# Chapter 34

# Assessment of Basilar Artery Reactivity in Stroke and Subarachnoid Hemorrhage Using Wire Myograph

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## Abstract

Blood flow regulation of normal cerebral arteries is a critical and important factor to supply the brain tissue with nutrients and oxygen. Stroke insult results in a disruption or reduction in cerebral arteries' blood flow with subsequent brain tissue damage. Hemorrhagic stroke is one type of stroke and accounts for about 13% of all of stroke insults. In this type of stroke, the cerebral artery breaks open and causes bleeding in or surrounding the brain. Subsequently, this bleeding causes blood vessels to constrict in a process called vasospasm, in which the vessels narrow and impede the blood flow to brain tissue. Hemorrhagic stroke is the major cause of prolonged constriction of cerebral arteries. This leads to partial brain damage and sometimes death in patients with aneurysmal subarachnoid hemorrhage. Among the key delicate techniques to assess small blood vessel functionality is the wire myograph, which can be utilized in several cerebral injury models including stroke. The wire myograph is a device that provides information about the reactivity, stiffness, and elasticity of small blood vessels under isometric conditions. In this book chapter, we describe the techniques involved in wire myography assessment and the different measures and parameters recorded; we describe the utility of this technique in evaluating the effects of subarachnoid hemorrhage on basilar artery sensitivity to different agonists.

**Key words** Basilar artery, Wire myograph, Vessel viability, Cumulative concentration-response curves

## 1 Introduction

### *1.1 Structure of Blood Vessels*

The wall of most blood vessels consists of three layers: the inner layer (intima) made of endothelial cells; the muscle layer (media), which is the thickest part and composed of vascular smooth muscle cells (VSMC); and the outer layer (adventia) containing fibroblasts and connective tissue. The main function of VSMC is to control blood flow, primarily by regulating the diameter of small resistance vessels. The VSMC can sense and react to changes in serum composition, extracellular fluid composition (due to bleeding), and intraluminal blood pressure [1].

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### 1.2 Microvasculature Structure and Function

It is well known that the main drop in hydrostatic pressure occurs at the level of resistance arteries (small blood vessels); these vessels have a crucial role in the regulation of blood pressure and blood flow. Resistance arteries comprise the small resistance arteries (<300 μm of lumen diameter), arterioles (<100 μm of lumen diameter), and capillaries (about 7 μm of lumen diameter) [2, 3]. Thus, any structural alteration that takes place at the level of these microvessels will have an impact on the regulation of blood pressure and blood flow. An increase in blood contraction or the thickness of the arterial wall with a reduced lumen causes an increase in vascular resistance [4, 5]; this alteration may define the remodeling process involved in blood remodeling. Poiseuille's law states that resistance is inversely proportional to the radius to the fourth power, which makes vascular resistance highly sensitive to small alterations in the lumen of arteries [6].

### 1.3 Tone of Cerebral Arteries

Being a highly metabolically active organ, the brain relies heavily on a constant delivery of nutrients and oxygen, whether in a state of wakefulness or sleep. Blood flow to the brain is maintained by the tone of cerebral arteries [7]. Tone is regulated by vasoconstriction or vasodilation; when the VSMCs of cerebral arteries contract, the arteries constrict and reduce blood flow to the region of the brain they supply. This could result in an immediate neural damage [8] which may be preceding the pathophysiology of certain neurological diseases, such as Alzheimer's disease or stroke [8].

### 1.4 Cerebral Artery Function

Cerebral arteries supply blood to the brain (Fig. 1). The main arteries that originally irrigate the brain are the anterior, middle, and posterior cerebral arteries. The anterior cerebral artery (ACA) brings blood to the medial surface of the frontal and parietal lobes, the anterior section of the basal ganglia and internal capsule, the olfactory bulb and tract, and the anterior part of the corpus callosum [9]. The middle cerebral artery (MCA), the largest of the cerebral arteries, irrigates almost all of the basal ganglia, the posterior and anterior internal capsules, and the lateral sides of the temporal, frontal, and parietal lobes [10]. The posterior cerebral artery (PCA) supplies most of the hypothalamus, thalamus, midbrain, splenium of the corpus callosum, medial part of the temporal lobe (including the hippocampal formation), posterior medial parietal lobe, and the medial and inferior parts of the occipital lobe [11].

The large cerebral arteries contribute significantly to the total cerebral vascular resistance, and their tone considerably determines the overall blood flow and the microvascular pressure [12]. Thus, abnormal changes in the tone of these arteries could contribute to the development of pathological conditions observed in several neurological conditions [12].

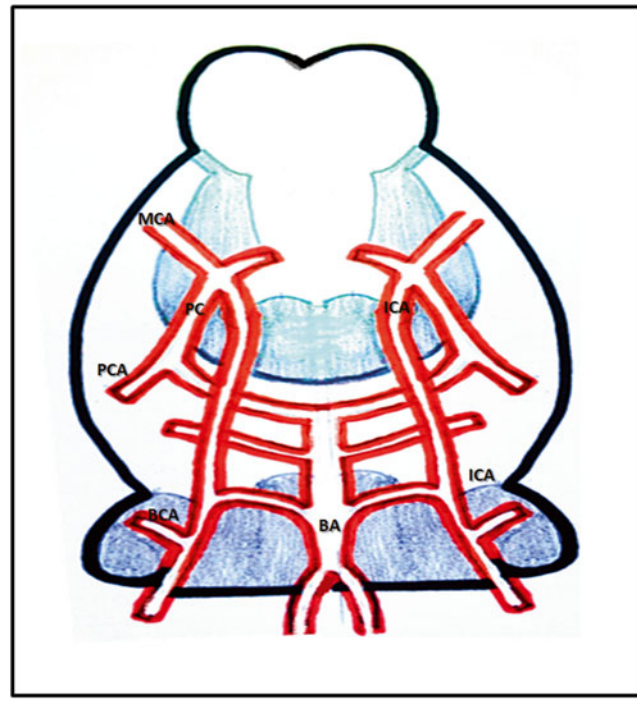


**Fig. 1** Schematic diagram of the ventral rat brain surface showing the location of the basilar artery. *MCA* middle cerebral artery, *PC* posterior communicating artery, *ICA* internal carotid artery, *PCA* posterior cerebral artery, *BA* basilar artery

