of anaesthesia on autonomic reflex control. Second, while decerebration is possible *in vivo*, the procedures are often complex and time-consuming, and the potential for haemorrhage is high. These problems are negated in this preparation. Third, peristaltic pump perfusion enables greater mechanical stability of the brainstem, which provides a better environment for cellular recordings. This allows for cellular recording techniques, including extracellular, intracellular and whole-cell patch recording, which can be maintained during repeated stimulation (13, 18, 19). Repeated stimulation can be difficult *in vivo*, due to mechanical movement of the brainstem, resulting from both ventilation and cardiac pulsations. Fourth, a number of peripheral afferent nerves can easily be stimulated in the same preparation, with little additional dissection and equipment. These advantages mean that the *in situ* arterially perfused preparation is ideal for the study of central integration of peripheral afferents and autonomic reflex control. This chapter will outline the procedure for preparation of the *in situ* arterially perfused preparation and several common techniques for stimulating peripheral afferents that evoke cardiorespiratory reflex.

2. Equipment Setup and Solutions

This section will outline all the equipment and steps in setting up prior to performing the in situ arterially perfused preparation. This section will outline equipment required for setting up the perfusion circuit (Sect. 2.1), general laboratory equipment (Sect. 2.3) and solutions required.

2.1. Perfusion Circuit Setup

1. A low-pulsation peristaltic pump (e.g. Watson-Marlow 505S, 520S) with a minimum of six rollers on the pump head will improve the mechanical stability of the brainstem.
2. Circulating water bath.
3. Water jacket heat exchanger (for countercurrent heat exchange) to warm the perfusate inline to 31–32°C (Fig. 1b).
4. Two bubble traps (Fig. 1c) are essential for removing air bubbles and are highly recommended for dampening the pulsations of the heart and the pump rollers to aid the mechanical stability of the brainstem.
5. Polypropylene filter screens (1 × 25 µm + 1 × 40 µm, 25-mm diameter, Millipore PP4502500, PP2502500) and filter holder. Filtration is important as it prevents blood clots and other tissue debris from entering the perfusion.
6. Double-lumen catheter (e.g. Braintree Scientific, DLR 4) that will allow perfusion through one lumen and recording of the perfusion pressure through the second cannula.
7. Thick wall Tygon tubing (Masterflex 06409-14) that is relatively impermeable to gases.
8. Plastic chamber to hold the preparation.

The circuit, as illustrated and described in Fig. 1, is used to carry the perfusate in this setup (reserved volume between 100 and 200 ml, depending on the amount of fluid trapped within the circuit). The perfusate is passed through an inline heat exchanger (Fig. 1b), a bubble trap (Fig. 1c), polypropylene filter screens and a second bubble trap. The perfusate finally enters the preparation through a double-lumen catheter. The perfusate is allowed to overflow from the preparation, where it is collected and returned to the perfusate reservoir through the base of the chamber and reused to prevent loss of nutrients, such as essential amino acids. The perfusate is continuously bubbled with carbogen (95% O₂/5% CO₂).

2.2. Equipment Required for Dissection

1. Low-melting point wax (e.g. Paramat or Paraplast embedding medium) or Silgel® (Wacker-Chemie, Germany)
2. Surgical tools
 - (a) Scissors (13–15 cm blade, straight or curved)

