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Ex vivo pressurized hippocampal capillary-parenchymal arteriole preparation for functional study

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Dear Aaron Berard, dear JoVE Editors,

We would like to thank the Editors and Reviewers of JoVE for the thorough review that our recently submitted manuscript JoVE60676 "*Ex vivo* pressurized hippocampal capillary-parenchymal arteriole preparation for functional study" received. We have addressed all of the comments (highlighted in yellow in manuscript) and include a detailed response to each of the referees' and editors' comments.

We appreciate this opportunity to resubmit our manuscript and look forward hearing from you.

Sincerely,

Sincerely,
Fabrice Dabertrand

TITLE:

Ex Vivo Pressurized Hippocampal Capillary-Parenchymal Arteriole Preparation for Functional Study

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KEYWORDS:

ion channel, brain capillary, cerebral parenchymal arterioles, pressure myography, myogenic reactivity, capillary to arteriole electrical signaling, neurovascular coupling, hippocampus, vessel cannulation, microcirculation

SUMMARY:

The present manuscript details how to isolate hippocampal arterioles and capillaries from the mouse brain and how to pressurize them for pressure myography, immunofluorescence, biochemistry, and molecular studies.

ABSTRACT:

From subtle behavioral alterations to late-stage dementia, vascular cognitive impairment typically develops following cerebral ischemia. Stroke and cardiac arrest are remarkably sexually dimorphic diseases, and both induce cerebral ischemia. However, progress in understanding the vascular cognitive impairment, and then developing sex-specific treatments, has been partly limited by challenges in investigating the brain microcirculation from mouse models in functional studies. Here, we present an approach to examine the capillary-to-arteriole signaling in an ex vivo hippocampal capillary-parenchymal arteriole (HiCaPA) preparation from mouse brain. We describe how to isolate, cannulate, and pressurize the microcirculation to measure arteriolar diameter in response to capillary stimulation. We show which appropriate functional controls can be used to validate the HiCaPA preparation integrity and display typical results, including testing potassium as a neurovascular coupling agent and the effect of the recently characterized inhibitor of the Kir2 inward rectifying potassium channel family, ML133. Further, we compare the

responses in preparations obtained from male and female mice. While these data reflect functional investigations, our approach can also be used in molecular biology, immunochemistry, and electrophysiology studies.

INTRODUCTION:

The pial circulation on the surface of the brain has been the object of much study, partly because of its experimental accessibility. However, the topology of the cerebral vasculature creates distinct regions. In contrast to the robust pial network rich in anastomoses with substantial capacity for redirecting the blood flow, the intracerebral parenchymal arterioles (PAs) present limited collateral supply, each of them perfusing a discrete volume of nervous tissue[1,2]. This creates a bottleneck effect on the blood flow which, combined with unique physiological features[3-8], makes intracerebral arterioles a crucial site for cerebral blood flow (CBF) regulation[9,10]. Despite the technical challenges inherent to the isolation and cannulation of PAs, the last decade has seen an increased interest in ex vivo functional studies using pressurized vessels[11-17]. One of the reasons for this increased interest is the considerable research effort conducted on neurovascular coupling (NVC), the mechanism sustaining the brain functional hyperemia[18].

Regionally, CBF can rapidly increase following local neural activation[19]. The cellular mechanisms and signaling properties controlling NVC are incompletely understood. However, we identified a previously unanticipated role for the brain capillaries during NVC in sensing neural activity and translating it into a hyperpolarizing electrical signal to dilate upstream arterioles[20-22]. Action potentials[23,24] and opening of large-conductance Ca^{2+}-activated K^+ (BK) channels on the astrocytic endfeet[25,26] increase the interstitial potassium ion concentration $[K^+]_o$, which results in activation of strong inward rectifier K^+ (Kir) channels in the vascular endothelium of capillaries. This channel is activated by external K^+ but also by hyperpolarization itself. Spreading through gap junctions, the hyperpolarizing current then regenerates in adjacent capillary endothelial cells up to the arteriole, where it causes myocyte relaxation and CBF increase[20,21]. The study of this mechanism led us to develop a pressurized capillary-parenchymal arteriole (CaPA) preparation to measure the arteriolar diameter during capillary stimulation with vasoactive agents. The CaPA preparation is composed of a cannulated intracerebral arteriole segment with an intact, downstream capillary ramification. The capillary ends are compressed against the chamber glass bottom by a micropipette, which occludes and stabilizes the entire vascular formation[20,21].

We previously made instrumental innovations by imaging CaPA preparations from the mouse cortex[20,21] and arterioles from the rat amygdala[13] and hippocampus[16,17]. As the hippocampal vasculature receives more attention due to its susceptibility to pathological conditions, here we provide a step-by-step method for CaPA preparation from the mouse hippocampus (HiCaPA) that can not only be used in functional NVC studies but also in molecular biology, immunochemistry, and electrophysiology.