## *1.5 Cerebrovascular Diseases Caused by Changes in Cerebral Artery Tone*

Maintaining a normal cerebrovascular tone is crucial for sustaining a sufficient perfusion to the brain. When blood flow to the brain is reduced, the lack of oxygen and nutrients may cause neurons to die. Thus, disruptions in tone contribute to the pathophysiology of several neurological conditions, like Alzheimer's disease and hemorrhagic and ischemic stroke. In this section, these conditions are discussed.

### *1.5.1 Hemorrhagic Stroke*

Intracerebral hemorrhage occurs when a weak blood vessel ruptures inside the brain. When blood starts to leak, the pressure inside the brain increases causing damage to the surrounding milieu. An adaptive mechanism, although an unsafe complication, is constriction of the involved cerebral artery and vasospasm [13]. These changes in the vascular tone reduce the blood flow to the part of the brain supplied by the injured artery, increasing the risk of ischemic stroke occurrence [14].

In the case of subarachnoid hemorrhage (Fig. 2), a blood vessel bursts outside the brain, causing a buildup of blood between the pia mater and the arachnoid membrane [13]. Vasospasm also occurs in this case and may also cause neuronal damage and ischemic stroke [14, 15].

Shortly after a hemorrhagic stroke, cerebral autoregulation is impaired, making cerebral vessels incapable of responding adequately

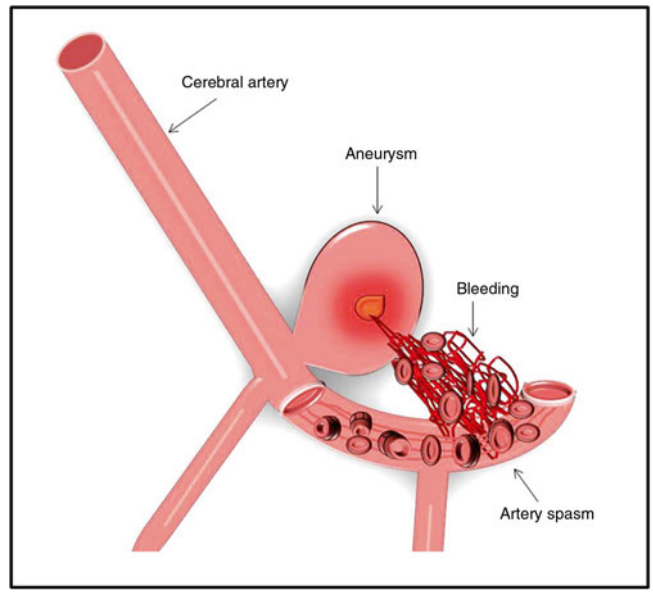


**Fig. 2** Subarachnoid hemorrhage leading to cerebral artery spasm. When blood vessel bursts outside the brain, the space surrounding the brain (the subarachnoid space) is filled with blood leading to cerebral blood vessel vasospasm (narrowing)

to changes in transmural pressure [16]. Moreover, vasoconstrictor receptors, like those for serotonin and endothelin, are upregulated [17]. These events, in turn, could contribute to subsequent cerebrovascular conditions like ischemia or hemorrhage.

### 1.5.2 Ischemic Stroke

Ischemic stroke takes place when a cerebral artery is obstructed, either by atherosclerosis or blood clots forming thrombosis or embolism [13]. When the blood flow to the particular part of the brain supplied by the blocked artery becomes drastically reduced, the lack of adequate oxygen supply causes neuronal damage and death.

In the acute phase after ischemic stroke, the VSMC and endothelial cells become less responsive [18, 19]. In the chronic phase, atherosclerosis may occur due to an upregulation in angiogenic and apoptotic factors in the endothelium [20]. Both of these events decrease blood flow to the brain, inducing even further damage and possibly increasing the risk of a secondary stroke.

### 1.5.3 Alzheimer's Disease

Although Alzheimer's disease develops as a result of neuronal injury induced by the accumulation of β-amyloid peptide [21], vasculopathy and changes in cerebrovascular tone also contribute to an increased susceptibility to this disease [22–25]. For example, changes in vascular tone caused by hypertension or atherosclerosis increase the risk for Alzheimer's disease [26]. Moreover, the β-amyloid peptide has been shown to be associated with vasculopathy by upregulating endothelial responses [27] and impairing cerebrovascular autoregulation [28].

### 1.6 Regulation of Cerebral Artery Tone

Regulating the tone of cerebral arteries is essential in maintaining sufficient cerebral blood flow. When the perfusion pressure increases in the brain, the cerebral vessels constrict in order to maintain a relatively stable blood flow [29]. This change in resistance is largely caused by the myogenic tone, which significantly affects autoregulation of the blood flow in the brain. Several mechanisms that govern regulation of the cerebrovascular tone include calcium-induced vasoconstriction, calcium sensitization, excitation-contraction coupling, endothelial responses, and potassium channels. Thus, understanding these mechanisms will pave the way to developing therapeutic strategies in preventing the pathological events that arise from a disruption in cerebrovascular tone.