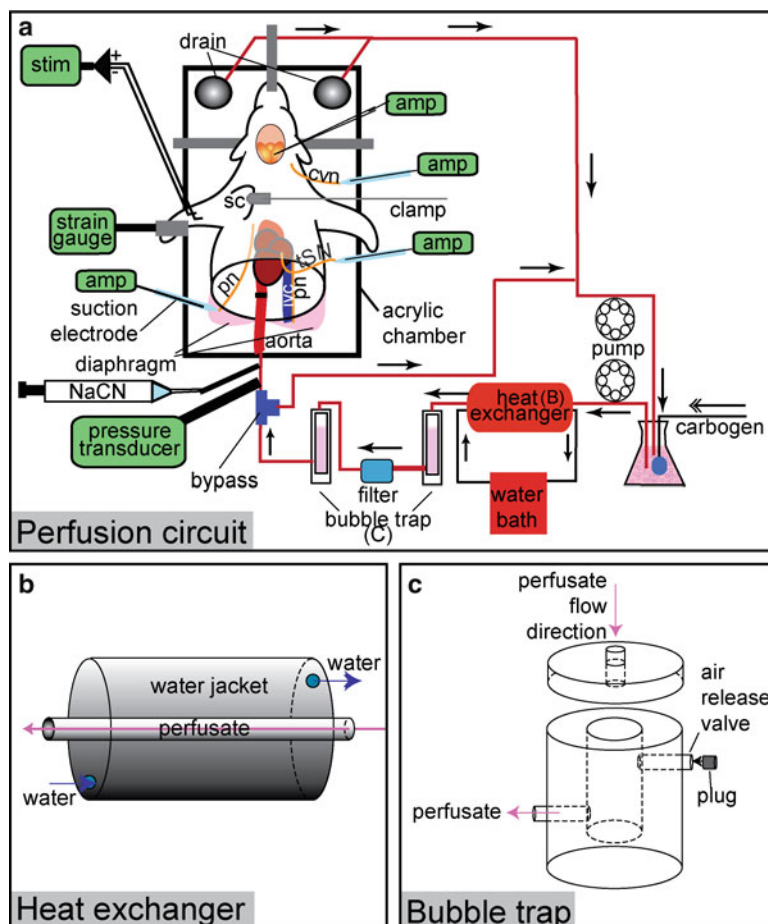


Fig. 1. Schematic diagram of the perfusion circuit and components used for the in situ arterially perfused preparation. (a) Schematic illustration of the decerebrated preparation, held in place by modified ear bars in an acrylic chamber. The preparation is perfused with a modified Ringer solution (Tables 1 and 2) through one lumen of the double-lumen catheter, retrogradely cannulated through the descending thoracic aorta. The second lumen is connected to a pressure transducer for recording of perfusion pressure. The perfusate is bubbled with carbogen (95% O₂ / 5% CO₂), warmed through an inline heat exchanger (b), through two bubble traps (c) and a nylon filter to trap debris. The overflow of the perfusate is collected from drains at the base of the acrylic chamber and recycled. The left phrenic nerves (pn) can be located by its insertion into the diaphragm and right pn can be found adjacent to the inferior vena cava (icv). Other efferent nerves that can be readily accessed in this preparation include the cervical vagus nerve (cvn) and thoracic sympathetic trunks (tsn). Peripheral somatic afferents can be activated by electrical stimulation of the forelimb, after stabilising the scapula (sc) and attaching the forepaw to a strain gauge. Peripheral chemoreceptors can be activated by direct inline injection of sodium cyanide (10–30 μ l of 0.03% NaCN).

- (b) Scalpel blades (#20 or #10)
- (c) Scalpel blade handle (#3)
- (d) Small dissection scissors
- (e) Straight forceps
- (f) Nerve hook

3. Decerebration aspirator (see Notes 6 and Fig. 4)
4. Dish with Silgel covering base

2.3. General Laboratory Equipment Required

1. Pressure transducer and bridge amplifier for perfusion pressure measurement
2. AC amplifiers for whole nerve and ECG recordings (e.g. Grass P511, Neurolog NL104A, or CWE BMA400)
3. Audio monitor
4. Data acquisition system, including an analogue to digital converter and computer for data acquisition (e.g. CED power1401 Mark II with Spike 2 version 7, or Powerlab Carbogen (95% O₂/5% CO₂))
5. Micromanipulator (e.g. Narashige MM-3)
6. Suture silk (4/0)
7. Pipette puller (e.g. Sutter P97, Sutter P2000)
8. Borosilicate glass capillary tubes (1.5 mm O.D., 0.86 mm I.D.)
9. Teflon-coated silver wire (0.003"–0.005" bare)
10. Electrode holder
11. Silastic tubing
12. Needles: 18G (blunt ends), 23G
13. PE 50 tubing
14. Syringes: 1cc and 30cc
15. Induction chamber for isoflurane anaesthesia
16. 3-way taps
17. Superglue

2.4. Chemicals and Salts

1. Salts for Ringer solution (see Table 1, Notes 1).
2. High-molecular-weight oncotic agent (see Table 2, Notes 2) is added to the Ringer solution to maintain osmolarity between the perfusate and the tissue. Incorrect oncotic pressure will not provide adequate brainstem perfusion and will reduce the longevity of the preparation.
3. Neuromuscular blocking agent: vecuronium bromide, rocuronium or pancuronium. Neuromuscular blockade is not required for the preparation but is essential for ensuring mechanical stability during neuronal recordings.

Table 1
Stock solutions for easy dilution to prepare Ringer solution for the in situ arterially perfused preparation

	Salt	Concentration
1	NaCl	5 M
2	KCl	0.12 M
3	KH ₂ PO ₄	50 mM
4	MgSO ₄ ·7H ₂ O	50 mM
5	NaHCO ₃	1 M
6	CaCl ₂	0.1 M

To make 2 L of Ringer solution for the in situ arterially perfused preparation, add 50 mL of stock solution 1–5 to 1.7 L of dH₂O and bubble with carbogen (95%O₂/5%CO₂) for 5 min before adding stock solution 6

Table 2
Oncotic agents used for maintaining osmotic pressure within the perfusate for the in situ arterially perfused preparation

Chemical	Concentration	Source	References
Ficoll type 70 (70 kDa)	1.25%	Sigma F2878	(16, 18, 20, 22)
Bovine albumin	2%	Sigma A7030	(11)
Polyethylene glycol 20,000	1–1.25%	Fluka 95172	(25, 26)
Dextran (260 kDa)	2.25–2.5%	Sigma D7265	(10)

- 4. Sodium cyanide (to activate chemoreceptors).
- 5. Arginine vasopressin—aids in maintaining perfusion pressure (see Notes 10).
- 6. Heparin.
- 7. Isoflurane.