PROTOCOL:

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of

the University of Colorado, Anschutz Medical Campus and were performed according to the guidelines from the National Institutes of Health.

1. Solutions

1.1. Use MOPS-buffered saline for the dissection and to keep samples at 4 °C before their utilization. Do not gas the solution. Prepare MOPS buffered saline with following composition: 135 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM glucose, 3 mM MOPS, 0.02 mM EDTA, 2 mM pyruvate, 10 mg/mL bovine serum albumin, pH 7.3 at 4 °C.

1.2. Use artificial cerebrospinal fluid (aCSF) as the bath solution and pipette solution. Gas both aCSF and Ca^{2+}-free aCSF with 5% CO_2, 20% O_2, and N_2 balance. Prepare the solution with the following composition: 125 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 4 mM glucose, 2 mM $CaCl_2$, pH 7.3 (with aeration with 5% CO_2, 20% O_2, and N_2 balance).

1.3. Obtain the maximal dilation in nominally Ca^{2+}-free aCSF (0 mM $[Ca^{2+}]_o$, 5 mM EGTA).

2. Organ chamber preparation

2.1. Insert borosilicate glass capillaries (outside diameter = 1.2 mm; inside diameter = 0.69 mm; length = 10 cm) into a glass puller. Pull the capillary to make a long, thin tip at one end.

2.2. To one side of the chamber, add a cannula that can connect to a miniature peristaltic pump to luminally pressurize the vessel. Under a dissection microscope, break the tip of the cannula so that it is small enough to fit the vessel of interest, but large enough to allow solutions to flow through the tip. Ensure that the tip is roughly 10–15 µm in diameter.

2.3. Fill the cannula using a syringe with an attached 0.22 µm filter with oxygenated aCSF. Make sure there are no air bubbles or debris in the cannula.

2.4. Add two more cannulas to the opposite side of the chamber. Do not break their tips.

3. Hippocampus dissection and isolation

3.1. Euthanize and decapitate a mouse. For this experiment, use an 8-week-old C57BL6/J mouse to compare differences between males and females. Inject the mouse with pentobarbital and decapitate with surgical scissors.

3.2. Using small dissection scissors, cut the skin along the midline at the top of its head. Move the skin off to the sides.

3.3. Starting at the caudal side of the skull, cut the skull along the midline until the olfactory bulbs are reached. Remove portions of the skull until the cerebrum is exposed.

3.4. Slowly remove the brain, starting near the nose of the mouse. Separate the brain from the olfactory bulbs, cranial nerves, and spinal cord by cutting through the structures with the small dissection scissors.

3.5. Place the brain in a dissection plate with enough MOPS solution to completely submerge it. Using a dissection microscope, place the brain in the center of the dissection plate with the ventral side facing down.

3.6. Using a razor blade, cut the brain in half along the longitudinal fissure. Hold the blade so that the sharp edge is parallel to the bottom of the dissection plate. Press the blade through the brain in one stroke. Move one hemisphere to the side of the plate.

3.7. Perform the following steps for each hemisphere separately or in parallel.

3.7.1. Place one hemisphere in the center of the plate so the midline is facing down. Then use the razor blade to cut along the transverse fissure to remove the cerebellum and brain stem. Push the blade straight through the tissue.

3.7.2. Rotate the hemisphere so that the medial side is facing up (**Figure 1A**). Use one spatula to hold the brain in place. Using a second spatula, insert the tip below the corpus callosum and scoop underneath to remove the thalamus, septum, and hypothalamus, covering the hippocampus (**Figure 1B**).

3.7.3. Ensure that the hippocampus is now visible as a curved structure near the posterior side of the cerebrum. Using one spatula to hold the cerebrum in place, use the second spatula to scoop the hippocampus out of the cerebrum (**Figure 1C**).

3.8. Transfer the hippocampi to a new dissection plate filled about halfway with MOPS solution. Discard the rest of the brain.

4. Hippocampal arteriole isolation

4.1. Complete the following steps for each hippocampus.

4.1.1. Pin down one of the hippocampi using small pins at each end of the section. The hippocampal artery is facing up.

4.1.2. Using very sharp forceps, gently stretch small sections of the hippocampus. This will loosen the tissue surrounding the arterioles, making it easier to dissect them.

4.1.3. Search through the dorsal hippocampal tissue to identify the external transverse artery (**Figure 1C**)[16,27].

4.1.4. Gently grab the external transverse artery and slowly pull it away from the tissue to collect the arterioles and capillaries perfusing the CA3 region of the hippocampus.

4.1.5. Once there are no more vessels to be removed from the tissue, discard the hippocampi. Keep the vessels on ice in plates while not in use.

5. Hippocampal capillary-parenchymal arteriole cannulation

5.1. Find an arteriole with a branch that ends with capillaries. Transfer it to the organ chamber (see diagram). Ensure that the arteriole is about 15–30 µm when fully dilated (**Figure 1D**).

