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Minimizing hypoxia in hippocampal slices from adult and aging mice

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Dear Dr. Bajaj,

I have revised the manuscript JoVE61377 "Minimizing hypoxia in hippocampal slices from adult and aging mice," per Editorial and Reviewers' comments from the March 26 2020 decision letter. My belief is that the comments raised by both Reviewers, as well as the Editors, are fully addressed. I am grateful for the entirety of Reviewers' comments and criticisms, as these have significantly strengthened the significance of the protocol, and ensured a proper context relative to other related methods. The rebuttal to the Editor's and Reviewers' comments is appended as a separate letter. The revised manuscript indicates new or altered text in red font. I hope that you will now find the manuscript acceptable for publication.

Yours sincerely,

M. Djurisic

Maja Djurisic, Ph.D

TITLE:

Minimizing Hypoxia in Hippocampal Slices from Adult and Aging Mice

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KEYWORDS:

Hippocampal slices, adult, aging, hypoxia, ATP, NMDG, CA1, patch-clamp, field-recordings

SUMMARY:

This is a protocol for acute slice preparation from adult and aging mouse hippocampi that takes advantage of transcardial perfusion and slice cutting with ice-cold NMDG-aCSF to reduce hypoxic damage to the tissue. The resulting slices stay healthy over many hours, and are suitable for long-term patch-clamp and field-recordings.

ABSTRACT:

Acute hippocampal slices have enabled generations of neuroscientists to explore synaptic, neuronal, and circuit properties in detail and with high fidelity. Exploration of LTP and LTD mechanisms, single neuron dendritic computation, and experience-dependent changes in circuitry, would not have been possible without this classical preparation. However, with a few exceptions, most basic research using acute hippocampal slices has been performed using slices from rodents of relatively young ages, ~P20-P40, even though synaptic and intrinsic excitability mechanisms have a long developmental tail that reaches past P60. The main appeal of using young hippocampal slices is preservation of neuronal health aided by higher tolerance to hypoxic damage. However, there is a need to understand neuronal function at more mature stages of development, further accentuated by the development of various animal models of neurodegenerative diseases that require an aging brain preparation. Here we describe a modification to an acute hippocampal slice preparation that reliably delivers healthy slices from adult and aging mouse hippocampi. The protocol's critical steps are transcardial perfusion and cutting with ice-cold sodium-free NMDG-aSCF. Together, these steps attenuate the hypoxia-induced drop in ATP upon decapitation, as well as cytotoxic edema caused by passive sodium fluxes. We demonstrate how to cut transversal slices of hippocampus plus cortex using a vibrating microtome. Acute hippocampal slices obtained in this way are reliably healthy over many hours of recording, and are appropriate for both field-recordings and targeted patch-clamp recordings, including targeting of fluorescently labeled neurons.

INTRODUCTION:

The advent of mammalian acute brain slice preparations facilitated experiments at the cellular and synaptic level that were previously possible only in invertebrate preparations like Aplysia[1].

Development of acute hippocampal slices was of particular significance, as it is a structure responsible for working memory and context formation, and has a specialized tri-synaptic circuitry that is amenable to easy physiological manipulation. However, the vast majority of acute brain slices are still prepared from relatively young mice and rats, as it is easier to preserve healthy neurons and circuits, and the slices remain viable for longer periods of time[2-4]. Here, we introduce modifications to standard slicing protocols that result in increased viability of acute hippocampal slices from adult and aging mice.

The major impediment to the long-term ex vivo viability of mammalian brain parenchyma is the initial hypoxic damage that occurs rapidly once blood flow to the brain stops following decapitation. Loss of oxygen results in fast metabolic consumption of major energy resources in the brain with the loss of phospho-creatine (P-creatine) being the most rapid, followed by glucose, adenosine triphosphate (ATP), and glycogen[4]. Preservation of ATP is of particular importance for the long-term health of brain slices, as ATP is needed to maintain the membrane potential via the Na-K ATPase, and consequently the neural activity[5,6]. The ATP level in the adult rodent brain is ~2.5 mM, and it drops precipitously within 20 s of decapitation to reach a basal steady state (~0.5 mM) at around 1 min post-decapitation[4,7,8]. In young animals, it takes longer to observe the same drop in ATP (~2 min); with phenobarbital anesthesia it is further slowed to 4 min[4]. These considerations show that preventing loss of ATP and other energy resources is a necessary strategy to prevent hypoxic damage to the brain and in turn to maintain the health of brain slices over longer periods of time, especially in adult animals.

Low temperatures slow down the metabolism. Consequently, it has been demonstrated that modest hypothermia protects brain energy reserves: in young animals, lowering body temperature by six degrees, from 37 °C to 31 °C, preserves ATP levels to around 80% of normal levels over 4 h of controlled hypoxia[9]. P-creatine levels are similarly preserved, as well as the overall phosphorylation potential[9]. This suggests that lowering body temperature prior to decapitation could be neuroprotective, as near-normal levels of ATP could be maintained through the slice cutting and slice recovery periods.

To the degree that an ATP drop cannot be completely prevented upon decapitation, a partially impaired function of the Na-K ATPase is expected, followed by depolarization via passive sodium influx. As the passive sodium influx is followed by water entry into cells, it causes cytotoxic edema and eventually pyknosis. In adult rats, replacing Na+ ions with sucrose in slice-cutting solutions has been a successful strategy to alleviate the burden of cytotoxic edema[10,11]. More recently, methylated organic cations that decrease sodium channel permeability[12] have shown to offer more effective protection than sucrose, especially in slices from adult mice, with N-methyl-D-glucamine (NMDG) being most widely applicable across different ages and brain regions[13-16].