*2.4.1. Additional
Equipment and Chemicals
(Depending on the
Peripheral Afferent
Stimulation Desired)*

- 1. Spinal/vertebral clamp
- 2. Force transducer (e.g. FT-100, CB Science)
- 3. Stimulator and isolation unit for control of electrical stimulus
- 4. Calibrated forceps for noxious pinch
- 5. Bradykinin, capsaicin for chemical stimuli

2.5. Solutions

The modified Ringer solution containing (in mM) 125 NaCl, 24 NaHCO₃, 3 KCl, 2.5 CaCl₂, 1.25 MgSO₄, 1.25 KH₂PO₄ and 1 D-glucose is made fresh on the day of use (see Notes 1). After bubbling with carbogen (95% O₂/ 5% CO₂), the pH should be ~7.4.

A high-molecular-weight compound, as listed in Table 2, is added to the 200 ml of Ringer solution as an oncotic agent (Notes 2) for the perfusate.

The neuromuscular blocking agent vecuronium bromide (3 $\mu\text{g}/\text{ml}$), rocuronium (10 $\mu\text{g}/\text{ml}$) or pancuronium (5 $\mu\text{g}/\text{ml}$) is usually added to the perfusate after the start of the perfusion, and a phrenic recording is successfully obtained.

2.6. Suction Electrodes for Whole Nerve Recording

The electrodes are borosilicate glass capillary tubes pulled on an electrode puller, with wide shoulders and a short taper. Strip the Teflon coating off the last 2–3 mm of a silver wire and place in the lumen. Seal the flat end of the pipette with silastic (silicone) tubing connected to a blunt needle attached to a 3-way tap and a 10-ml syringe (Fig. 2). Silastic tubing is preferred due to the flexibility of the material and its ability to form a tight seal around the glass capillary and silver wire. If an air leak develops around the silastic tubing, a small drop of superglue can be used to form a tight seal. The small amount of perfusate drawn up into the syringe when aspirating the nerve ending will provide the conductive bridge between the nerve and the silver wire. A second Teflon-coated silver wire is wrapped around the outside of the electrode and held in

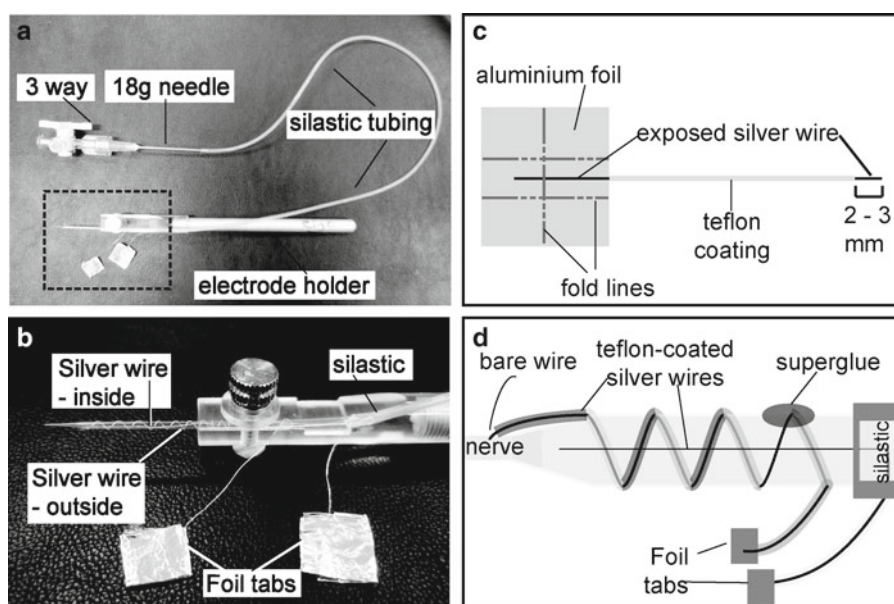


Fig. 2. Photos and schematic illustration of suction electrodes and stimulating electrodes commonly used in the in situ arterially perfused preparation. (a) Photograph of the suction electrode, connected to a silastic (silicone) tubing attached to an 18-G needle and 3-way tap, mounted on an electrode holder. The suction electrode (*dashed box*) is highlighted in (b), showing the Teflon-coated silver wire both inside and coiled around the outside of the borosilicate glass capillary. The ends of the silver wires are folded into 'foil tabs' (c) for easy connection to alligator clips. (d) Schematic representation of the construction of a suction electrode with the nerve aspirated inside the capillary and the correct placement of the reference wire (outside silver wire).

place with a drop of superglue (Fig. 2d). This is the reference wire for the bipolar nerve recording.