#### 1.6.1 Calcium-Mediated VSMC Contraction

VSMC contraction is dependent on calcium. When agonists that promote contraction, such as angiotensin II, endothelin I, or norepinephrine bind to their G-protein-coupled receptors, phospholipase C (PLC) is activated. This enzyme catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into the secondary messengers inositol trisphosphate ($IP_3$) and diacylglycerol (DAG).

$IP_3$ binds to ligand-gated calcium channels on the sarcoplasmic reticulum, resulting in calcium release into the cytosol. Calcium then binds to calmodulin to form a complex that activates myosin light chain (MLC) kinase, which in turn phosphorylates myosin light chain. Phosphorylated light chain of myosin then binds to the actin filament in a crossbridge and causes contraction. Hence, the degree of VSMC contraction is dependent on the level of myosin phosphorylation.

To stop the contraction of VSMC, the enzyme MLC phosphatase is activated. In turn, the MLC phosphatase removes the phosphate group of the light chain of myosin and thus terminates the VSMC contraction [30]. Of note, calcium also enters VSMC from extracellular stores through ligand-gated calcium channels in the plasma membrane.

DAG also promotes VSMC contraction by activating protein kinase C (PKC), which in turn phosphorylates specific proteins like L-type calcium channels that further regulate the crossbridge cycle. The L-type calcium channels are voltage-gated channels that open in response to membrane depolarization, which is brought on by stretch of the VSMC.

#### 1.6.2 Calcium Sensitization via the RhoA/ROCK Pathway

When the blood pressure against VSMC increases, mechanotransduction results in the activation of the RhoA/ROCK pathway, which in turn promotes remodeling of the actin cytoskeleton and calcium sensitization. RhoA is a guanine nucleotide (GTP)-binding protein that is activated by guanine nucleotide exchange factors (GEF) that convert the inactive GDP-bound RhoA into the active GTP-bound form. RhoA activates Rho kinase (ROCK), which in turn activates LIM kinase-2 (LIMK2). This leads to the

phosphorylation and subsequent inactivation of cofilin, and thus a depletion of G-actin and accumulation of F-actin [31–34]. These changes in the actin cytoskeleton cause VSMC remodeling.

VSMC contraction is regulated by either the cytosolic calcium concentration or the calcium sensitivity. Calcium sensitivity is regulated by inhibition of MLC phosphatase, thus promoting the phosphorylated form of the light chain of myosin. After getting activated by RhoA, ROCK phosphorylates MLC phosphatase and inactivates it, thus increasing calcium sensitization and promoting vasocontraction. Specific inhibitors of ROCK, such as Y-27632 or fasudil, induce VSMC relaxation and reduce blood pressure and cerebral resistance [35, 36].

### 1.6.3 Excitation-Contraction Coupling in VSMC

Unlike skeletal muscle cells, VSMC do not require neuronal input or an action potential to contract. VSMC contract to other kinds of stimuli like hormones (angiotensin II, endothelin I, epinephrine), metabolic substances (adenosine, hydrogen ions, potassium ions), or physical interactions (mechanical stretch, shear stress). In these cases, there is no excitation-contraction coupling.

Neuronal stimuli that stimulate VSMC contraction are autonomic. VSMC are innervated mainly by the sympathetic nervous system. The main neurotransmitter used by the sympathetic nervous system is norepinephrine, which binds to its metabotropic adrenergic receptors to activate VSMC to contract by raising intracellular calcium concentrations. This pathway then involves binding to calmodulin and activating MLC kinase as described in the previous section. The parasympathetic input employs the neurotransmitter acetylcholine which binds to muscarinic receptors on the VSMC to promote contraction. However, both sympathetic and parasympathetic inputs could promote vasodilation, depending on the receptors activated on the VSMC.

### 1.6.4 Endothelium-Mediated Regulation of Cerebrovascular Tone

The endothelial cells of the cerebral arteries release vasoactive factors that regulate the tone of the vessels. The most important ones are endothelium-derived hyperpolarizing factor (EDHF), prostacyclin ($PGI_2$), and nitric oxide (NO).

EDHF is secreted by endothelial cells after endothelial intracellular calcium increases [37] or through myoendothelial gap junctions [38, 39]. It causes VSMC to relax and thus causes vasodilation. On the other hand, $PGI_2$ is a prostanoid produced by the enzyme cyclooxygenase (COX) from arachidonic acid. $PGI_2$ binds to its cell surface receptor on VSMC to induce an increase in 3′–5′-cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA) and causes vasodilation [40, 41].

NO is a small molecule produced by endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) nitric oxide synthase. This gaseous molecule activates guanylate cyclase to form cyclic guanosine monophosphate (cGMP), which in turn activates

protein kinase (PKG) [42]. PKG activates MLC phosphatase [43, 44] and induces VSMC relaxation and vasodilation [30, 45, 46].

#### 1.6.5 Potassium Channels Involved in Regulation of Cerebrovascular Tone

When potassium channels open and potassium ions start to efflux out of the cell, the plasma membrane undergoes hyperpolarization, inducing the closure of voltage-gated calcium channels, like the L-type calcium channels, and vasorelaxation. Potassium channel activity majorly influences vascular tone and vasorelaxation [47, 48].

Adenosine triphosphate (ATP)-dependent potassium channels ($K_{ATP}$) are linked to metabolic activity. When the intracellular ratio of ATP to adenosine diphosphate (ADP) decreases, the $K_{ATP}$ channels open and induce vasorelaxation [29]. This adaptive mechanism is needed in cases of insufficient oxygenation, and inhibition of $K_{ATP}$ prevents hypoxic vasorelaxation in cerebral and peripheral blood vessels [49, 50].