Numerous brain-slicing protocols involve using cold temperatures only during the slice-cutting step, sometimes in combination with Na^+ ion replacement strategy[16,17]. In young animals, these protocols appear to offer sufficient neuroprotection since the brains can be extracted quickly after decapitation because the skull is still thin and easy to remove[3]. However, this strategy does not produce healthy slices from adult animals. Over time, a number of laboratories studying adult

rodents have introduced transcardial perfusion with an ice-cold solution to decrease the animal's body temperature, and therefore hypoxic damage to the brain, prior to decapitation. This procedure was successfully applied across different brain regions: cerebellar slices[18], midbrain slices[19], neocortical slices[11,20], perirhinal cortex[21], rat hippocampus[10,22,23], olfactory bulb[24], ventral striatum[25], visual cortex[26].

In spite of the advantages offered by transcardial perfusion and Na^+ ion replacement in preparing slices from rat and in some brain regions in mice, mouse hippocampus remains one of the most challenging areas to protect from hypoxia[13,20]. To date, one of the most common approaches to slicing hippocampus from aging mice and mouse models of neurodegeneration involves the classical fast slicing of the isolated hippocampi[27]. In the protocol described here, we minimize the loss of ATP in the adult brain by introducing hypothermia prior to decapitation by transcardially perfusing the animal with ice-cold Na^+- free NMDG-aCSF. Slices are then cut in ice-cold Na^+-free NMDG-aCSF. With this enhanced protocol we obtain acute hippocampal slices from adult and aging mice that are healthy for up to 10 h after slicing and are appropriate for long-term field-recordings and patch-clamp studies.

PROTOCOL:

The protocol is carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Stanford University Institutional Animal Care and Use Committee. Methods are also in accordance with the Policies of the Society for Neuroscience on the Use of Animals and Humans in Neuroscience Research.

NOTE: All mice were maintained in a pathogen-free environment. Wild-type mice on mixed C57Bl/ 6 x SV/ 129J genetic background were used here, unless otherwise noted.

1. Setup

1.1. Prepare 1 L of 1x aCSF using the following reagent (in mM): 125 NaCl, 26 $NaHCO_3$, 2.3 KCl, 1.26 KH_2PO_4, 1.3 $Mg_2SO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 25 glucose (**Table 1**). Use this solution for the recovery chamber and subsequent recordings.

1.1.1. Store aCSF as a 10x stock solution that contains NaCl, $NaHCO_3$, KCl, and KH_2PO_4. Store $Mg_2SO_4 \cdot 7H_2O$ and $CaCl_2$ as 1 M stock solutions. Prepare the working solution on the day of experiment from the above stock solutions, and add glucose before adjusting the final volume with 18 MΩ water.

NOTE: Depending on the brain region or mouse strain, chilled aCSF can be also used for transcardial perfusion with success[11,15,26].

1.2. Prepare 300 mL of NMDG-aCSF using the follow reagents (in mM): 135 NMDG, 1 KCl, 1.2 KH_2PO_4, 1.5 $MgCl_2$, 0.5 $CaCl_2$, 20 choline bicarbonate, 10 glucose (**Table 1**). This volume of NMDG-aCSF is sufficient for both transcardial perfusion and cutting steps.

1.2.1. Store NMDG-aCSF as a 3x stock solution at 4 °C. Prepare working solution on the day of the experiment; add choline bicarbonate and glucose before adjusting the final volume with 18 MΩ water. Bubble the solution with 95%O_2/5%CO_2 to buffer it, and saturate with oxygen.

NOTE: NMDG stock starts as highly alkaline, and requires concentrated hydrochloric acid (HCl) to adjust pH to ~7.4. Addition of hydrochloric acid should be slow once below pH 8, as even a small surplus of HCl can precipitously acidify the solution to pH 3. This adjustment should be done prior to adding divalent cations. This NMDG-aCSF recipe is sodium-free. Previously published NMDG recipes use 30 mM $NaHCO_3$ for buffering, which results in 30 mM sodium present in NMDG-aCSF[13,20].

1.3. Set the vibrating microtome cutting tray and mounting disk to -20 °C.

1.4. Prepare the recovery chamber.

1.4.1. Fill the recovery chamber to just above the slice-holding mesh, keep it on the bench at room temperature, and bubble.

NOTE: The slice recovery chamber used here is very similar to the classical chambers described by previously by Edwards and Konnerth[3]. aCSF is kept in a glass beaker (400 mL) that holds a round acrylic frame with black nylon mesh glued to the bottom. The acrylic frame is suspended in the middle of the beaker via an acrylic hook resting over the edge of the beaker. The glass bubbler is inserted all the way to the bottom. The design allows for oxygenation of both sides of slices. Bubbling also provides constant mixing of aCSF in the recovery chamber. The black mesh provides a high contrast against the off-white slices, which are then easier to see.

1.5. Prepare the transcardial perfusion and cutting solution.

1.5.1. Chill the entire 300 mL of NMDG-aCSF in a freezer, until ice crystals start to form on the surface and the walls of the bottle. DO NOT over-freeze!

1.5.2. Place the bottle with chilled NMDG-aCSF on ice and bubble. The solution should be between 0–2 °C.

NOTE: Slushy (NMDG-) aCSF implies having most of the solution as liquid, while a small fraction of slushy ice present will keep the solution near 0 °C throughout perfusion and cutting. Care should be taken to keep away ice crystals from the brain during cutting.

1.6. Prepare the tissue-mounting disk.

1.6.1. Take the disk out of the freezer, wipe it dry if needed.

1.6.2. Cut out a block of 5% agar about the size of a mouse brain from the previously prepared agar plate, and glue it in the center of the disk using a thin layer cyanoacrylate glue. Place the disk with glued agar on ice, and cover with paper towels until ready to use.

1.6.3.1. For 5% agar plate, dissolve 5 g of agar in 100 mL of 18 MΩ water by microwaving, and pour into a clean petri dish. Keep at 4 °C.

1.7. Take the cutting tray out of the freezer, place it into the microtome, surround it with ice, and load the blade.

2. Transcardial perfusion and brain extraction

2.1. Overdose mice via an intraperitoneal injection of rodent cocktail. Check for the anesthesia depth by checking the pain reflex (toe pinch); a mouse should not exhibit the reflex once it reaches deep anesthesia.