2.6.1. Potential Problems

1. If the nerve continuously slips out of the suction electrode:
 - The pipette tip is too large. Change to a different electrode with a smaller opening.
 - The seal between the silastic tubing at the end of the glass capillary or at the 3-way tap may be leaking. Check and reseal with superglue or trim the end of the silastic and reattach.
2. The nerve signal is weak:
 - Check to see if the nerve is drawn into the pipette properly. If only a very short piece of nerve is drawn into the electrode, the tip is too small. Either change to another electrode with a larger opening or gently file back the glass using emery paper.
 - The nerve may be damaged. Release the nerve from the electrode and trim to make fresh cut end.

Connect the loose ends of the wire to the preamplifier directly by clipping on alligator clips. The thin silver wires can be difficult to connect and this can be overcome by wrapping the wire in small pieces of aluminium foil to increase contact area (Fig. 2c). This needs to be checked and rewrapped regularly as the aluminium oxidises over time and this will reduce the conductivity between the wire and the foil.

2.7. Stimulation

Electrodes for Whole Nerve Stimulation

Stimulation electrodes used in the in situ arterially perfused preparation differ from the stimulation electrodes used in in vivo anaesthetised rats. Due to the size of the preparation and continuous perfusion, it is not always possible to isolate the nerve on bipolar hook electrodes. To overcome this problem, take two lengths of silver wire (0.005" diameter, Teflon-coated) approximately 5–10 cm long and strip away the Teflon coating over the last few millimetres of the wires at both ends to allow electrical contact. The other loose ends of the wire will be connected to the two poles of the stimulator. To improve the ease of connecting to stimulators, the loose ends of the silver wires can be wrapped in aluminium foil to increase the surface area for contact (Fig. 2c). Wrap one end of each of the two silver wires around the nerve that you wish to stimulate (e.g. vagus nerve) approximately 3–5 mm apart, making sure the wire is tightly wrapped around the nerve but without crushing the nerve. Excess lengths of exposed wires should be trimmed. The wires must then be secured in place and electrically isolated using either histology (low melting point) wax or Silgel. Low-melting point wax is ideal as it sets hard quickly but is fluid at

relatively low temperature with a melting point of 55°C. This will prevent damage to the nerve. Silgel is another good option, although it will take longer for Silgel to set.

3. Methods

3.1. General Preparation of the In Situ Arterially Perfused Preparation

The following procedures and equipment are required for the in situ arterially perfused preparation:

1. Pretreat the animals with heparin sodium (<1,000 units) by intraperitoneal injection approximately 10–15 min prior to anaesthesia (see Notes 3).
2. Anaesthetise the animal using isoflurane (3–5%) in air. Wait until the animal is deeply anaesthetised, as determined by an absence of withdrawal to pinch and corneal reflex (see Notes 4).
3. Transect the animal just below the diaphragm (sub-diaphragmatically) using large scissors and immediately plunge the torso into cold Ringer solution bubbled with carbogen (see Notes 5).
4. Remove the scalp to expose the skull and slice off the top of the skull using the tip of a fresh scalpel blade, fitted onto a standard scalpel handle. Cut open the dura to expose the brain.
5. Remove or destroy the cortex of the rat, taking care not to damage the colliculi that lie directly below the cortex, just rostral of the cerebellum. Decerebration or decortication can be achieved either by aspiration or dissection (see Notes 6).
6. Once the decerebration/decortication is completed, remove the skin. Removing the skin is important to prevent hair and other debris that can clog the perfusion circuit.
7. Transfer the preparation to a dish containing Sylgard®/Silgel on the bottom on which you can securely pin down the preparation using pins. Cover the preparation with cold Ringer solution bubbled with carbogen (see Notes 7).
8. Pull down the diaphragm on the left to identify the insertion of the *left* phrenic nerve. Carefully lift the lobes of the left lung to find the phrenic nerve between the lobes. The lobes of the lung may be removed or left intact, as the lungs will remain deflated. However, removing the lungs will make it easier to find and record from the phrenic nerves. Gently rub the phrenic nerve with a pair of forceps, or nerve hook, to remove the connective tissue surrounding the nerve. This is important as excessive connective tissue will make it more difficult to obtain a good seal and hold the nerve in the suction electrode later. DO NOT cut the phrenic nerve at this point. The *right* phrenic nerve runs along the inferior vena cava and can be dissected

away at this point or later. The lobes of the right lung can be removed or left intact.