The inward-rectifier potassium ion channels ($K_{ir}$) selectively allow potassium to pass more easily into the VSMC cell than out of the cell [51, 52] and promote vasorelaxation. In fact, $K_{ir}$ has been shown to couple cerebral blood flow to neuronal activity in rats since active neurons release potassium ions into the extracellular space [53].

Voltage-gated potassium channels ($K_V$) also help regulate cerebral artery tone. $K_V$ channels are opened by depolarization of the VSMC and promote vasorelaxation. This negative feedback mechanism occurs in response to arterial constriction [48]. Inhibition of $K_V$ induces vasoconstriction of cerebral arteries [54, 55].

### 1.7 Assessment of Blood Vessel Reactivity

Small blood vessels' (less than 100 μm) isometric responsiveness to hormones and other agonists can be measured by using the wire myograph (Mulvany myograph). This device was developed by Mulvany and Halpern [56] to investigate the active and passive tension of blood vessels from a variety of arteries, including cerebral arteries [57, 58]. The wire myograph system is a highly sensitive tool used to investigate the responsiveness of blood vessels to different stimuli and hormones. In this chapter, we explain the steps of myograph measurement which consist of blood vessel isolation, mounting, normalization, assessment of tissue viability, and construction of a cumulative concentration response curve [59].

## 2 Materials

### 2.1 Buffers and Solutions (See Note 1)

1. Physiological salt solution (PSS): mmol/L: 115 NaCl, 4.7 KCl, 1.4 $MgSO_4$, 7.0 $H_2O$, 5 $NaHCO_3$, 1.2 $K_2HPO_4$, 1.1 $Na_2HPO_4$, 1.0 $CaCl_2$, 20 HEPES, and 5 glucose; pH 7.35.
2. High-potassium solution (123 mmol/L $K^+$): KCl 123.70 mmol/L, $MgSO_4 \cdot H_2O$ 1.17 nmol/L, $KH_2PO_4$ 1.18 mmol/L, $CaCl_2$ $2H_2O$ 2.5 mmol/L, $NaHCO_3$ 25 mmol/L, EDTA 0.03 mmol/L, glucose 5.5 mmol/L.

3. Acetylcholine (C3389, Sigma) 10 mmol/L stock solution.
4. Norepinephrine (A7257, Sigma) 1 mmol/L stock solution.

### 2.2 Tools and Instruments

#### 2.2.1 Dissection Tools (Please Refer to Fig. 3)

1. Large dissection dish (93 mm diameter × 22 mm H; DD-90-S-BLK, Living Systems Instrumentation) (*see* **Note 2**).
2. Deep dissection dish (100 mm diameter × 50 mm H; DD-100-D-BLK, Living Systems Instrumentation).
3. Stereo microscope (C-FMBN, Nikon) (*see* **Note 3**).
4. Cold Light Source/Fiber Optic Illumination (NCL 150, Volpi).
5. Multi-purpose Iris scissors (11 cm long, straight).
6. Vannas scissors (8 cm long, straight, 3 mm blades).
7. Spring scissors (12.5 cm long, 9 mm, straight).
8. Dressing tissue forceps.
9. Dumont Tweezers #5 (11 cm long; 0.025 × 0.005 mm).
10. Dissection pins (0.1, 0.2, and 0.5 mm).

#### 2.2.2 Multi-Wire Myograph System and Data Recording

1. Multi-wire myograph system (620 M, DMT, Aarhus, Denmark) (Fig. 4).
2. Stainless steel wires (diameter = 40 μm) (*see* **Note 4**).
3. Mounting jaws (one jaw is connected to the false transducer, the other one is mobile and is connected to a micrometric screw). The head is suspended in the myograph chamber (Fig. 5).
4. PowerLab 4/35 (AD Instruments).

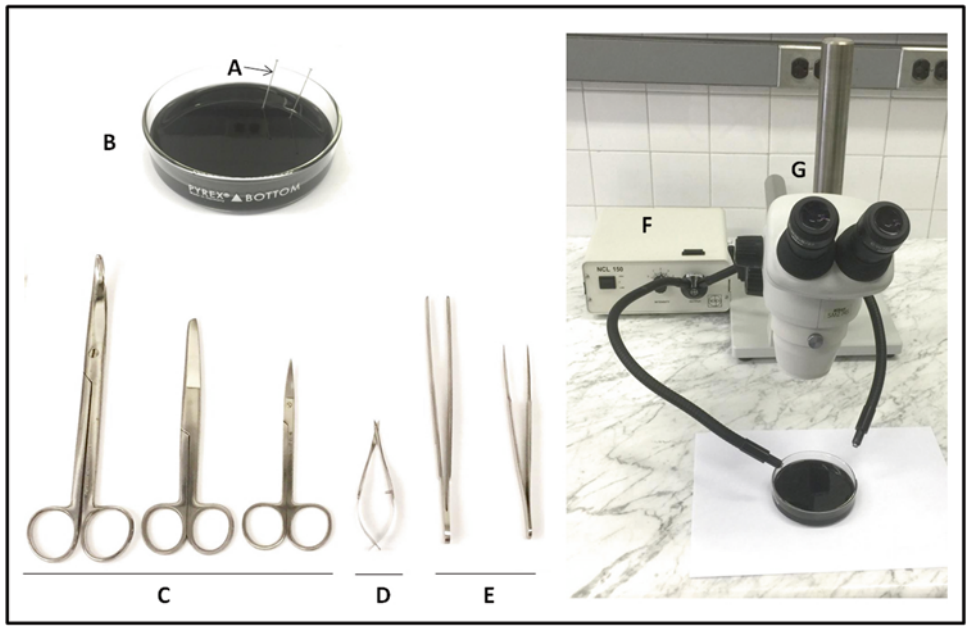


**Fig. 3** Dissection tools and microscope. (**a**) Dissection pin; (**b**) coated plate; (**c**) scissors; (**d**) micro-scissors; (**e**) forceps; (**f**): fiber-optic illuminator; (**g**) stereo zoom microscope