NOTE: Rodent cocktail recipe: Ketamine HCl (66 mg/mL), xylazine HCl (6.6 mg/mL), acepromazine maleate (0.1 mg/mL), 18 MΩ water to final volume. Dose: 0.4 mg/g body weight, 0.04 mg/g body weight, 6 x 10^{-4} mg/g body weight for ketamine, xylazine, and acepromazine, respectively.

2.2. Set-up the peristaltic pump for transcardial perfusion. Insert one side of the pump tubing into the bottle with iced NMDG-aCSF. Fit the other side of the tubing with the 27 G needle that will be inserted into the left ventricle.

2.3. Set the pump speed at approximately 3.5 mL/min. At this speed, the outflow of NMDG-aCSF is a fast drip, not continuous flow.

NOTE: Perfusion by gravity is a good substitute for the peristaltic pump perfusion, as long as the same approximate flow-rate can be achieved. A perfusion at high flow-rate will result in burst blood vessels in the brain. A telltale sign of an overly fast perfusion and increased pressure in blood vessels, is solution coming out of the nose of the animal.

2.4. Perfusion

2.4.1. Place the properly anesthetized mouse on its back on a diaper. Using paper tape, tape down its front and hind legs so that the chest and abdomen are exposed.

2.4.2. Cut out a large patch of the skin atop of the chest, going from below the sternum to the throat; this should provide a large working area. Grab the sternum with forceps, lift gently, and start cutting through the rib cage on both sides until the chest cavity is exposed.

2.4.3. Cut through the diaphragm in order to expose the chest cavity. The flap of the rib cage should be left attached via a thin piece of muscle. It should be possible to set it on a side without

having it fall back onto the exposed chest cavity. Check that the heart is still beating. Ensure that most of the liver is visible.

2.4.4. Insert the 27 G needle into the left ventricle; the mouse's left ventricle looks lighter in color than the right. Locate the dark red-colored right atrium. Cut through the right atrium with small scissors; the blood should start flowing out.

2.4.5. Start the pump that has been preset to the correct flow speed. If everything was done correctly, the liver should change color from red to brown soon after the start of perfusion. Monitor the liver color to determine the length of the perfusion; the liver should turn pale brown.

2.4.6. Run the pump for few more minutes. If using rectal thermometer, the body temperature should fall down to 28–29 °C, and animal's nose should be cold to the touch

2.5. Brain extraction.

NOTE: For this step, have the following dissection tools ready: decapitation scissors, small scissors with straight or angled blade, scalpel and #10 blade, single edge blade, #3 forceps, spatula with one side bent to 90°, a spatula, a "60°" tool (**Figure 1A, B**), and a small soft brush.

2.5.1. Decapitate the mouse with large decapitation scissors. Using a scalpel with #10 blade, cut open the skin on top of the skull. With small angled scissors, cut the skull at the midline. Next, using the #3 forceps, pry away the right and left halves of the skull, being careful to take the dura away with it. The brain should be exposed now.

NOTE: If the dura detaches from the skull, it will stay over the brain and has to be removed separately. The edges of the remaining dura can tighten and slice deep through the brain, potentially damaging the brain region of interest.

2.5.2. Remove the brain by scooping it out with a small spatula. Drop the brain into the NMDG-aCSF solution placed in a separate small beaker on ice. Leave it there for up to a minute.

NOTE: In addition to hypothermia, exposure to the ice-cold saline firms up the brain, which is necessary for even cutting. The entire procedure from decapitation to the brain extraction should be under 30 s.

3. Slicing

3.1. Take the brain out of the NMDG-aCSF and place it on a piece of filter paper.

3.2. Cut and remove a 60° wedge of tissue using a "60°" tool centered at the midline from the rostral end of the forebrain. Use this as the mounting surface as it provides the proper angle for hippocampal slices as discussed below (**Figure 1A, B**).

NOTE: Two single-edged blades held together via a home-made holder make an easy-to-use tool for making the 60° cut (**Figure 1A**).

3.3. Separate hemispheres down the midline with the scalpel.

3.4. Glue the hemispheres onto the mounting disk as follows. Take the mounting disk that has been on ice until now. If needed, wipe it dry again. Glue each hemisphere in front of the agar block, cut side down.

3.5. Ensure that the ventral side of the both hemispheres are touching the agar block. The agar block provides additional support during cutting and is essential for even slices. Ensure that the dorsal sides of both hemispheres are facing the blade. When glued on the cut side, each hemisphere is oriented relative to the blade in a way that ensures transverse slices of dorsal hippocampus in situ (**Figure 1B**).

3.5. Submerge the disk with hemispheres into a cutting chamber containing ice-cold carbogenated NMDG-aCSF.

3.6. Cut 400 µm sections. Cutting should be done in less than 10 min. Different microtomes will require different settings to achieve this time. A total of 8–10 slices from the dorsal hippocampus region will be obtained.

4. Recovery

4.1. Transfer slices to a recovery chamber containing carbogenated aCSF at room temperature using disposable transfer pipettes with cut-off tips (**Figure 1C**).

NOTE: It is highly recommended to fortify recovery chamber-aSCF with 5 mM Na-ascorbate and 3 mM Na-pyruvate. Pyruvate is an energy substrate shown to boost the production of ATP in slices[28], while Na-ascorbate is a free-radical quencher[29].

4.2. Incubate at room temperature (22–24 °C) for approximately 2 h before recording (up to 4 h).

NOTE: A longer incubation at room temperature results in healthier slices for longer periods of time, relative to the standard incubation at 37 °C for 30 min followed by room temperature incubation. Rewarming at 37 °C can introduce cytotoxic edema[13].

REPRESENTATIVE RESULTS:

We applied the above protocol to generate hippocampus slices from CamKIIa-Cre+; PirB+/+ mice on a mixed genetic background C57Bl/ 6 x SV/ 129J, at P > 120. Large numbers of pyramidal cells in the CA1 field (**Figure 2A**) and subiculum (**Figure 2B**) appear in low contrast when observed under infrared differential contrast microscopy (IR-DIC), a hallmark of healthy cells in a slice preparation. With this preparation, a high rate of giga-ohm seals (>90%) is routinely achieved when targeting the healthiest cells approximately 20–50 microns beneath the surface. For this

success rate, it is important to use a high NA objective for IR-DIC, such as a 60x water-immersion objective, in order to achieve adequate visualization of pyramidal cells at this depth (**Figure 2A, B**).