9. Separate the aorta from the vertebrae to facilitate cannulation (see Notes 8).
10. This is the end of the most basic components of the dissection for the in situ arterially perfused preparation. The dissection time up to this point should be kept to a minimum. Once you are proficient with this method, this dissection should be achievable within 10 min. If required, additional dissections (as discussed in Sect. 3.2) can be performed at this point.
11. Transfer the preparation to the perfusion chamber and retrogradely cannulate the descending aorta with the double-lumen cannula (see Notes 9). Advance the cannula 5–10 mm from the cut end and secure tightly with suture silk. The preparation can be placed onto modified ear bars (if required) or simply left in the bottom of the chamber. It is crucial that you make sure there are no points for leakage (e.g. torn arterial branches) between the suture and the tip of the cannula.
12. Arterial perfusion of the preparation can now begin, using carbogenated Ringer solution containing an oncotic agent. Perfusion to the preparation can slowly increase (over 1–2 min) until the flow rate is around 25–30 mL/min (for most preparations). This slow increase is important as it allows the vasculature to adapt to the change in perfusion pressure and for the preparation to rewarm progressively.
13. Cut the phrenic nerve at the insertion to the diaphragm. Using a suction electrode, tip diameter 200–300 μm , held on a micro-manipulator, position the tip of the electrode next to the cut end of the phrenic nerve and draw back on the plunger of the attached syringe (Fig. 2d). The nerve should be drawn into the pipette and form a tight plug. Close the 3-way tap at this point to maintain the seal.
14. The heart will start beating soon after the perfusion begins and the preparation starts to rewarm (see Notes 10). Rhythmic respiratory muscle activity will usually begin within 5 min of the start of the perfusion, and the perfusion pressure has reached approximately 50 mmHg. To achieve this perfusion pressure, some preparation may require some ‘fine-tuning’ at this point, either by altering flow rate or by addition of AVP (see Notes 11).

3.2. Additional Dissections for Recording and Stimulation

Brainstem exposure: The dorsal surface of the brainstem can be exposed by cutting away the occipital bone and cerebellum. When removing the cerebellum, take care not to cut or damage the dorsal pontine respiratory group, located below the cerebellar peduncles. Damage to this region will produce apneustic bursting patterns. *This step is only required if direct brainstem microinjections or cellular recordings are to be performed.*

Sympathetic chain: The sympathetic chain can be found at the caudal levels of the thoracic vertebra running diagonally from the spinal processes towards the midline. Access to the sympathetic trunk can be gained by removing the last 3–5 ribs. However, take care not to cut too close to the vertebrae or you will damage the sympathetic chain.

Vagal nerve dissection: Turn the preparation over to expose the ventral side of the neck. This dissection can be made easier by placing a small cylinder, e.g. a piece of Tygon tubing used in the perfusion circuit (Masterflex 06409-14), under the neck to elevate and extend the neck. Cut away the *sternomastoid* muscle and blunt dissect through the *omohyoid* muscle to expose the common carotid artery and the adjacent vagus nerve. Blunt dissect the vagus nerve away from the common carotid artery. The recurrent laryngeal nerve can also be found running adjacent to the trachea.

For efferent vagal nerve recordings (16, 20): cut the vagus nerve as distal as possible and drape over the side of the neck for later recording with a suction electrode. Try to make the free section of nerve as long as possible to help placement of the electrode tip.

For vagal nerve stimulations (20): wrap two lengths of silver wires snugly around the vagus nerve, as described for stimulating electrodes.

4. Peripheral Afferent Stimulations to Evoke Cardiorespiratory Reflexes

As described above, a key advantage of this preparation is the ability to stimulate a variety of peripheral afferent nerves that can excite or inhibit central neural networks. The following describes a number of methods for stimulating and inhibiting these systems.

4.1. Baroreceptor Reflex Activation

Baroreceptors can be stimulated via graded increase in perfusion flow rate (21, 22), either computer or manually controlled. Perfusion pumps with serial input (e.g. Watson-Marlow 520DU) that allow for computer-controlled changes in perfusion pressure ramps will be more reproducible than manually controlled changes. In either method, the baroreceptors are stimulated by maximally increasing the pump flow rate for a few seconds, until the desired change in perfusion pressure is obtained. The rate of rise of perfusion pressure should be consistent between trials within the same preparation. This increase in perfusion pressure should lead to bradycardia, bradypnoea and sympathoinhibition.

4.2. Chemoreflex Activation

1. *Inline (intra-aortic) bolus injection of sodium cyanide* (11, 13, 21, 22): 10–30 µl of 0.03% solution of sodium cyanide. Connect a side port, constructed from a length of PE50 tubing and 23-G needle, to the inflow tube just distal of the