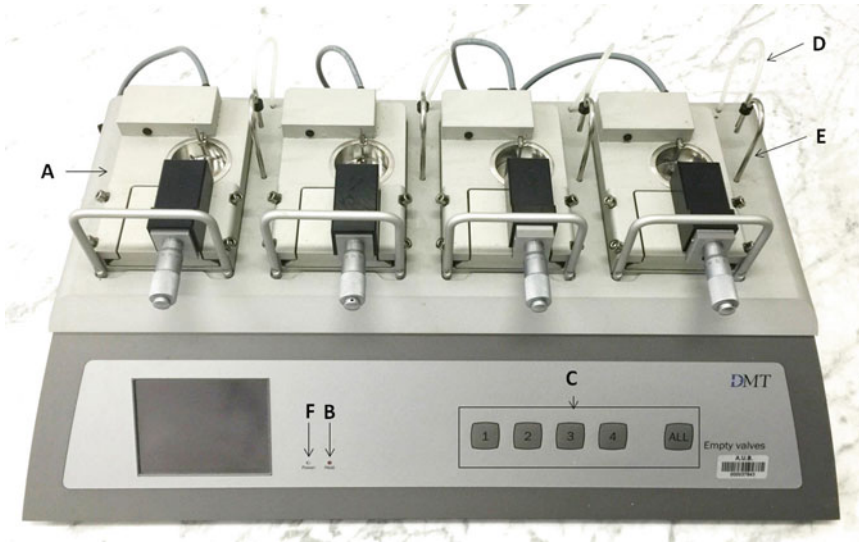


**Fig. 4** Multi-wire myograph system (620 M, DMT, Aarhus, Denmark). The system is composed of (**a**) multi-wire myograph unit, (**b**) heat indication, (**c**) valve buttons myo-interface display, (**d**) gas tube, (**e**) suction tube

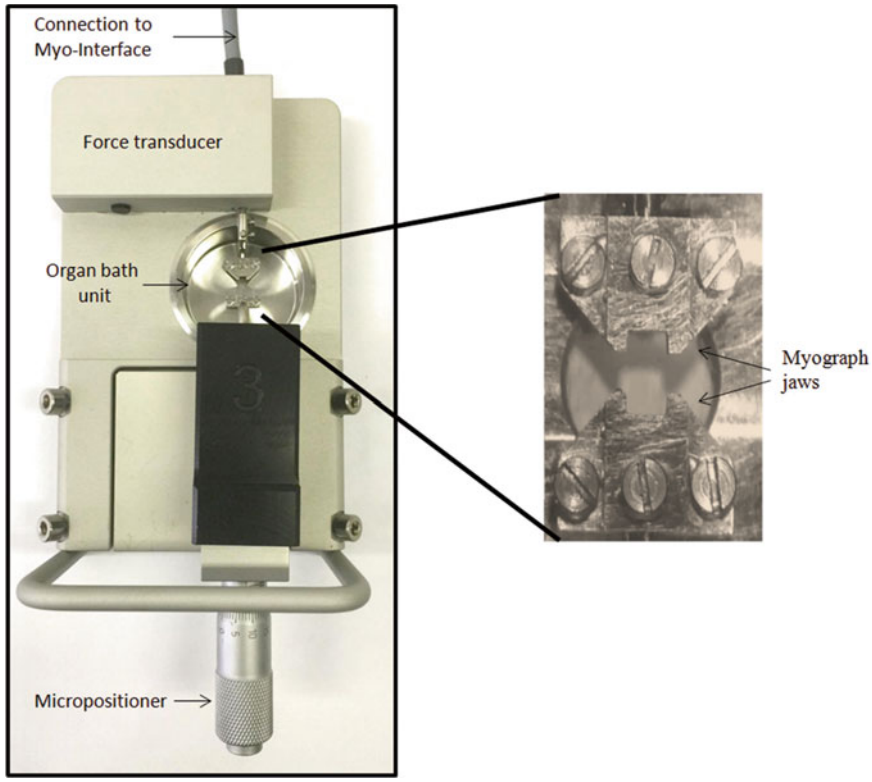


**Fig. 5** Multi-wire myograph unit

## 3 Methods

### 3.1 Dissection Procedure for Rat Basilar Artery

1. Male Sprague-Dawley rats (200–250 g) are used for the study (*see* **Note 5**).
2. Cerebral injury rat models are used or subarachnoid hemorrhage is induced in rats by injection of 250 μL autologous blood into the basal cisterns [60].
3. After 3 days, the rats are euthanized by $CO_2$.
4. The brains are quickly removed and placed in deep dissection dish containing cold PSS (*see* **Note 6**).
5. The basilar artery (Fig. 1) is isolated under the microscope and then transferred into 5 mL large dissection dish containing ice-cold PSS (*see* **Notes 7–10**).
6. The artery is pinned up on a coated bottom Sylgard material (*see* **Note 11**).
7. The dissection and cleaning are done with the help of a pair of trabecular scissors and a pair of fine forceps (*see* **Notes 12** and **13**).
8. The basilar arteries are cut into 1.5–2 mm ring segments (three to four segments from each animal) (*see* **Note 14**).

