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Preparing acute brain slices from the dorsal pole of the hippocampus from adult rodents

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Cover Letter

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27/07/2020

Dear Nam Nguyen, Kyle Jewhurst, and Felix Leroy,

Thank you for reviewing my protocol manuscript for the planned methods collection of "Preparation of Acute Hippocampal Slices" in the Journal of Video Experimentology (JoVE). I submit to you my revised manuscript titled: "*Preparing acute brain slices from the dorsal pole of the hippocampus from adult rodents"* for further consideration for publication.

In the revised protocol text, I have addressed all the reviewer's and editorial comments as requested. These changes and corrections are outlined in the Response to Editor and Reviewer file, submitted with the manuscript.

In addressing the comments, I have substantially revised the text of the original manuscript as well as including a new figure 1, which encompasses elements of the protocol and experimental set-up, as requested by the reviewers. Given the current phase of lock-down in the United Kingdom, in response to the ongoing Covid-19 pandemic, it was not possible to add new data comparing the efficacy of this method to other similar methods as requested by Reviewer 4 (Major point 1). However, I have used an alternative approach to validate this method to that of previous reports.

Regarding the editorial changes requested, all figures and tables have now been separated from the manuscript text file. Changes to the manuscript text have been highlighted in yellow, and protocol steps that are required for filming are indicated in green. If this is not suitable, please let me know and I can provide a version of the manuscript with an alternative highlighting approach.

I hope that you find the manuscript suitable for publication at JoVE and please do not hesitate to contact me if you require any further information.

Yours sincerely,

Sam A. Booker

TITLE:

Preparing Acute Brain Slices from the Dorsal Pole of the Hippocampus from Adult Rodents

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KEYWORDS:
Electrophysiology; Acute slice; Whole-cell patch-clamp recording; Hippocampus; Dorsal; Coronal

SUMMARY:
The purpose of this protocol is to describe a method to produce slices of the dorsal hippocampus for electrophysiological examination. This procedure employs perfusion with chilled ACSF prior to slice preparation with a near-coronal slicing angle which allows for preservation of healthy principal neurons.

ABSTRACT:
Whole-cell patch-clamp recordings from acute rodent brain slices are a mainstay of modern neurophysiological research, allowing precise measurement of cellular and synaptic properties. Nevertheless, there is an ever increasing need to perform correlated analyses between different experimental modes in addition to slice electrophysiology, for example: immunohistochemistry, molecular biology, in vivo imaging or electrophysiological recording; to answer evermore complex questions of brain function. However, making meaningful conclusions from these various experimental approaches is not straightforward, as even within relatively well described brain structures, a high degree of sub-regional variation of cellular function exists. Nowhere is this better exemplified than in the CA1 of the hippocampus, which has well-defined dorso-ventral properties, based on cellular and molecular properties. Nevertheless, many published studies examine protein expression patterns or behaviorally correlated in vivo activity in the dorsal extent of the hippocampus; and explain findings mechanistically with cellular electrophysiology from the ventro-medial region. This is further confounded by the fact that many acute slice electrophysiological experiments are performed in juvenile animals, when other experimental modes are performed in more mature animals. To address these issues, this method incorporates transcardial perfusion of mature (>60 day old rodents) with artificial cerebrospinal fluid followed by preparation of modified coronal slices including the septal pole of the dorsal hippocampus to record from CA1 pyramidal cells. This process leads to the generation

of healthy acute slices of dorsal hippocampus allowing for slice-based cellular electrophysiological interrogation matched to other measures.

INTRODUCTION:

The hippocampus is arguably the most well studied structure in the mammalian brain, due to its relatively large size and prominent laminar structure. The hippocampus has been implicated in a number of behavioral processes: spatial navigation, contextual memory, and episode formation. This is, in part, due to the relative ease of access to the dorsal portions of the hippocampus in rodents for in vivo analysis. Indeed, the major output cells are typically less than 2 mm from the pial surface.