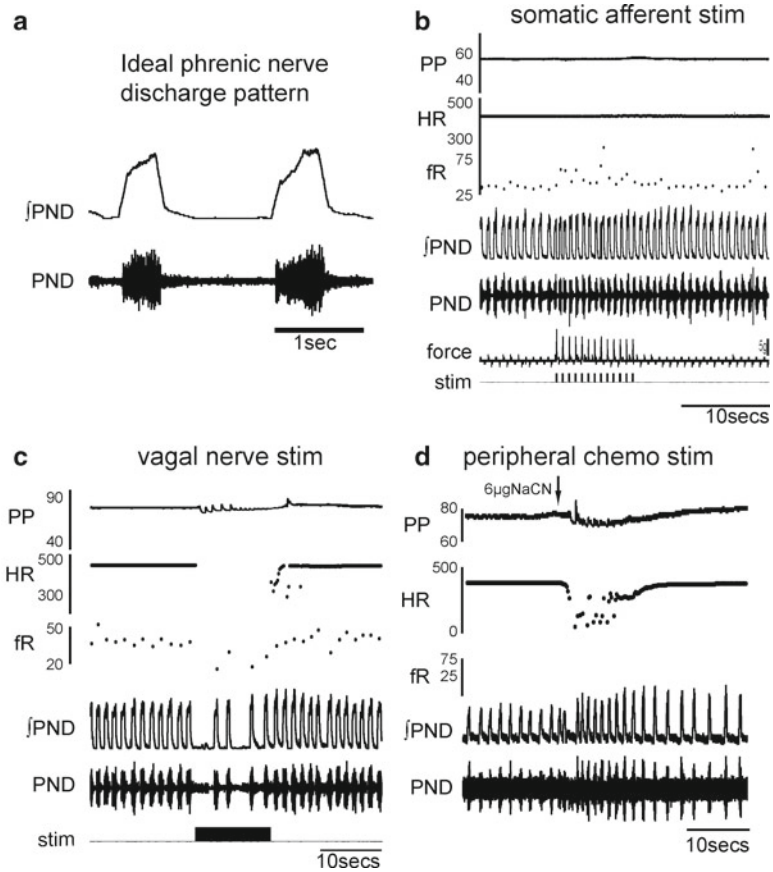


Fig. 3. Ideal phrenic nerve discharge (PND) and example effects of cardiorespiratory reflexes following activation of peripheral afferents. (a) Example of raw and rectified and smoothed PND in the working heart-brainstem preparation demonstrates a rapid onset with a ramping discharge pattern. (b–d) Example of peripheral afferent stimuli that excite and inhibit cardiorespiratory parameters measured in the in situ arterially perfused preparation. Train stimulation of somatic afferents (b) and peripheral chemoreceptor activation (d) produces tachypnoea, while vagal nerve stimulation (c) produces apnoea that mimics Hering-Breuer reflex activation. Vagal nerve stimulation (c) and chemoreceptor activation (d) also produce profound bradycardia and decrease in perfusion pressure.

connection to the double-lumen catheter. A bolus injection of between 10 and 30 μ l of 0.03% sodium cyanide should elicit a rapid chemoreflex response characterised by bradycardia, tachypnoea and hyperpnoea (Fig. 3d).

2. Switching the perfusion mix to one bubbled with low O_2 (10% O_2 , 5% CO_2 , balanced N_2) will produce a more sustained peripheral chemoreceptor activation.
3. Switching the perfusion mix to one bubbled with high CO_2 (7–10% CO_2 , balanced with O_2) will activate central chemoreceptors.

4.3. Somatic Afferent Stimulation

Somatic afferent nerves innervating the forelimbs can be activated by direct electrical stimulation of the forelimb flexor muscles in the limbs to produce isometric contraction (12, 18, 19, 21). The ipsilateral scapula is stabilised using a vertebral clamp and the distal end of the forelimb attached to a force displacement transducer connected to a recording amplifier to measure the tension generated by the electrically evoked forelimb contraction (see Notes 12). The electrical stimuli used to evoke muscular contractions are delivered using two silver wires (0.7 mm OD) introduced into the *biceps brachii* and *coracobrachialis* muscle groups running parallel to the *humerus*. The wires are then attached to a stimulation isolation unit. The following stimulation parameters are used to produce sufficient muscle contraction: pulse trains (0.4–1.3 Hz, 50- to 75-ms train duration, 1- to 3-ms pulse duration, 250 μ A to 1 mA). Successful activation of somatic afferents is evidenced by a resetting of respiratory rhythm that results in tachypnoea, using train stimulation (Fig. 3b) occurring in late expiration, with pressor and tachycardic responses also apparent in sustained (tetanic) contractions (12, 18, 19, 21).

4.4. Nociceptors

Nociceptive afferents can be stimulated in a variety of ways:

Noxious pinch: The forelimb (23), hindlimb or tail, the latter two can only be done on the decerebrated arterially perfused rat preparation (11), can be stimulated with either a manually operated calibrated forceps or a pneumatic pincher. The design will need to provide accurate control of the distance between the ends of the forceps to allow for measurement and reproducibility of the stimulus intensity. The duration of the stimulation is between 4 and 10 s and will vary between preparations and is determined by the start of cardiorespiratory reflex responses (tachycardia, pressor response and bradypnoea).