5.2. Carefully mount the blood vessel by pushing the cannula tip through the arteriole wall below the target area. Carefully slide the vessel onto the cannula until there is enough tissue to place the tie on.

5.3. Make a loose knot with 12-0 nylon sutures so that it fits over the blood vessel and cannula. Use a half-hitch knot to secure ties. Then pull the ends to tighten the knot and secure the arteriole to the cannula. Remove any extraneous vessel branches below the tie by gently pulling them with forceps.

5.4. Make another tie and secure it on the other end of the arteriole to seal it.

5.5. Lower the cannula with the attached vessel until it is flat against the coverslip on the bottom of the chamber. Be careful not to lower the cannula too much or it will break.

5.6. Using one cannula on the opposite side of the chamber, lower it so that the point of it pins down the tie on the end of the arteriole.

5.7. Use the third cannula to pin down the capillary branch to the coverslip. Place the tip close to the end of the branch while leaving the ends of the capillaries exposed (not underneath the cannula).

6. Pressure myography

6.1. Move the chamber from the dissection scope to the microscope with the recording software.

6.2. Connect the inflow and outflow tubing to the chamber for perfusion. Start the perfusion with heated aCSF (37 °C) at a flow rate of 4 mL/min.

6.3. Attach the pressurizing cannula to a peristaltic pump paired with a pressure transducer and bring the internal pressure to 20 mm Hg.

6.4. Start the recording software. Adjust the microscope and imaging settings to achieve the clearest image possible. Begin the recording once the settings are optimized for the detection software.

6.5. Increase the pressure of the vessel up to 40 mm Hg while recording the arterial diameter with an edge detection software.

6.6. Allow ~15–20 min for the chamber to perfuse aCSF, washing out the MOPS solution, and preparing to equilibrate and develop myogenic tone.

6.7. To test the viability of a vessel, apply 1 µM NS309 solution to the bath perfusion (see **Figure 2A**,**B** and the Representative Results section). The arteriolar segment must dilate, demonstrating about 30–40% myogenic tone as previously described[3,14,20].

7. Focal stimulation of capillary ends

7.1. Once the baseline tone for the arteriole has been established and the endothelial function assessed, test the response of capillary stimulation.

7.2. Using a glass puller, make cannulas so that there is a fine point at one end. Break the tip off a cannula so that the drug tested can flow through the tip smoothly at 5 psi.

7.3. Fill the cannula with the drug solution of interest and add it to a 3-axis micromanipulator attached to the microscope. Connect the tubing from the pressure ejection system to the cannula.

7.4. Slowly lower the cannula into the bath near the capillaries, being careful not to hit any part of the vessel or hardware in the chamber. Maneuver the tip of the cannula next to the ends of the capillaries without touching them. Keep the tip of the cannula just off the coverslip to avoid the vessel from being stimulated if the cannula leaks.

7.5. When ready to stimulate the capillaries, lower the cannula to the coverslip and just next to the capillaries. Activate the pressure ejection system with the desired ejection time (here 20 s). Once the stimulation is finished, raise the cannula slightly to avoid further stimulation.

7.6. Repeat stimulation as necessary. Change the pressure ejection system cannula to test different drug compounds.

7.7. To confirm that only the capillaries are being stimulated, fill the cannula with 1 µM NS309 solution and repeat the above steps.

NOTE: Capillary endothelial cells do not express K^+ channels activated by NS309, so the arteriole must not respond to the stimulation. If the arteriole dilates, then the cannula will need to be

repositioned or the diameter of the hole will need to be smaller (see **Figure 2A**,**B** and the Representative Results section).

REPRESENTATIVE RESULTS:

Endothelial small-conductance (SK) and intermediate-conductance (IK) Ca^{2+}-sensitive K^+ channels exerted a dilatory influence on the diameter of PAs. Bath application of 1 µM NS309, a synthetic IK and SK channel agonist, caused near maximal dilation (**Figure 2A**,**B**). However, capillary endothelial cells lack IK and SK channels and did not hyperpolarize in response to NS309[20]. As a result, stimulating capillary ends with 1 µM NS309 by focal pressure ejection (20 s, 5 psi) did not cause upstream arteriolar dilation (**Figure 2A**,**B**). This result indicates that NS309 did not reach the arteriole in the HiCaPA preparations and could be used as a control to assess the spatial restriction of the compound applied onto capillaries by pressure ejection.

This preparation was fundamentally designed for the measurement of inside-out electrical signaling from capillaries to PAs. Using the HiCaPA preparation, we applied aCSF containing 10 mM K^+ onto the capillary ends and measured an upstream arteriolar dilation (**Figure 2A**,**C**) as we previously did in CaPA preparations from the cortical vasculature[20]. We then investigated, for the first time to our knowledge, capillary-to-arteriole electrical signaling in female mice using HiCaPA preparations. Arteriolar dilation evoked by capillary stimulation with 10 mM K^+ did not differ between preparations from male and female mice (**Figure 2A**,**C**).