### 3.2 Mounting of the Vessel on the Wire Myograph

1. The chamber bath is washed three times with distilled $H_2O$ and twice with PSS.
2. PSS is added to each chamber bath and maintained at 37 °C and perfused with 5 % $CO_2$.
3. Two 40 μm stainless steel segments (4 cm) of the wires are isolated (*see* **Notes 15** and **16**).
4. The wires are fixed with screws on two mounting hits. One is connected to a force transducer while the other is connected to a micrometer screw, so it can move (to stretch the blood vessels) (Figs. 4 and 5).
5. The heads are suspended in a myograph chamber which is initially filled with cold PSS. Keep the wire as straight as possible.
6. The basilar artery is transferred to the myograph chamber.
7. The mounting jaws are widely spread apart. With fine forceps, insert one wire into the lumen of the basilar artery (*see* **Note 17**). Maintain the filled wire vertical in the gap (*see* **Notes 18** and **19**).
8. In order to have enough space for the artery to pass, the heads are moved apart and wires bent out. The heads are now gently pulled further up the wire (Fig. 5).
9. The vessel is adjusted to bring the distal part into the gap between the mounting heads. With the screwdriver pressed against the wire, the vessel is held back and positioned in the

gap. The artery should be able to move freely in the longitudinal direction and therefore should not be clamped between the lower parts of the support.

10. The wire is tightened with a pole bent to the lower left screw and fixed in place. It is important that the wire is tight and follows the support all the way (*see* **Note 20**).
11. In order to get a second wire into the lumen from the top end, remove the part of the vessel that is not in the gap between the jaws.
12. In order to close the jaws, use a pair of forceps to rub the tissue away.
13. The wires are adjusted in order to have parallel wires and simultaneously horizontal levels (*see* **Note 21**).
14. Turn on the heating and gas pump. The system is now ready for use.
15. Rotate the micrometer counterclockwise until the pre-tension is achieved.

### *3.3 Measuring Isometric Response*

Contractile responses of basilar artery are examined in a wire myograph (Danish Myo Technology, 620) by recording isometric tension (*see* **Notes 22** and **23**) (Fig. 6).

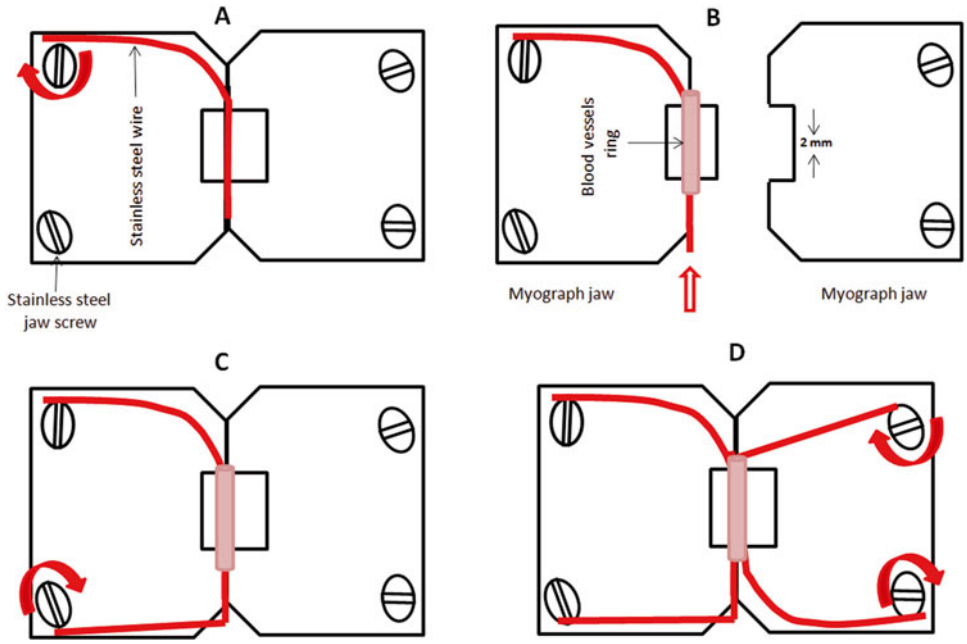


**Fig. 6** Mounting of basilar artery: (**a**) Stainless steel wire is attached to the stainless steel jaw screw. (**b**) Stainless steel wire is inserted through basilar artery. (**c**) The wire is attached to the second screw. (**d**) Repeat same procedure with the second wire

### 3.3.1 Resting Tension Internal Circumference Relationship

1. The sensitivity of blood vessels to different agonists is dependent on the degree of muscle stretch, so it is important to normalize the blood vessel dimensions.
2. It is useful to have optimal internal circumference (optimal stretch) that gives the maximum contraction. This can be calculated using active tension/internal circumference relationship.
3. Blood vessel lumen circumference is considered $L_{100}$ when it is fully relaxed under a transmural pressure of 100 mmHg. $L_{100}$ is calculated using resting tension-internal circumference curve.
4. Another advantage of resting tension-internal circumference curve is that the blood vessel's wall thickness at $L_{100}$ (μm) can be calculated using microscopy. The wall thickness of the mounted basilar artery is measured using maximum magnification of the dissection microscope.
5. Resting tension-internal circumference curve is used also to normalize the vessel's dimensions.
6. The maximum active tension can be achieved at $0.9 \times L_{100}$. Any segment giving more than 10 mN is used in the experiments.

*Procedure*

1. Fill the chamber with 1 mL PSS maintained at 37 °C and perfused with 5% $CO_2$ (*see* **Notes 24** and **25**).
2. Basilar artery is subjected to stretch using the micropositioner (about 10 μm) after being held for 1 min.
3. Repeat step 2 until the wall tension gives 1 mN/mm.
4. Basilar artery is subjected to relaxation (as done in **steps 2** and **3** but the muscle is relaxed) until the tension becomes 0 mN/mm.
5. The points between tension and internal circumference are recorded and expressed as an exponential curve.