Corneal stimulation: The cornea can be mechanically or chemically stimulated. For mechanical stimulation, the stimulus duration is between 1 and 2 s and the pressure ranges between 0.4 and 1.4 bar of air pressure (23). Chemical stimulation of the cornea can be achieved by applying a drop of bradykinin (10–20 μ l of 1 μ M) or capsaicin (10–20 μ l of 0.12%, or 1 mM). The level of stimulation should be determined prior to paralysis (23).

Subcutaneous noxious stimulation: subcutaneous injections of bradykinin (5–10 μ l of 1 μ M) or capsaicin (5 μ l of 0.12%, or 1 mM) can be used to stimulate subcutaneous chemoreceptors.

Thermal stimulation: Thermal sensors can be evoked by dripping heated saline (50–56°C) onto the skin of the forepaw (and hindpaw or tail in the decerebrated arterially perfused rat (11)), until a reflex cardiorespiratory response is obtained (11, 23).

Electrical stimulation: A branch of the brachial plexus, or the sciatic nerve in the decerebrated arterially perfused rat (11), can be electrically stimulated (10–40 V, 1–2 ms, 8–30 Hz). The nerves

that form the brachial plexus emerge between the *scalene* muscles in the rat to form the brachial plexus in the axilla. The plexus can be accessed ventrally by cutting and reflecting the *clavicle* and the *pectoralis major* and *pectoralis minor* muscles. The *subclavian artery* runs within the axilla and care needs to be taken during the dissection not to damage the artery. Once the plexus is exposed, a branch can be isolated and a silver wire can be wrapped around the nerve for stimulation, as described above.

4.5. Pulmonary Stretch Receptor Activation (Hering-Breuer Reflex)

The pulmonary stretch receptors can be activated either via lung inflation (if left intact) or electrical stimulation of the vagus nerve (Fig. 3c) (20, 24). The parameters used for electrical stimulation of the vagus nerve are between 25 and 200 μA , at 50 Hz, with a pulse duration of 1–3 ms for 12 s. Electrical stimulation of the vagus slows phrenic bursting by increasing phrenic interburst interval (expiratory time). The threshold current required to produce a detectable increase in phrenic interburst interval should be determined in each preparation and the stimulus current chosen is normally 1.5–2 times threshold.

5. Notes

1. The Ringer solution can be readily made up using the following stock solutions listed in Table 1. To make 2 L of perfusate, measure out 1.7 L of distilled water and add 50 mL of stock solutions #1–5 and the D-glucose. Bubble with carbogen for at least 15 min before adding stock solution #6. It is important to bubble the Ringer solution with carbogen to adjust the pH to 7.3–7.4, prior to addition of stock solution #6 to prevent formation of precipitates. The Ringer solution can become cloudy after sitting on the bench for some time, but this can be reversed by bubbling the solution with carbogen again.
2. The high-molecular-weight compound, such as Ficoll® 70 (e.g. Sigma #F2878, Table 2), can be added to the perfusate only, rather than the entire volume of Ringer solution. The oncotic agent is not required for the Ringer solution used in the dissection stages. In general, 200 mL of Ringer solution is sufficient for one preparation. A fresh 200 mL of perfusate is prepared for each preparation. Once the oncotic agent is dissolved in the Ringer solution, the perfusate is filtered through a Whatman #1 filter to remove any particulates.
3. Heparin pretreatment is not necessary but is recommended by some researchers as it will improve the perfusion of the preparation by minimising blood clots in microvasculature. Heparin pretreatment can extend the amount of time spent

dissecting before the perfusion begins, allowing for more complex procedures to be carried out.

4. While all care should be taken to ensure that the animal is deeply anaesthetised before any surgical procedure begins, it is important that the heart continues to beat when the incisions are made. This is crucial as it will reduce the formation of clots in the microvasculature that may cause impairment in tissue perfusion.
5. Plunging the torso into cold Ringer solution will help rapidly reduce metabolism and aid in preserving the tissue. The Ringer solution is best chilled by placing the glass container in ice. The ideal temperature range for the Ringer solution is between 4 and 10°C.
6. An aspirator for decerebration can be made using a 5-mL syringe with a 200-μL pipette tip fitted over the syringe luer. Trim the opening of the 200-μL pipette tip to approximately 2–3 mm diameter to allow for easy suction of the cortex (Fig. 4). To aid suction, plunge the skull under the surface of the Ringer solution, then carefully and gently aspirate to remove the cortex. To assist in locating the colliculi, use the tip of the aspirator to gently separate the cortex from the cerebellum and push rostrally before aspirating.
7. Securely pinning down the preparation will help with the remaining fine dissection. The preparation can be pinned using any type of pins, from drawing pins, sewing pins, insect pins to syringe needles. The dish with Silgel/Silgard should be placed on ice to keep cold. The silicone compound in the dish will take longer to chill and can be stored in the refrigerator and placed into the ice just prior to when it is required.
8. In preparations where you do not wish to record thoracic sympathetic nerve activity, isolation of the thoracic aorta can be achieved by taking hold of the cut end of the aorta and tugging down sharply but firmly until most of the aorta is separated from the thoracic vertebra. Retrim the end of the aorta to get a nice, flush, cut end to make cannulation easier. However, if you wish to record from the thoracic sympathetic nerve trunk, then it is important to maintain the circulation to the spinal cord, as this is derived from dorsal penetrating arteries arising from the back of the aorta. Thus, you will need to maintain this circulation by cannulating the descending aorta distal to the level of T10, approximately at the level of the diaphragm.
9. The double-lumen cannula allows for perfusion and measurement of the perfusion pressure (Fig. 1). It is critical that a slow but continuous flow through the cannula tip is maintained to prevent the development of air bubble in the perfusion line.
10. On occasion, the heartbeat will be irregular or arrhythmia will develop. In most cases, the rhythm will settle down and become regular as the preparation is 'tuned'. On other occasions, a bolus