Finally, another fundamental benefit of this approach is the possibility to apply pharmacological tools in the bath before capillary stimulation. Here we tested the effect of ML133, a recently developed Kir2 inhibitor[28]. Addition of 10 µM ML133 to the bath perfusion virtually abolished capillary-induced arteriolar dilation in response to 10 mM K^+ in HiCaPA preparations from both male and female mice (**Figure 2A**,**C**). This last result suggests that the Kir2.1 channel mediates electrical signaling in female cerebral vasculature as we previously described in the cortical microcirculation of the male brain.

FIGURE LEGENDS:

Figure 1: Methodology for isolation and pressurization of hippocampal capillary-parenchymal arterioles (HiCaPA) preparation from mouse. (**A**) Freshly isolated brain is cut in half in the sagittal plane following the interhemispheric fissure and placed with the medial side facing up. (**B**) The thalamus, septum, and hypothalamus are gently removed to reveal the hippocampus. (**C**) The hippocampus is carefully removed. (**D**) Arterioles with capillary trees are isolated from the hippocampus and one end of the arteriolar segment is cannulated with a micropipette connected to a pressurizing system, and the other end is occluded. Capillary ends are sealed and maintained against the coverslip with the tip of a glass pipette. Internal diameter is monitored with an edge detection system in one or several regions of the arteriole.

Figure 2: Focal stimulation of capillaries with 1 µM NS309 has no effect on upstream arteriolar diameter, unlike stimulation with aCSF containing 10 mM K^+. (**A**) Representative recording of the upstream arteriolar diameter showing the effect of bath application of 1 µM NS309 followed by successive capillary ends stimulation (20 s, 5 psi) with 1 µM NS309 and with aCSF containing

10 mM K^+ in the absence or presence of the Kir2 channel inhibitor ML133. Application of 10 mM K^+ onto capillaries produced a rapid upstream arteriolar dilation that was blocked by 10 µM ML133. NS309 did not cause dilation. The absence of upstream arteriolar dilation in response to capillary stimulation with NS309 illustrates that pressure-ejected compounds do not reach the arteriole. (**B**) Summary data showing diameter changes induced by 1 µM NS309 applied in the bath or on the capillary ends (n = 14; ****$p < 0.0001$, paired t-test). (**C**) Summary data showing arteriolar diameter changes induced by 10 mM K^+ applied directly onto the capillaries in HiCaPA preparations from male (n = 6) or female (n = 8) mice before and after 10 µM ML133 was applied in the bath (***$p < 0.0005$, n.s. = nonsignificant, unpaired t-test).

DISCUSSION:

The pressurized HiCaPA (hippocampal capillary-parenchymal arteriole) preparation described in the present manuscript is an extension of our well-established procedure to isolate, pressurize, and study parenchymal arterioles[29]. We recently reported that Kir2.1 channels in brain capillary endothelial cells sense increases in $[K^+]_o$ associated with neural activation, and generate an ascending hyperpolarizing signal that dilates upstream arterioles[20]. Revealing this previously unanticipated role for the capillaries has been possible in part by developing the CaPA preparation from cortical microcirculation[20,21]. This manuscript presents a similar experimental approach but from a deeper and more restricted structure of the mouse brain to describe a simple and reproducible approach to investigate capillary-to-arteriole signaling during neurovascular coupling.

The brain microcirculation is exquisitely fragile and certain practices, specially minimizing stretching and handling of the vessels, must be used to ensure the survival of the arterioles and capillaries. The spontaneous development of the myogenic tone is the first indicator of a preparation's viability[30]. The endothelial function can then be assessed by adding the SK and IK channels' agonist NS309 to the bath solution, which should cause near maximum dilation. In case of a failure to develop tone or response to the bath application of NS309, the preparation should be replaced with another one. NS309 is also used to test the spread of the focal capillary stimulation. Because capillary endothelial cells lack SK and IK channels[20], local delivery of NS309 onto capillaries by pressure ejection should have no effect on upstream arteriolar diameter as displayed in **Figure 2**, illustrating that compounds do not accidentally stimulate the arteriole. Once these steps are validated, capillary-to-arteriole signaling can be tested.

Here we examined electrical signaling by stimulating capillaries with aCSF containing 10 mM K^+. However, different signaling modalities can be explored using the present approach by stimulating capillaries with different known vasoactive agents or neurotransmitters. Another benefit of this preparation is the possibility to investigate and eventually compare NVC between different animals and between different brain regions. This is particularly interesting because the brain is not uniformly targeted by cerebrovascular pathologies[31,32]. A general limitation of the approach presented here is that by isolating the microcirculation, crucial components of the neurovascular unit, such as neurons and astrocytes, are lost. Other preparations, such as the cranial window for in vivo CBF imaging, maintain the structure of the intact neurovascular unit and are more appropriate to study NVC in an intact system. However, in the cranial window

preparation, parenchymal arterioles are difficult to image without specific equipment, like a multiphoton microscope, and deeper regions, such as the hippocampus, remain difficult to image. In this regard, the approach developed in the Filosa laboratory using luminal flow to induce myogenic tone in brain slices represents an elegant link between brain slice and in vivo approaches[33]. However, the surrounding nervous tissue can limit the penetration of a drug applied topically, increasing its off-target potential and making interpretations difficult, because several cell types are exposed to the drugs. We primarily developed our ex vivo approach to address these potential issues. In conclusion, multiple approaches should be used in conjunction to fully study NVC.