In rodents, the hippocampus is a relatively large structure, formed of an invagination of the telencephalon extending from the dorsal septum to the ventral neocortex. It is composed of 2 major regions: the dentate gyrus and the *cornu ammonis* (CA); the latter of which is divided into 3 well-described sub-regions (CA1-3) that extend into the dentate gyrus hilus (formerly known as CA4), based on connectivity, cellular anatomy, and genetic properties[1]. This structure is maintained along the dorso-ventral extent of the hippocampus, albeit with major variations in synaptic properties[2-4], anatomy[5], genetic diversity[6-8], and behavioral function[9,10]. Of the CA regions, the CA1 subfield is composed largely of glutamatergic CA1 pyramidal cells (CA1 PCs), for which 3 subtypes have been defined[11], and inhibitory interneurons that make up ~10% of neurons, but are highly diverse with over 30 subtypes defined[12-14]. In addition to regional specific differences, normal aging has been shown to have dramatic effects on synaptic transmission[15-17], anatomy[18], and genetic profile[19]. The current gold-standard method to assess the intricacies of cellular and synaptic properties in a controlled manner is through the use of whole-cell patch-clamp recordings from acute brain slices[20].

The understanding of hippocampus function is based largely on dorsal manipulation due to the ease with which it is accessed surgically or anatomically for behavioral tasks, implantation of electrodes or imaging windows, or viral plasmid expression. In many studies additionally, these procedures are performed with late-juvenile or adult rodents to prevent variability in brain structure during development. Despite this, many approaches to examine cellular and subcellular electrophysiology are performed in early- to mid-juvenile rodents, from mostly the ventro-medial portion of the hippocampus in its transverse plane[21-25]. Where the whole dorso-ventral extent has been assessed, a tissue-chopper is used to maintain the transverse extent[4,26], or the experiment has been performed in young rats[27] or mice[28]. Furthermore, cooling of tissue prior to dissection of the brain is known to preserve hippocampal structure in rats[29] and neocortical neurons in mice[30,31]. Nevertheless, there is a paucity of detail regarding the production of brain slices from the dorsal transverse axis of the hippocampus, as generated by modified coronal slices, in mature rats.

This protocol describes an approach by which whole-cell patch-clamp recordings can be obtained from single or pairs of neurons in modified coronal slices of dorsal hippocampus from aged rats, followed by post-hoc morphological identification. Healthy

brain slices are obtained following transcardial perfusion of chilled artificial cerebrospinal fluid (ACSF), facilitating measurement of electrophysiological properties from CA1 PCs and local interneurons.

PROTOCOL:

All animals were generated and maintained according to the Home Office and Institutional guidelines (HO# P135148E). All rats were maintained on a 12 h light/dark cycle and given *ad libitum* access to food and water.

1. Transcardial perfusion of chilled ACSF

1.1. Prior to all experiments, place ~200 mL of sucrose-ACSF (**Table 1**) in the freezer at -20 °C (until semi-frozen, for slicing) and a further ~100-200 mL of filtered sucrose-ACSF on ice (for perfusion), bubbling with carbogen (95% O_2/5% CO_2).

1.2. Collect an adult rat from its home cage and place it for ~30 minutes in a holding cage in the procedure room to acclimatize to noise and light levels.

1.3. Prepare the dissection tools (**Figure 1A**), the perfusion area and the injectable anesthetic (approx. 1 mL of 200 mg/mL sodium pentobarbital for a final concentration of 100 mg/kg).

1.4. Prepare an appropriate anesthesia chamber by placing a small swab of tissue paper or cotton wool inside. Introduce 1-2 mL of volatile isoflurane anesthetic to the absorbent material in the chamber.

1.5. Place the rat in an anesthesia chamber to sedate. Monitor breathing until the breathing rate drops to ~1 shallow breath per second.