Patch-clamp recordings from single neurons are easily achievable using this hippocampal slice preparation even in mice over six months of age. In **Figure 2C**, using mEPSC recordings in CA1 neurons, we demonstrate that induction of chemical NMDA-LTD with 3 min bath application of 20 µM NMDA results in lower mEPSC frequency in CA1 cells when assessed 60 min post-NMDA treatment. This finding suggests that NMDA-LTD causes activity-dependent pruning of synapses in CA1 in older mice (results adapted from Djurisic et al.[15]). Change in mEPSC amplitude was not detected. During mEPSC recordings, CA1 cells were also filled with biocytin. **Figure 2D** reveals an intact dendritic arbor and healthy cell habitus of filled CA1 pyramidal neurons. A robust distribution of the fluorescent dye throughout the cell allows for routine evaluation of dendritic spine properties under different conditions.

Using field-recordings, we readily observe long-term potentiation (LTP) of CA3-CA1 synapses of ~170% in slices from adult mice, suggesting maintenance of signaling cascades needed for LTP (**Figure 2E, F**). Network connectivity needed for a robust field excitatory postsynaptic potential (fEPSP) signal is also preserved (**Figure 2E**). The ability to assess synaptic plasticity in hippocampal slices from adult or aging mice is especially relevant for mouse models of neurodegenerative diseases as their hallmark synaptic dysfunction develops later in life.

Together, our results demonstrate that an acute hippocampal preparation from adult and aging mice allows assessment of synaptic function at the level of both single cells and population of cells routinely, as long as transcardial perfusion and ice-cold NMDG-based solutions are used to minimize hypoxic damage.

FIGURE LEGENDS:

Figure 1: Cutting method and recovery of transversal hippocampal slices from adult and aging mice. (**A**) A "60°" tool used for removing the 60° wedge from the rostral end of the brain, centered on the midline. (**B**) Positioning the hemispheres for cutting of transversal slices. An illustration of a 60° cut is shown on the left and the upper right panels; positioning of the two hemispheres on the surface with glue in manner shown in the lower right panel ensures a perpendicular orientation of dorsal hippocampus relative to the blade. This orientation results in transversal slices of hippocampus. Yellow structures are a 3D rendering of hippocampi within the rodent brain (gray). This illustration is adapted from SynapseWeb[30]. All the views are of the dorsal side of the brain. (**C**) Transversal hippocampal slices cut from a 4-month-old mouse in a recovery chamber.

Figure 2: Patch-clamp and synaptic plasticity measurements in hippocampal slices from mice at P120–180. (**A**) Example IR-DIC micrographs of CA1 region. (**B**) Example IR-DIC micrographs of subiculum. In both A and B, images are taken 3 h after cutting with a 60x water-immersion objective. Calibration bar is 10 µm. (**C**) The effect of chemical NMDA-LTD on mEPSCs in CA1

neurons from P120–180 mice. Upper panel are example traces of mEPSCs recorded at baseline and after NMDA-LTD pulse. Lower left panel: NMDA-LTD resulted in lower mEPSC frequency. Lower right panel: mEPSC amplitude after NMDA-LTD is not detected. N = 17 cells at baseline and n = 15 cells for NMDA-LTD, 6 mice. P = 0.004, t-test. This figure has been modified from Djurisic et al.[15]. (**D**) Example of biocytin filled CA1 pyramidal cell. (**E**) Example of fEPSP signal from CA1 stratum radiatum after Schaffer collateral stimulation. The gray trace is obtained during baseline recording, and the black trace is observed 20 min after tetanic stimulation. Each trace is an average of 30 consecutive traces. (**F**) Cumulative average of long-term potentiation of CA3-CA1 synapses after 4 trains of 100 Hz induction; n = 23 slices from 8 WT mice at approximately P90.

Table 1: Media formulations.

DISCUSSION:

The protocol described here demonstrates that hippocampal slices obtained from adult and aging mice can remain healthy and viable for many hours after cutting. The protocol is appropriate for patch-clamp recordings, as well as long-lasting field-recordings in the CA1 regions.

There are two critical steps in this protocol. First step is the transcardial perfusion step with an ice-cold solution. Fast clearance of blood is signaled by rapid change of liver color. Extracted brain should be off-white in color. If the brain remains pink, it means that systemic blood was not replaced with the cold aCSF, and the drop in body temperature was not achieved. This could be caused by improper placement of the needle into the heart, or because the heart was punctured through. Brains from poorly perfused mice should not be used for slicing. The second critical step is the use of NMDG as a sodium ion substitute. A well-known earlier variation of the protocol that successfully uses sucrose as substitute for Na^+ in rat brains[10,31] does not produce sufficiently healthy hippocampal slices in mice (also see Ting et al.[13]). The use of NMDG as a sodium ion substitute is critical for mouse hippocampal slices.

While we get reliably good hippocampal slices for electrophysiology using the described protocol, the hippocampal preparation from adult and aging animals remains challenging. Its difficulty also changes with different mouse lines and genetic backgrounds. Potential modifications to consider are NMDG-aCSF and recovery-aCSF additives like taurine, D-serine, or N-acetylcysteine (NAC) that could augment neuronal and synaptic function[13,29], increase oxygenation[29], as well as Cl^- ion substitutions during perfusion and cutting[32]. Use of an interface recovery chamber is another way to maintain increased oxygenation, which is especially relevant for long-term plasticity field-recordings. The described recovery chamber (**Figure 1C**) is modifiable for that purpose (e.g., a culture insert dish that is moistened from below by aSCF, could substitute the submerged mesh). The slice-cutting step using sapphire or ceramic blades instead of steel ones, in combination with microtomes designed to minimize the vertical vibration (e.g., zero-z in Leica VT1200S), is another modifiable step. It could further improve the quality of the slice surface as the sharp blades decrease tissue compression exacerbated by heavy myelination of white matter around hippocampus.