### 3.3.2 Vessel Viability

This is an important step and is necessary to test whether the vascular muscle is intact and contracts normally. Different vasoconstrictors can be used, such as high-potassium solution (123 mmol/L; NaCl replaced by KCl on a molar basis), norepinephrine (10 μmol/L), or high-potassium solution containing 10 μmol/L noradrenaline. In order to test the ability of vessel to contract normally, five blood vessel stimulations are applied (lasting 2 min each), each followed by a 5-min washout period with PSS (*see* **Notes 26–29**).

*Procedure*

1. Fill the chamber with 1 mL PSS maintained at 37 °C and perfused with 5% $CO_2$.
2. A pre-tension of 2 mN/mm is applied to each basilar artery (*see* **Note 30**).

3. Basilar artery is subjected to a 45-min period of normalization.
4. High-potassium solution (123 mm/L $K^+$) is added to the baths. Wait for 2 min (*see* **Note 29**).
5. Wash the blood vessel with PSS two times and incubate it in PSS to allow the blood vessel to relax and reach the baseline.
6. Repeat **steps 4** and **5**.
7. Add norepinephrine (10 μmol/L) to the chamber bath for 2 min.
8. Repeat **step 5**.
9. Add high-potassium solution containing 10 μmol/L noradrenaline to the chamber and wait for 2 min.
10. All contractions are recorded and measured using a PowerLab unit and LabChart software (ADInstruments).

### 3.3.3 Checking Endothelial Function (Vasodilator Response Curve)

It is very important to test the integrity of endothelium in the basilar artery after blood vessel isolation, cleaning, or mounting (*see* **Note 31**). This procedure is used to test the success of endothelium removal (*see* **Note 32**). In order to have vasodilator response curve, blood vessels should be pre-contracted using a suitable agonist such as noradrenaline (0.1–3 μM) or (*see* **Notes 33** and **34**) to produce 60–80% of the response.

*Procedure*

1. Fill the chamber with 1 mL PSS maintained at 37 °C and perfused with 5% $CO_2$.
2. Slowly stretch the vessel to its normalized micrometer setting (pretension of 2 mN) and let the vessel equilibrate for 45 min.
3. Pre-constriction with a sub-maximal concentration (3 μmol/L) of noradrenaline is induced (*see* **Note 35**).
4. 2 μL of acetylcholine (5 μmol/L) is added in order to have $10^{-8}$ mol/L acetylcholine.
5. Once the vessel starts to relax (after 2–3 min), 0.18 μL of acetylcholine (50 μmol/L) is added to give a final concentration of 0.1 μmol/L acetylcholine in total volume within the chamber.
6. Add 0.18 μL of acetylcholine (0.5 mmol/L) to give 1 μmol/L acetylcholine and wait for 3 min.
7. Add 0.18 μL of acetylcholine (5 μmol/L) stock solution to give 10 μmol/L.
8. After 3 min, add 0.9 μL of acetylcholine (10 mmol/L; stock solution in freezer) to give 0.1 mmol/L acetylcholine. Wait for 4 min.
9. Wash the blood vessels four times with PSS to reach initial tension.

10. Relaxation responses of basilar artery are recorded using a PowerLab 4/35 unit and LabChart software (ADInstruments) and expressed as a percentage of the induced noradrenaline-induced contraction.

### 3.3.4 Cumulative Concentration-Response Curves

A standard cumulative concentration-response curve is defined by four parameters: the baseline tension, the maximum tension (*see* **Note 36**), the slope of the curve, and the agonist concentration that provokes a response halfway between baseline and maximum tension ($EC_{50}$). The $EC_{50}$ is the molar concentration of an agonist that produces 50% of the maximum contraction for that agonist. Different agonists with different concentrations are used in order to create cumulative concentration-response curves (*see* **Notes 37** and **38**). The well-known vasoconstrictors are 5-hydroxytryptamine type 1B (5-$HT_{1B}$ receptor agonist), angiotensin II ($AT_1$ and $AT_2$ receptor agonist), and endothelin-1 type A and B ($ET_A$ and $ET_B$ receptor agonist, $10^{-14}$–0.3 μmol/L).

*Procedure*

1. Fill the chamber with 1 mL PSS maintained at 37 °C and perfused with 5% $CO_2$.
2. Allow the basilar artery to equilibrate in PSS for 45 min.
3. Blood vessels are normalized. $L_{100}$ is calculated and the internal circumference of 0.9 $L_{100}$ is used in order to have maximum active tension.
4. Cumulative concentration-response curves are obtained to the following agonists: 5-$HT_{1B}$ ($10^{-11}$–30 μmol/L), $AT_1$ and $AT_2$ ($10^{-12}$–3 μmol/L), and $ET_A$ and $ET_B$ ($10^{-14}$–0.3 μmol/L).
5. Following each concentration-response curve, the basilar artery is washed with PSS and allowed to relax (around 30 min) before the next vasoconstrictor is applied.
6. Contractile responses of basilar artery are recorded using a PowerLab unit and LabChart software (ADInstruments). This software will automatically generate the $EC_{50}$.
7. The contractile responses to different agonists are expressed as increase in isometric tension divided by the normalized lumen diameter of blood vessels (active wall tension (mN/mm)).
8. The $EC_{50}$ for each cumulative concentration-response curve is calculated.
9. The sensitivity of basilar artery to each vasoconstrictor is expressed as the - log $EC_{50}$.
10. It is very important to check the integrity of the endothelium at the end of the experiment with the silver nitrate stain (or repeat Subheading 3.3.2).