1.6. At this point, start bubbling semi-frozen sucrose-ACSF with carbogen on ice for use during slicing.

1.7. Weigh the rat and note its weight.

1.8. Terminally anesthetize the rat by injecting the prepared sodium pentobarbital into the intraperitoneal cavity. The dose of sodium pentobarbital should be 100 mg/kg, calculated from the previously taken weight and stock concentration of drug. Place the rat in a holding chamber and allow 0.5-5 minutes for the onset of terminal anesthesia.

1.9. Confirm cessation of reflexes: test both corneal blink (touch the pupil) and hind-paw pinch (lift the leg and pinch the hind-paw) reflexes using a blunt probe (i.e., rounded forceps). Once reflexes have ceased, pin the rat to the polystyrene or cork surgical board using hypodermic needles.

1.10. Open the chest cavity and place the cannula at the base of the left ventricle of the heart. Puncture the right atrium and immediately start perfusion with the ice-cold (0 - 1 °C) sucrose-ACSF (using a peristaltic pump at 50 mL/minute).

1.11. Once full exchange of fluids and cooling of the body has occurred (<5 minutes), remove the cannula and pins, and then decapitate using a guillotine.

1.12. Carefully and rapidly remove the skull using bone scissors and Rongeur bone tools (**Figure 1A,1B**).

1.12.1. Start by making 2x bilateral cuts through the foramen magnum using the bone scissors and remove the skull to the lambda suture with the Rongeurs. Cut carefully along the midline suture with the bone scissors to just behind the eyes.

1.12.2. Make 2 bilateral cuts through the skull, perpendicular to the midline. Using the Rongeurs, open the skull along the midline. Take extra care to remove pia mater using fine scissors or a hooked needle.

1.13. Scoop the brain out of the skull using a blunt spatula, severing the cranial and optic nerves with side to side compression. Place the brain into carbogenated, semi frozen (0 – 1 °C) sucrose-ACSF for 1-2 minutes prior to slicing.

2. Preparation of brain slices from dorsal hippocampus

2.1. Remove the perfused brain from the semi-frozen (0 – 1 °C) sucrose-ACSF and place in a glass Petri dish lined with filter paper. Place the brain onto its ventral surface.

2.2. Using a scalpel (No. 22 blade), remove the posterior portion of the brain at ~10° from vertical to create a flat surface to glue the brain to the stage (**Figure 1C**).

2.3. Apply a small amount of cyanoacrylate glue to the stage of the vibratome. Spread the glue to make a thin film approximately 50% larger than the cross-sectional area of the cut surface of the brain.

2.4. Lift the brain out of the glass dish onto a spatula, cut side down, using a paintbrush to guide the tissue. Blot the brain with a piece of tissue to remove excess ACSF and slide the brain, cut surface down, onto the center of the glue. Using a Pasteur pipette, add 1-2 mL of ice-cold sucrose-ACSF over the brain to remove glue away from brain block.

2.5. Place the brain into the slicing chamber and flood with semi-frozen sucrose ACSF. Use a spoon or spatula to keep excess ice away from brain block, and then carbogenate (**Figure 1D**).

2.6. Move the blade of the vibratome into position: ~1 mm from the dorsal surface of the brain and vertically ~1 mm anterior to bregma. Ensure that the blade is fully submerged, and remove bubbles using a paintbrush.

2.7. Start slicing the brain. To trim down to the dorsal hippocampus, use a speed of 0.1 - 0.2 mm/s, with a horizontal blade movement of 1 - 1.5 mm and a reciprocal oscillatory rate of ~90 Hz. When slicing the dorsal hippocampus, reduce the speed to 0.05 - 0.1 mm/s.

2.8. Collect slices of dorsal hippocampus (nominally 3-4 full slices or 6-8 hemisected slices) per brain. If longitudinal slices of ventro-medial hippocampus are required, continue slicing. Once the dorsal hippocampus has been sliced, there is no need cut extra tissue beyond approximately the position of the 3rd ventricle. Stop the vibratome, separate the slice with a bent hypodermic needle, and collect in the base of the slicing chamber.