This protocol is applicable to any brain region of interest, with appropriate modifications in cutting angles. Different brain regions are sensitive to hypoxia to different degrees. Thus, the stringency of the slice preparation protocol will matter. In fact, a protocol that uses NMDG-aSCF has been reported for adult mouse neocortex and striatum slice preparations[13,20]. This set of protocols uses an NMDG-aCSF recipe that is not sodium-free (it contains 30 mM $NaHCO_3$)[20], a potentially critical factor for regions as susceptible to hypoxia as hippocampus. Moreover, transcardial perfusion has been conducted with NMDG-aCSF at room temperature[13]. As discussed above, without lowering the body temperature via cold transcardial perfusion, the ATP levels will drop to about 20% of normal value within a minute[4,7]. This loss of ATP could be hard to recover in brains from aging animals. It follows then that re-introduction of Na^+ ions with regular aCSF during the recovery step will result in more cytotoxic edema.

This protocol is applicable to the vast array of transgenic mouse lines modeling neurodegenerative diseases. Moreover, the protocol could be further modified to brain slices from other mammalian model species, as well. Together, this protocol could serve as a basis for a standardized preparation for acute hippocampal slices from aging animals, and thus facilitate comparisons across studies in the context of disease mechanisms.

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DISCLOSURES:

The author has nothing to disclose.

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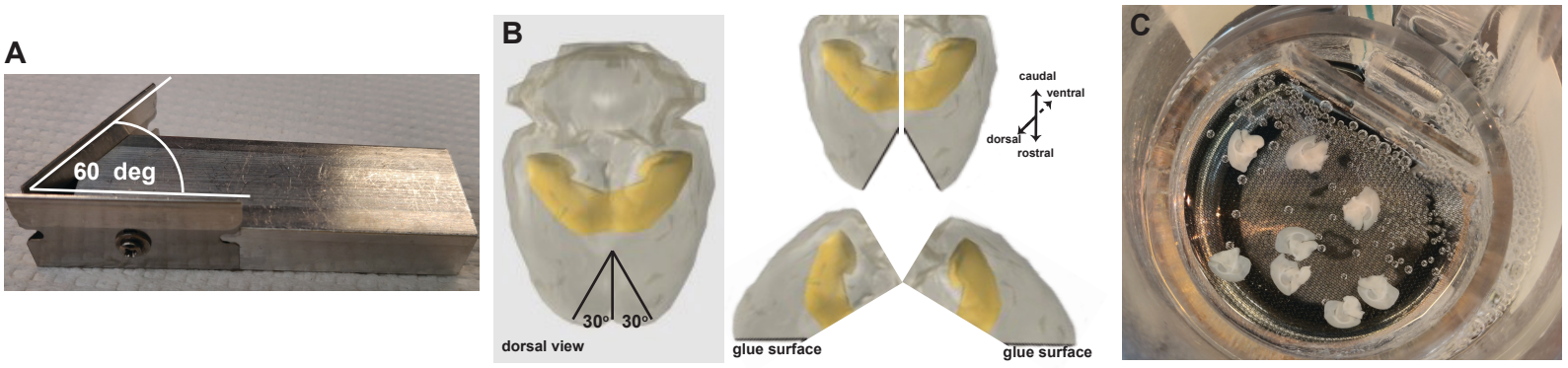
Figure 1