In summary, the present report describes an ex vivo intact preparation of pressurized hippocampal arterioles and capillaries that allows the effects of pharmacological and biological agents to be tested on functional parameters at discrete positions along the capillary-arteriole continuum.

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DISCLOSURES:

The authors have nothing to disclose.

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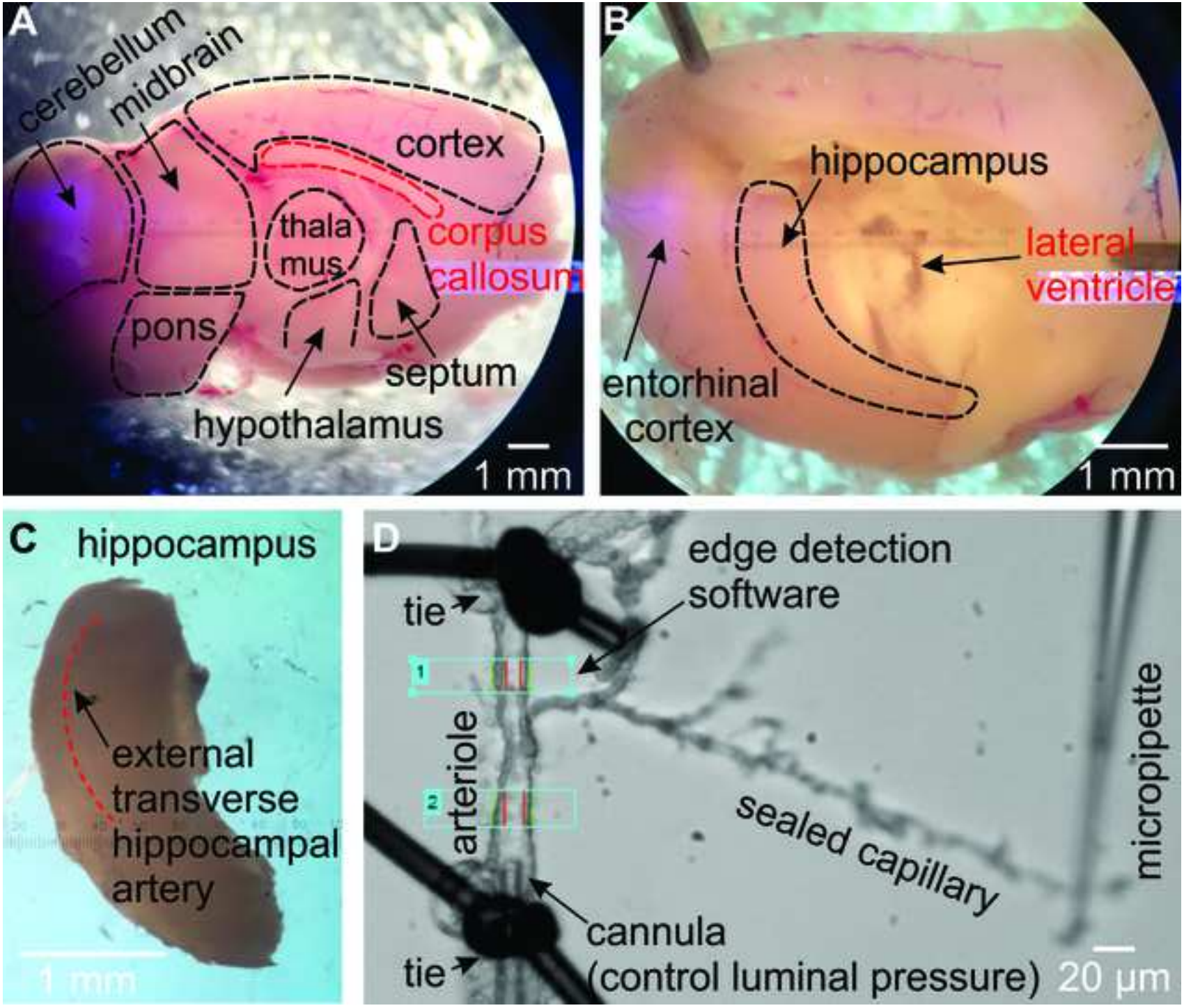