NOTE: Slices can be 250-500 µm thick, depending on experimental requirements. For recordings from the dorsal hippocampus, typically use 400 µm thick slices to preserve as much of the local network, whilst allowing the suitable microscopy conditions.

2.9. Trim the slices to contain only the hippocampus and overlying cortex under a dissecting microscope. Transfer slices to the pre-warmed 35 °C chamber with the anterior surface facing up.

2.10. Determine the storage chamber based on the experiment to be performed. To obtain high quality whole-cell patch-clamp or extracellular field recordings close to the slice surface in submerged recording chambers, store in submerged chambers. Alternatively, store in a liquid/gas interface chamber for recordings of oscillatory network activity or interface extracellular field recordings.

2.11. For the submerged storage conditions, allow slices to recover at 35 ºC for 30 minutes from the time of the last slice entering the storage chamber. This allows for reactivation of metabolic processes and re-sealing of cut neurites. After 30 minutes, transfer the storage chamber to room temperature.

3. Recording the dorsal hippocampal neurons

3.1. Fabricate recording patch pipettes from capillary glass. This protocol uses 1.5 mm outer diameter, 0.86 mm inner diameter borosilicate glass with filament, which yields a tip resistance of 3-5 MΩ when filled with intracellular solution (**Table 1**). Keep intracellular solutions chilled on ice to prevent degradation of energetic components and filtered prior to use (syringe filter, pore size: 0.2 µm).

3.2. Carbogenate recording ACSF and pre-warm in a water bath (35-40 ºC). Deliver the carbogenated and pre-warmed ACSF to the recording chamber via perfusion tubing

assisted by a peristaltic pump. Start perfusion several minutes prior to transferring a slice into the chamber.

3.3. Stop the perfusion and transfer a brain slice to the recording chamber with the anterior surface facing up. Hold the slice in place with a platinum ring with single fibers of silk attached to form a “harp” shape. Position the slices so that *stratum pyramidale* of CA1 runs perpendicular to the axis of the first recording pipette.

3.4. Restart the flow of carbogenated and pre-warmed (35-40 ºC) recording ACSF (**Table 1**) at an optimal rate of 6-8 mL·min^{-1}.

NOTE: High flow rates (6-8 mL·min^{-1}) are optimal for maintaining network activity in slices[32]. Lower flow rates (i.e., 2-3 mL·min^{-1}) can be used to maintain slice stability for imaging experiments or where biologically relevant network activity is not required.

3.5. Assess slice quality using infrared differential inference contrast (IR-DIC) optics with 40x objective magnification (visualized with a CCD camera). Assume good slice quality if a large number of ovoid-shaped, moderately contrasted CA1 PCs can be seen in *str. pyramidale* at depths of 20-30 µm below a smooth and lightly dimpled surface (**Figure 1A**). Poor quality slices contain large numbers of highly contrasted, shrunken or swollen cells, with an uneven slice surface.

3.6. Fill patch pipettes with an intracellular solution (e.g., based on an intracellular [Cl$^-$] of 24 mM) to allow comparison with other published data.

3.7. Perform whole-cell patch-clamp recordings as previously described[22]. Exclude cells from analysis if the membrane potential (V_M) on break-through is more depolarized than -50 mV, the series resistance is >30 MΩ; or the series resistance changes by >20% over the course of the recording. Under these recording conditions, series resistance is typically in the range of 8 – 25 MΩ and stable for up to 1 hour.

3.8. To examine CA1 intrinsic excitability from the dorsal hippocampus, test intrinsic physiological properties with whole-cell recordings with the following protocols in current-clamp configuration:

3.8.1. From the resting membrane potential with no bias current applied, apply small (-10 pA, 500 ms) current steps repeated 30 times.

3.8.2. From -70 mV with bias current applied, apply hyper to depolarizing current steps of 500 ms duration (-100 to +400 pA, 25 pA steps) with 3 repetitions of a family of traces.