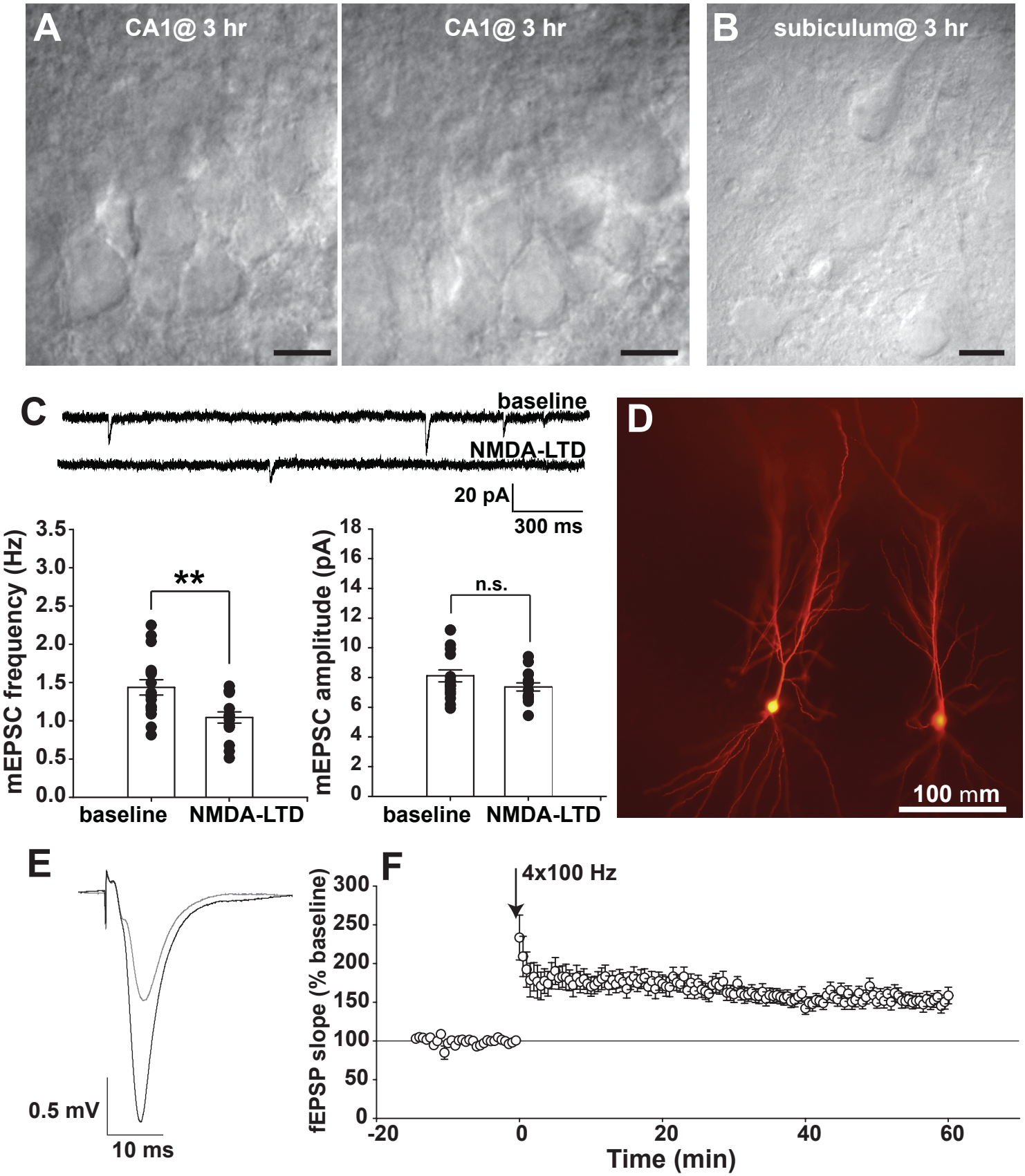


Table 1

Artificial cerebrospinal fluid (aCSF)

Ingredients	MW	Final conc. (mM)	g/2 Liters (10X)	g/Liter (1X)
NaCl	58.44	125	146.1	-
$NaHCO_3$	84.01	26	43.68	-
KCl	74.55	2.3	3.43	-
KH_2PO_4	136.1	1.26	3.44	-
Mg2SO4*7H2O	203.3	1.3	-	1.3 ml of 1M stock
$CaCl_2*2H_2O$	147.02	2.5	-	2.5 ml of 1M stock
Glucose*H_2O	180.2	25	-	4.5 g

a) Add $MgCl_2*6H_2O$ and $CaCl_2*2H_2O$ from 1 M stocks

b) aCSF 10x keep @RT

c) aCSF 1x @4 °C for 24h

NMDG-aCSF

Ingredients	MW	Final conc. (mM)	g/2 Liters (3X)	g/300 mL (1X)
NMDG	195.22	135	158.13	-
pH = 7.4 with HCl (equimolar to NMDG)				
KCl	74.55	1	0.4473	-
KH_2PO_4	136.1	1.2	0.9799	-
$MgCl_2*6H_2O$	203.3	1.5	1.8297	-
$CaCl_2*2H_2O$	147.02	0.5	0.4411	-
Choline Bicarbonate	80%	20	-	1238 µL
Glucose*H_2O	180.2	10	-	0.54

a) Filter and store 3x stock solution at 4 °C

Table of Materials

Name of Material/Equipment	Company	Catalog number	Comments/Description
“60 degree” tool	made in-house		
#10 scalpel blade	Bard-Parker (Aspen Surgical)	371110	
1M $CaCl_2$	Fluka Analytical	21114	
95%O2/5%CO2	Praxiar or another local supplier		
Acepromazine maleate (AceproJect)	Henry Schein	5700850	
Agar	Fisher	BP1423-500	
Beakers, measuring cylinders, reagent bottles			
Brushes size 00-2	Ted Pella		Crafts stores are another source of soft brushes, with larger selection and better quality than Ted Pella.
CCD camera	Olympus	XM10	
Choline bicarbonate	Pfalz & Bauer	C21240	
Cyanoacrilate glue	Krazy glue		Singles
Decapitation scissors	FST	14130-17	
Feather blades	Feather	FA-10	
Filter paper #2	Whatman		Either rounds or pieces cut from a bigger sheet work well.

Forceps	A. Dumont & Fils	Inox 3c	
Glass bubblers (Robu glass borosillicate microfilter candles) - porosity 3	Robuglas.com	18103 or 18113	Glass bubblers are more expensive than bubbling stones used in aquaria. However, they are easy to clean and sterilize, and can last a long time.
Glucose	Sigma-Aldrich	G8270	
HCl	Fisher	A144SI-212	
Ice buckets			
KCl	Sigma-Aldrich	P4504	
Ketamine HCl (KetaVed)	VEDCO	NDC 50989-996-06	
KH_2PO_4	Sigma-Aldrich	P0662	
Leica Tissue slicer VT1000S			The cutting settings are 1 mm horizontal blade amplitude, frequency dial at 9, and speed setting at 2
Magnetic stirrers and stir bars			
Mg_2SO_4 x $7H_2O$	Sigma-Aldrich	230391	
$MgCl_2$	Sigma-Aldrich	M9272	
MilliQ water machine	Millipore		Source for 18 Mohm water
Na-ascorbate	Sigma-Aldrich	A4035	
Na-pyruvate	Sigma-Aldrich	P8574	
NaCl	Sigma-Aldrich	S3014	
$NaHCO_3$	EMD	SX0320-1	
Needle 27G1/2			
NMDG	Sigma-Aldrich	M2004	
Paper tape			
Peristaltic pump	Cole-Parmer	#7553-70	
Peristaltic pump head	Cole-Parmer	Masterflex #7518-00	

Personna blades		Personna double edge	Amazon
pH meter			
Recovery chamber	in-house made		
Scalpel blade handle size 3	Bard-Parker (Aspen Surgical)	371030	
Scissors angled blade	FST	14081-09	
Single edge industrial razor blade #9	VWR	55411	
Spatulas			
Transfer pipettes	Samco Scientific	225	
Upright microscope	Olympus BX51WI		
Xylazine HCl (XylaMed)	VetOne	510650	

April 13, 2020

Dear Dr. Bajaj,

Please, find the point-by-point rebuttal to both the Editorial and Reviewers' comments below in red font.