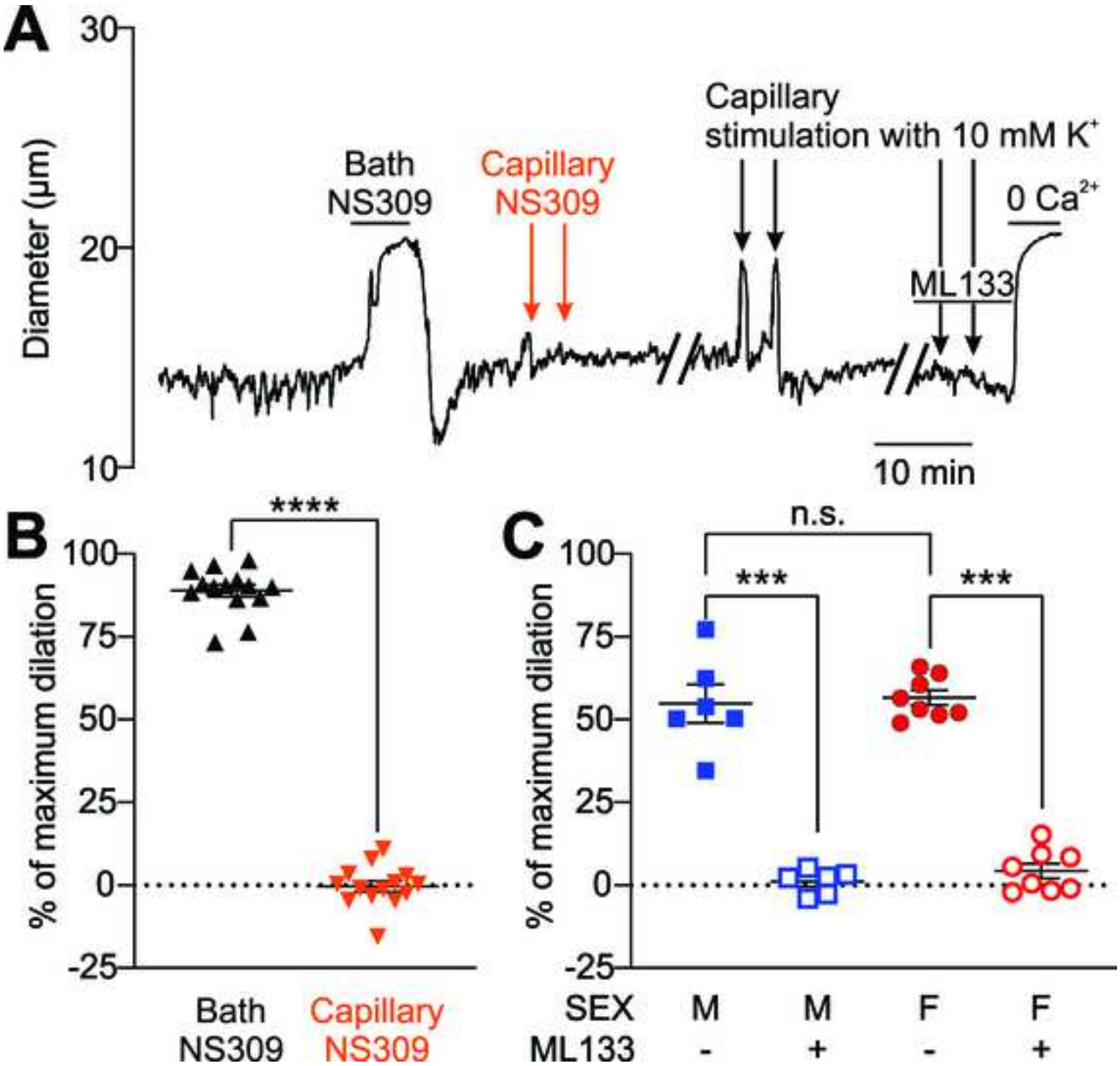

Name of Material/Equipment	Company
0.22µm Syringe Filters	CELLTREAT Scientific Products
12-0 Nylon (12cm) Black	Microsurgery Instruments, Inc
Automatic Temperature Controller	Warner Instruments
Borosilicate Glass O.D.: 1.2 mm, I.D.: 0.68 mm	Sutter Instruments
Bovine serum albumin	Sigma-Aldrich
$CaCl_2$ dihydrate	Sigma-Aldrich
D-(+)-Glucose	Sigma-Aldrich
Dissection Scope	Olympus
ECOLINE VC-MS/CA 4-12 — complete Pump with Drive and MS/CA 4-12 pump-head	Ismatec
EGTA	Sigma-Aldrich
Fine Scissors - Sharp	Fine Science Tools
Inline Water Heater	Warner Instruments
Integra™ Miltex™ Tissue Forceps	Fisher Scientific
KCl	Sigma-Aldrich
KH_2PO_4	Sigma-Aldrich
Magnesium sulfate heptahydrate	Sigma-Aldrich
MgCl Anhydrous	Sigma-Aldrich
Micromanipulator	Narishige
ML 133 hydrochloride	Tocris
MOPS	Sigma-Aldrich
NaCl	Sigma-Aldrich
NaH_2PO_4	Sigma-Aldrich
$NaHCO_3$	Sigma-Aldrich
NS309	Tocris
Picospritzer III - Intracellular Microinjection Dispense Systems, 2-channel	Parker Hannifin
Pressure Servo Controller with Peristaltic Pump	Living Systems Instrumentation
Sodium pyruvate	Sigma-Aldrich
Super Fine Forceps	Fine Science Tools
Surgical Scissors - Sharp-Blunt	Fine Science Tools
Vertical Micropipette Puller	Narishige