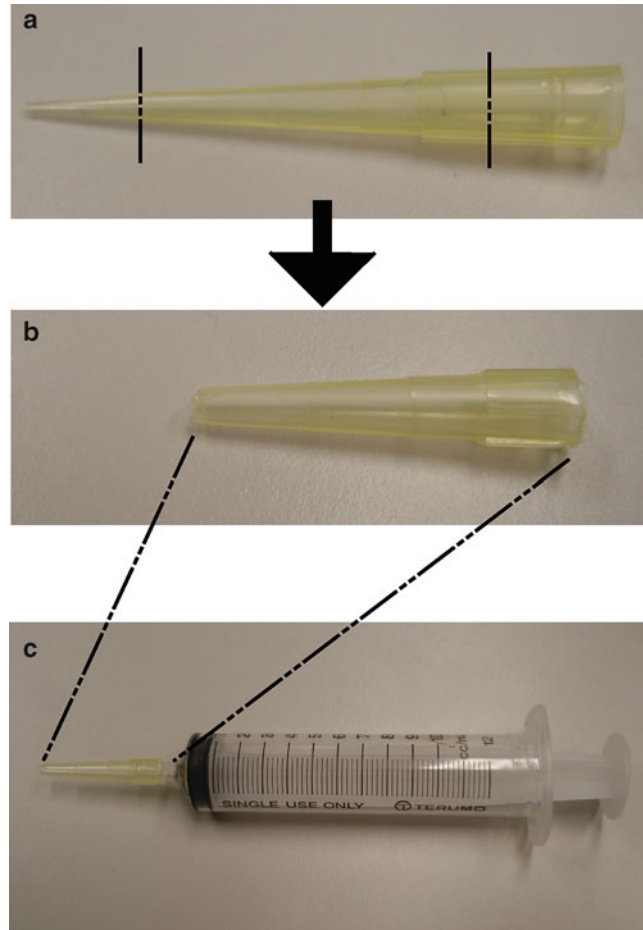


Fig. 4. Construction of aspirator used for decerebration. Trim a 200- μ L pipette tip as illustrated by the *dashed lines* (a) to increase the opening. Trim the large end of the pipette tip to allow for a tight fit onto the luer end of a 10-mL syringe (b). Finally, firmly attach the trimmed pipette tip to a 10-mL syringe (c).

injection of sodium cyanide to evoke a severe but transient bradycardia may allow the rhythm to reset to a regular one.

11. You will need a perfusion pressure of approximately 50–70 mmHg to obtain sufficient perfusion to the brainstem. In some preparations, this is achieved when an adequate flow rate is reached (~ 30 ml/min). However, in some preparations, despite an adequate flow rate, the perfusion pressure remains low. Adequate perfusion is required for the establishment of the eupneic bursting pattern of the respiratory discharge (Fig. 3a) and will increase sympathetic activity, which will assist in producing vasomotor tone to help maintain the perfusion pressure. There are two ways to increase perfusion pressure. The simplest option is to increase flow rate until a ramping phrenic discharge pattern is established. Once the desired bursting pattern is established, slowly decrease the flow rate

while observing the bursting pattern. If the ramping discharge begins to revert to an apneustic or flat discharge pattern, slightly increase the perfusion rate again until the ramping discharge pattern is re-established (Fig. 3a). The second option is to induce pharmacological vasoconstriction by adding, for example, 200–400 pM arginine vasopressin (AVP) to the perfusate (10).

12. Typically, a force production of ~5–10 g of isometric contraction is required for activation of somatic afferents. Thus, it is vital that the forelimb is mechanically stable. The scapula can be secured using a small vertebral clamp, and the forepaw held on a strain gauge approximately parallel to the table. The forelimb will also need to be pre-tensioned to >10 g of force prior to any electrical stimulation of the forelimb muscles.

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