Editorial comments:

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. DONE.
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. DONE.
3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …" DONE.
4. Please define all abbreviations during the first-time use. DONE.
5. JoVE cannot publish manuscripts containing commercial language. 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. The only place where commercial language appears in this revision is in the Table of Materials. DONE.
6. Please move all the materials and equipment section to the table of materials. We need only action steps in the video. The Protocol section now starts with the "Setup". DONE.
7. Please use S.I units throughout e.g., M for moles/L etc. DONE.
8. For solutions and recepies, please include a separate table as a .xlsx file and upload it separately to your editorial manager account. Please do not embed the table in the manuscript. DONE.
9. 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." DONE
10. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). DONE
11. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes. DONE.
12. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. A paragraph at

the beginning of the Protocol section has the ethics and IACUC statements. Lines 103-108. DONE.
13. Please use complete sentences throughout. The incomplete sentences were corrected throughout the text and Figure Legends. DONE.
14. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. Individual protocol steps contain 3 actions at most. DONE.
15. Please ensure you answer the "how" question, i.e., how is the step performed? I believe this has been accomplished.
16. Please include the strain of mouse used? The mouse genetic background and strain information is now in the Protocol section (line 107-108), as well as Representative Results (line 245-246). DONE.
17. Please move the summary to the discussion section. The summary section from the Protocol in the prior version of the manuscript has been moved to the beginning of the Discussion section (lines 302-305). DONE.
18. 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 highlighted text is less than 2.75-page limit. DONE.
19. Please ensure that representative result is described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title, how do these results show the technique, suggestions about how to analyze the outcome, etc. The Representative Results section is re-written to match the above requirements. DONE.
20. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol. The Figure Legends have been edited as requested. DONE.
21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." The License to Publish file from Molecular Psychiatry has been uploaded with the manuscripts files. The relevant language on Copyright is in Section 3. DONE.
22. 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 DONE
b) Any modifications and troubleshooting of the technique DONE.
c) Any limitations of the technique. Implicit limitation in the method is that it is ex-vivo, which is generally accepted, so it has not been discussed explicitly. Discussion on the difficulty of the preparation has been folded into the discussion on modifications above.
d) The significance with respect to existing methods DONE

e) Any future applications of the technique DONE
23. Please do not abbreviate the journal titles in the reference section. DONE

Reviewer #1:
Manuscript Summary:
The possibility of performing experiments in brain slices from adult animals is crucial in many studies, but transcardiac perfusion is necessary to minimize hypoxia which is mostly lethal in mature tissues. This procedure has been adopted in several laboratories and it is therefore not original, but after checking in the JoVE database I did not find the approach explained in detail, i.e. there is not a detailed and reviewed visual demonstration available. Thus, I expect that an original video reporting the protocols would be very popular. I have however some remarks that Dr Djurisic should address to improve this work.

I thank Reviewer #1 for the constructive comments. This version of our protocol was focused on hippocampal slices because it was an invited contribution for the acute hippocampal slice collection. However, the comment on the use of transcardial perfusion for slice preparations from different brain regions of adult rodents is well taken. Modifications to the manuscript have been made accordingly, which together should provide a necessary context for the protocol under revision here. Below are the point-by-point answers to the two major requests.

Major Concerns:
Most of the protocols described in the manuscript and that will be described in the video are not important only to prepare hippocampal slices but also brain slices from other areas. Just to cite three examples, transcardiac perfusion with different variants was used to prepare cerebellar slices (Blot and Barbour, Nat Neurosci 17 289-95, 2014), cortical slices (Thomson and Bannister, J Physiol 519 57-70, 1999), and midbrain slices (Lammel et al., Neuron, 70, 855-62, 2011). This publication should analyze the most important applications of the transcardiac perfusion approach of the last 20-25 years and fully acknowledge the associated literature. My expectation is that most of potential users will adopt the approach of transcardiac perfusion to prepare brain slices from other areas in a variety of applications in adult rodents. Obviously, hippocampal slices is still the specific preparation used in Djurisic and Schatz laboratories, but this should be shown as an application example.

I thank Reviewer #1 again for this comment. The Introduction section has been expanded to provide a very brief overview of the use of transcardial perfusion in slice preparation from adult rodents (both rats and mice) over the last three decades. The hope is that this addition provides the necessary context for the protocol presented here, as well as contrast to an ongoing difficulty of preparing hippocampal slices from adult mice. Lines 85-95 in Introduction:

> "…Over time, a number of laboratories studying adult rodents have introduced transcardial perfusion with an ice-cold solution to decrease the

animal's body temperature, and therefore hypoxic damage to the brain, prior to decapitation. This procedure was successfully applied across different brain regions: cerebellar slices [17], midbrain slices [18], neocortical slices [10,19], perirhinal cortex [20], rat hippocampus [9,21,22], olfactory bulb [23], ventral striatum [24], visual cortex [25].

In spite of the advantages offered by transcardial perfusion and Na^+ ion replacement in preparing slices from rat and in some brain regions in mice, mouse hippocampus remains one of the most challenging areas to protect from hypoxia [12,19]. To date, one of the most common approaches to slicing hippocampus from aging mice and mouse models of neurodegeneration involves the classical fast slicing of the isolated hippocampi [26]…"

Although JoVE did not provide a publication for the transcardiac perfusion approach, there is a publication describing the use of NMDG for the preparation of acute brain slices (Ting et al. JoVE, 2018 doi: 10.3791/53825) that might partially overlap with the present work. This publication, that does not utilize transcardiac perfusion, is cited by Dr Djurisic, but it might be useful to address more in detail the differences or the common protocols at the stage of slice preparation which is supposed to be described again in the present work. While watching the video by Dr Djurisic, scientists might find also useful to watch the Ting et al. video to develop their own protocol adapted to the specific preparation.

I thank Reviewer #1 for this comment. To address the comment, in this revised version of the paper there is a new "Significance" section in the Discussion, where the Ting protocol(s) have been discussed in greater detail relative to the protocol in this manuscript. Lines: 330-341.