Catalog Number	Comments/Description
229751	
S12-0 NYLON	
TC-324B	
B120-69-10	
A7030	
C3881	
G5767	
SZ11	
ISM 1090	
E4378	
14063-09	
SH-27B	
12-460-117	
P9333	
P5379	
M1880	
M8266	
MN-153	
4549	
M1254	
S9625	
S9638	
S8875	
3895	
052-0500-900	
PS-200	
P3662	
11252-20	
14001-13	
PP-83	

Dear Dr. Dabertrand,

Your manuscript, JoVE60676 "Ex vivo pressurized hippocampal capillary-parenchymal arteriole preparation for functional study," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Sep 20, 2019**.

We are delighted to see that our manuscript was well received, and we deeply thank the Editors and the Reviewers for their suggestions and comments.
Below is a point-by-point response as well as a detailed description of the changes that we made in the revised manuscript.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript has been thoroughly proofread.

2. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Pressure Servo Control, Living Systems 109 Instrumentation Inc., Burlington, VT, USA, IonOptix, Milton, MA, USA, Picospritzer III (Parker Hannifin, 207 Hollis, NH, USA), etc.
We have removed all commercial language and added the products to the Table of Materials and Reagents.
L115: Removed “(Pressure Servo Control, Living Systems Instrumentation Inc., Burlington, VT, USA)”
L194: Removed “(IonOptix, Milton, MA, USA)”
L213: Replaced “Picospritzer III (Parker Hannifin, Hollis, NH, USA)” with “pressure ejection system”
L220: replaced “Picospritzer” with pressure ejection system
L223: replaced “Picospritzer” with pressure ejection system

3. Please reword lines 30-32, 81-82, 225-227, as it matches with previously published literature.
These lines have been reworded.

4. Please include a single line space between each step, substep, and note in the protocol section.
This has been changed.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
The protocol section is now entirely written in imperative tense:
L117: “should” has be replaced by “must”
L150: “should now be” has been replaced by “is now”
L159: “should be” has be replaced by “is”
L171: “Ideally, the arteriole will be” has been replaced with “The arteriole must be”
L204: “should” has be replaced by “must”
L216-218: the sentences “The tip of the cannula should be next to the ends of the capillaries without touching them. It should be just off of the cover slip to avoid the vessel being stimulated if the cannula leaks” have been changed to “Maneuver the tip of the cannula next to the ends of the capillaries without touching them. Keep the tip of the cannula just off of the cover slip to avoid the vessel being stimulated if the cannula leaks”
L226-227: “Capillary endothelial cells do not express K^+ channels activated by NS309, so there should be no response of the arteriole” has been changed to “Capillary endothelial cells do not express K^+ channels activated by NS309, so the arteriole must not respond to the stimulation”.
L228: “may” has been changed to “will”
L229: “may” has been changed to “will”

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
Personal pronouns have been removed:
L129: “Starting at the caudal side of the skull, cut the skull along the midline until you reach the olfactory bulbs” has been changed to “Starting at the caudal side of the skull, cut the skull along midline until the olfactory bulbs are reached”.
L182: “Be careful not to lower the cannula too much or you will break it” has been replaced by “Be careful not to lower the cannula too much or it will break”.

7. The Protocol should contain only action items that direct the reader to do something. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

We have removed sentences that were not actions
L109: “Cannulas will be used in multiple steps and can be stored for later experiments when not in use.” has been removed.
L118: “The points are flexible and will help hold down the capillaries.” has been removed.
L162: “Be careful to minimize stretching and handling the vessel. They are sensitive and it can damage the endothelium, making the vessel unresponsive.” has been removed
L173: “(This loose knot can be made in a separate dissection plate and then transferred into the chamber.)” has been removed
L180: Removed “This will stabilize the vessel to make recording easier.”

8. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
An ethics statement has been added at the beginning of the protocol L94.

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
L132: “by cutting through the structures with the small dissection scissors” was added for clarification.
L138: “Hold the blade so that the sharp edge is parallel to the bottom of the dissection plate. Press the blade through the brain in one stroke.” was added for clarity.
L144: “Push the blade straight through the tissue.” was added.
L166-167: “Once there are no more vessels to be removed from the tissue, discard the hippocampi. Keep the vessels on ice while not in use.” was added.