*3.3.5 Denuding the Endothelium*

In order to assess the endothelial dependence of the actions of different vasoconstrictors on the basilar artery, the endothelium is removed mechanically by passing a 50–75 μm human hair through the basilar artery lumen and rubbing the intimal surface. Endothelial denudation is considered successful if the precontracted basilar artery has no response to 10 μmol/L acetylcholine.

## 4 Notes

1. The solutions should be prepared freshly for each experiment. If left for several weeks (even at 4 °C), the buffers might produce fungi, which will affect the experiment and results.
2. Dissection dishes are lined with black Sylgard material for tissue fixation during dissection procedures.
3. It is preferable to use an inverted microscope instead of an upright microscope, since accessories like pH meter or oxygen electrode might be used in the bath. An inverted microscope has more space for these accessories.
4. Stainless steel wires are better than tungsten wires due to oxidation.
5. The rats are maintained at stable room temperature, humidity, and sleep cycle, with regular access to uncontaminated drinking water and food in order to maintain stable physiologic cycles of metabolism.
6. After euthanizing the animal, remove the brain and isolate the basilar arteries as quickly as possible. If cerebral arteries are left on the brain for too long, toxins released from the dying neurons will affect the blood vessels and reduce blood vessel reactivity.
7. It is easy to differentiate between arteries and veins. The wall (tunica media) of arteries is thicker than that of veins. The lumen of an artery is narrower than that of a vein.
8. This technique requires a careful grip of the scissors. Move the blades directly up and make the cuts parallel to the vessel wall, in order to avoid cutting the vessel itself.
9. Work fast and carefully to avoid damaging endothelial cells in the vessels.
10. Branched blood vessels are challenging to work with, so the middle cerebral arteries are not often used. The superior cerebellar arteries or posterior cerebral arteries (specifically the third branch) are easier to isolate because they have longer segments that are not branched. However, if a branched vessel is the one you need to isolate, it is possible, but tedious. Ligatures may be required to isolate a non-branchy segment and more effort will be made in the mounting process.

11. It is important and necessary to stretch the blood vessels during dissection to aid the microdissection.
12. Most of the fat is removed firstly from one side of the vessel and then from the other side, and finally from the smaller branches.
13. The vessel should be as clean as possible because excessive fat on the vessel affects the muscle contractility.
14. Interestingly, the blood vessels can be left in the fridge for a couple of days and then actually be used in the wire myograph experiment. However, it is crucial to leave the vessels in the fridge at 4 °C, and not at –20 °C or –80 °C.
15. In general, small arteries with internal diameters between 100 and 500 μm can be mounted on 40 μm stainless steel wires. In case the arterioles have an internal diameter of less than 60 μm, the 25 μm tungsten wires are used.
16. Two wires of approximately 2 cm length, with blunt ends and as straight as possible, are required.
17. The lumen of the vessel often closes when it is cut. To open the blood vessels, grab it firmly with the forceps as close to the proximal end as possible. The wire tip can also be used to open the lumen.
18. Once the wire is inside the lumen, the vessel is slowly and carefully drawn up the wire to avoid damaging the endothelial layer and vascular wall. Take special care when the wire tip is passing the branching points.
19. Remember that the transducer is very sensitive to pressure at the transducer head level, so do not apply too much force when you tighten the screws. This also applies any time you have the heads together.
20. Screws are turned in clockwise so as to tighten the wires. When tightening the screws, always place the wire clockwise around the screw head.
21. If the wires are not parallel, move the wires with the use of forceps. Moving the heads back and forth makes it easier to see how the wires are positioned.
22. For beginners that are new to this technique, take your time to practice the entire procedure before beginning the actual research experiments.
23. In the process of practicing, it is advisable to use mesenteric arteries and to keep repeating the same experiment, such as KCl-induced constriction or phenylephrine concentration-response curve, until consistent results are obtained.

24. If you note any contraction in the vessel, do not force it; let it relax, and then change the PSS in the chamber bath.
25. In case problems arise, first check the pH, oxygenation, and temperature in the bath.
26. Since force production and sensitivity of vessels to different agonists are dependent on the extent of stretch (according to active tension-length relationship), it is essential to conduct contraction studies under isometric conditions to prevent compliance of the mounting wires.
27. You can use only segments that developed more than 2 mN force to high potassium with intact endothelium.
28. In the case of endothelium-denuded segment, you can use segments that developed 1.5 mN force to high-potassium solution.
29. It is very important to have a fixed time interval between each dose.
30. Basilar arteries may vary with length and size, and it is important to have maximum contraction for normalization. High-potassium solution (123 mmol/L) or norepinephrine (10 μmol/L) can be used to normalize experimental data as a percent of high potassium or norepinephrine-induced contraction.
31. If the endothelium is intact, acetylcholine will induce relaxation on the blood vessels (vasodilator curve).
32. Before starting vasodilator response curve experiments, make sure that the blood vessel has cholinergic receptors (placental vessels lack cholinergic receptors).
33. Phenylephrine or norepinephrine produces a sustained constriction (tonic) which is stabilized after 3 min.
34. This tonic contraction is uniform along the length of the basilar artery.
35. This will produce a contraction of 80% of the maximum response to high-potassium solution.
36. Concentration-response curve can be obtained by using large ranges of concentrations (until receptor saturation) of an agonist.
37. Concentration-response curve is usually logarithmic. The *X*-axis plots the concentration of an agonist while the *Y*-axis plots response (tension mN/mm).
38. Some agonists bind irreversibly to their receptor and cannot be washed out with physiological salt solution. Irreversible agonists should be used at the end of experiments.

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*Conflict of interests*

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