"**Significance.** … In fact, a protocol that uses NMDG-aSCF has been reported for adult mouse neocortex and striatum slice preparations [12,19]. This set of protocols uses an NMDG-aCSF recipe that is not sodium-free (it contains 30 mM $NaHCO_3$) [19], a potentially critical factor for regions as susceptible to hypoxia as hippocampus. Moreover, transcardial perfusion has been conducted with NMDG-aCSF at room temperature [12]. As discussed above, without lowering the body temperature via cold transcardial perfusion, the ATP levels will drop to about 20% of normal value within a minute [4,6]. This loss of ATP could be hard to recover in brains from aging animals. It follows then that re-introduction of Na^+ ions with regular aCSF during the recovery step will result in more cytotoxic edema. "

Reviewer #2:

Manuscript Summary:

This manuscript by Dr. Djurisic describes the technique to cut hippocampal slices. This technique will be especially very helpful for researchers working with brain tissues from

older animals and can be applied to different brain regions with changes in the initial blocking/cutting angle.

Many thanks to Reviewer #2 for the constructive comments. All of the Reviewer #2's requests have been addressed, either in text or in rebuttal.

Major Concerns:
I only have a major concern. What is the advantage of a perfusion made with normal ACSF with respect to the NMDG-one? From all the reasons brought up in the manuscript to remove Na+ (and possibly Cl-, see below) from the cutting solution, it would appear that it would be beneficial during the transcardiac perfusion as well. Therefore, it is unclear why the perfusion is presented as performed with normal ACSF.

The Reviewer #2 makes a valid point. Previously published work from our laboratory reports data from slices prepared after transcardial perfusion with regular aCSF (Djurisic et al 2019); this approach has been described in the first version of this manuscript. However, we have recently switched to all-NMDG protocol in the lab. Even though we have not published any work yet using the all-NMDG version of the slice method, per Reviewer #2's suggestion, the revision of this protocol now entails transcardial perfusion, as well as cutting in NMDG-aCSF. We believe that this version of the protocol should be more uniformly successful across different mouse lines and brain regions, as was implicit in the Reviewer #2's comment. The reference to the "all-NMDG" approach is referenced throughout the manuscript, in addition to the Setup section of the protocol (lines 16, 33-34, 97-98, 111-131, 145-146, 169-171, 212, 226-227).

At the same time, a possibility of transcardial perufion with regular aCSF has been mentioned. Subsection 1.1.2. in Setup (lines 118-119) references few papers from different laboratories, in addition to ours, where ice-cold aCSF was successfully used for transcardial perfusion, suggesting that under certain circumstances cold temperature on its own provides sufficient protection from hypoxia:

"1.1.2. Note: Depending on the brain region or mouse strain, chilled aCSF can be also used for transcardial perfusion with success [11,15,26]."

Minor Concerns:
Lines 44-45: a reference should be added for this statement
This statement has been deleted. However, a reference to the older work by Lipton and Whittingham (1979) that demonstrated the loss of neural activity in hippocampal slice when O2 concentrations in the bath was lowered from 80% to 20% is cited in line 57 of Introduction.

Line 50: a reference for a review that mentions that electrophysiology experiments are performed mainly on younger animals would be helpful. Lines 48-49

Line 75 In addition to substitution of Na+, the absence of Cl- appears to be essential

because passive Cl- influx also cause swelling and excitotoxicity (Rothman SM (1985) J Neurosci 5:1483-1489).
I really appreciate this comment. However, unlike sucrose-aCSF, the use of NMDG-aCSF does entail physiological Cl- concentration, as the NMDG has to be buffered with equimolar HCl, thus introducing Cl- ions. However, finding suitable Cl- substitutions could be potential new modification to the protocol. A line has been added in the Discussion, to suggest that new modification (line 321). Again, many thanks for this comment.

Lines 133-134 Can a gravity system be used for the perfusion, or a peristaltic pump is essential for the perfusion?
It can. This possibility is now clearly stated in lines 174-177.

"2.3.1. Note: Perfusion by gravity is a good substitute for the peristaltic pump perfusion, as long as the same approximate flow-rate can be achieved. ..."

Line 212 NMDA should be changed to NMDG. Corrected.

Lines 239- 244 More details about the making of the recovery chamber would be helpful.

Done. Lines 136-143.

"1.4.2. Note: The slice recovery chamber used here is very similar to the classical chambers described by previously by Edwards and Konnerth (1992) [3]. aCSF is kept in a glass beaker (400 ml) that holds a round acrylic frame with black nylon mesh glued to the bottom. The acrylic frame is suspended in the middle of the beaker via an acrylic hook resting over the edge of the beaker. The glass bubbler is inserted all the way to the bottom. The design allows for oxygenation of both sides of slices. Bubbling also provides constant mixing of aCSF in the recovery chamber. Black mesh provides a high contrast against the off-white slices, which are then easier to see."

Lines 314-318 It might be a matter of semantics, but I wouldn't define the perfused brain as white; maybe beige/cream color instead?

Point well taken. Since there is a range of shades in brain color that can occur after perfusion, "off-white" has been chosen, as it is less specific. I hope this is acceptable. Line 308.

Lines 337-339 Given that the concentration of ascorbate and pyruvate is usually 1-3 mM, I wouldn't think that the increase in Na+ would be a concern.

I thank the Reviewer for noticing this discrepancy. The comment was aimed at the NMDG-aCSF solution by Ting et al (2014, 2018), that contains 30 mM sodium-bicarbonate, thus making their NMDG-aCSF NOT sodium-free. This has been now clearly stated at multiple instances in the paper: e.g. lines 130-131 in the Setup:

> "1.2.3. Note: … Previously published NMDG recipes use 30 mM $NaHCO_3$ for buffering, which results in 30 mM sodium present in NMDG-aCSF [13,20]."

See also lines 334-336 of Discussion:

> "This set of protocols uses an NMDG-aCSF recipe that is not sodium-free (it contains 30 mM $NaHCO_3$) [20], a potentially critical factor for regions as susceptible to hypoxia as hippocampus."

The reference to ascorbate and pyruvate introducing 1-3 mM Na is now removed.

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Author(s) [Please list all named authors, continuing on a separate sheet if necessary]: Maja Djurisic, Barbara K. Brott, Nay L Saw, Mehrdad Shamloo, Carla J. Shatz (the "Author(s)")

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