10. 3.1: Age, sex, strain of the mouse used? How do you perform euthanasia? Do you clean the dissection area, prior to performing incision?
This is now detailed in the manuscript
L120: “For this experiment, an 8-week-old C57BL6/J mouse is used to compare differences between males and females. The mouse was injected with pentobarbital and decapitated with surgical scissors.” was added to protocol

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
The protocol is slightly over 3 pages long. Steps 3 to 7 of the protocol would represent the filmable parts.

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All figures are original content.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The discussion follows this order.

14. Please sort the materials table in alphabetical order.
Commercial items from protocol have been added to the materials table. All items have been listed in alphabetical order.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the Roserhart et al., study the authors provide a detailed description of how to pressurized and functionally test capillary-parenchymal arteriole vessels from a mouse preparation. I found the description easy to follow and the technique of extreme value to the field. The possibility of translating this approach to numerous vessels across brain regions is of great value. Moreover, the use of this approach to test functional vessel responses (both for capillaries and arterioles) in female vs. male brains is timely and also important. The only section I believe could use some clarification is that for the solutions and corresponding gasses. For example, it would be helpful to better defined which solutions are used for the dissection, within the cannula solution vs. aCSF and which solutions are gassed with O2 and CO2. The O2 concentrations have been a matter of debate and thus, a further descriptions and justifications may be needed. The concentration of O2 was not specified.

In addition, while the authors describe an important limitation of the current approach "lack of neurons and astrocytes" I was surprise the authors did not reference the work of Kim et al., who developed a technique to pressurized and also study the functional responses of parenchymal arterioles (with corresponding capillaries downstream) in a brain slice preparation. J Physiol. 2012 Apr 1;590(7):1757-70. doi: 10.1113/jphysiol.2011.222778. Epub 2012 Feb 6.

Overall, I believe this is an excellent article of great value to the field.

Major Concerns:
none

Minor Concerns:
details for solutions

We thank the referee for the great and constructive review, which helped us to improve the manuscript.

L99-105 We have expanded the Solutions section to detail how solutions are used. Briefly, MOPS-buffered saline is used for the dissection and to keep samples at 4°C before their utilization. It is not gassed. The artificial cerebrospinal fluid (aCSF) is used as bath solution and pipette solution for consistency with the literature. Both aCSF and Ca^{2+}-free aCSF are gassed with 5% CO_2, 20% O_2, and N_2 balance. This gas composition has been chosen to provide physiological environment of O_2 and CO_2.

We particularly thank the reviewer for suggesting to add the study from Kim et al. to the Discussion. The method developed in the Filosa lab is really unique, using luminal flow to induce myogenic tone in brain slices represents an elegant link between brain slice and *in vivo* approaches. However, using pharmacological tools with this technique can lead to multifaceted interpretations as several cell types are exposed to the drugs. We primarily developed our *ex vivo* approach to have a better control over the experimental conditions and particularly the use of pharmacological tools. We have added these considerations to the Discussion (L320-326).

Reviewer #2:
In this study, the Authors presented an approach to examine capillary-to-arteriole signaling in an ex vivo hippocampal capillary-parenchymal arteriole (HiCaPA) preparation from mouse brain. They described how to isolate, cannulate and pressurize the microcirculation to measure arteriolar diameter in response to capillary stimulation. The Authors showed which appropriate functional controls can be used to validate the HiCaPA preparation integrity, and displayed typical results that can be obtained, including testing potassium as a neurovascular coupling agent. They also compared the responses in preparations obtained from male and female mice.
In conclusion, the Authors described an ex vivo intact preparation of pressurized hippocampal arterioles and capillaries that allows the effects of pharmacological and biological agents to be tested on functional parameters at discrete positions along the capillary-arteriole continuum. While their data reflect functional investigations, the Authors suggest that their experimental approach can also be used in molecular biology, immunohistochemistry, and electrophysiology studies.
The manuscript has been constituted logically, the findings mentioned above are quite impressive. In addition, this experimental approach could be important to understand the interaction between capillary and arteriolar microvessels and could also be used to get more knowledge on drug delivery into the brain at the capillary level. Therefore, the mentioned paper does deserve to be published in this Journal.

GENERAL VIEW
1. The questions suggested by the Authors are original, important, and well defined.
2. The data of the mentioned manuscript are well defined and controlled.
3. The methods and experimental design they performed are well organized.
4. They used sufficient current methods to get their data.

5. The quality of written English is quite well.
6. All tables and figures in the manuscript are well designed.
7. This study meets the ethical standards of scientific/medical research.

We deeply thank the referee for supporting our manuscript.

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Author(s):	Amanda C. Rosehart, Abbie C. Johnson, Fabrice Dabertrand

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CORRESPONDING AUTHOR

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Institution:	University of Colorado Anschutz Medical Campus
Title:	Assistant Professor
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