3.8.3. Apply a sinusoidal wave of 100 pA peak-to-peak amplitude, with variable frequency from 0.1 to 20 Hz. Repeat 3 times.

3.8.4. Apply a 5x 2 nA, 2 ms stimuli to drive action potentials at 20, 40, 60, 80, 100 Hz. 10 sweeps per frequency.

3.8.5. From a -70 mV voltage-clamp, apply 5 minutes of spontaneous excitatory postsynaptic currents (EPSC) for recording.

3.9. To reseal cells for histological analysis following successful recording, produce outside-out patches by slowly retracting the patch pipette. When an increase in series resistance is observed from experimental levels to >1 GΩ as measured by a -5 mV test pulse, raise the holding potential to -40 mV and retract the pipette fully.

3.10. Perform additional recordings in the same slice to satisfy the required statistical power of the experimental design.

3.11. Remove brain slices containing recorded neurons from the recording chamber, place in 24-well plate, replace the ACSF with 4% paraformaldehyde (in 0.1 M phosphate buffer) and leave overnight.

3.12. The next day, replace the PFA with 0.1 M phosphate buffer and store until histological processing. Visualize cells with fluorescent-conjugated streptavidin as previously described[22].

REPRESENTATIVE RESULTS:

The protocol described above allows for the preparation of viable slices from the septal pole of the dorsal hippocampus in mature rats. A key factor in this protocol is the perfusion of chilled sucrose-ACSF, prior to slice preparation, resulting in healthy CA1 PCs proximal to the slice surface. The quality of the slice produced is assessed visually under IR-DIC optics, and healthy cells identified as having large, ovoid-shaped cell bodies are located throughout the full extent of *stratum pyramidale*, from the compact layer, into *stratum oriens* (**Figure 2A, black arrow**). Unhealthy slices are identified as having dead cells on the surface and rarely in the depths of the slice (**Figure 2A, red arrow**), which are identified on the basis of having either condensed and highly contrasted somata, or large “ballooned” somata with condensed nuclei.

Confirmation of slice health is achieved by performing whole-cell patch clamp recordings from putative healthy neurons. Whole-cell patch-clamp recordings are achieved with rapid, spontaneous gigaohm seal formation (15.5 ± 2.9 s; **Figure 2B**), comparable to those previously reported[31]. When the membrane is ruptured, healthy neurons in mature rats possess hyperpolarized resting membrane potentials (Mean: -65.6 ± 1.5 mV, Range: -55.6 to -73.9 mV; 15 PCs from 4 rats) and relatively low input resistances (Mean: 90.3 ± 5.2 MΩ, Range: 54.9 to 134.2 MΩ; 19 PCs from 4 rats). General slice quality is confirmed by high-fidelity spontaneous EPSCs (**Figure 2C,2D**), given low electrical noise (<10 pA peak-to-peak) when filtered at 10 kHz. Furthermore, stable cell recordings of hyperpolarized neurons require typically a <200 pA holding current, which is stable over long periods, due to the absence of network activity in the submerged recording conditions of this slice preparation.

Whole-cell patch clamp recordings from dorsal CA1 PCs allow for direct measurement of action potential discharge properties. Provided that the slice quality is sufficiently high, many cells can be recorded from a single slice within a short time frame (~1 hour). A key determinant of cell viability is the presence of an intact dendritic tree, and the axon surviving beyond the initial segment. The slicing angle of 10° from vertical allows for the preservation of this cellular anatomy, with cells preserved within the plane of slicing (**Figure 3A**). Healthy CA1 PCs from adult rats typically have a hyperpolarized membrane potential of -60 mV to -70 mV, input resistances of 100-200 MΩ and membrane time-constants of 20-40 ms when measured at the soma (**Figure 3B**). A key requirement for neuron inclusion in datasets is the presence of action potentials in response to depolarizing stimuli. CA1 PCs in adult rats present increasing numbers of action potentials to depolarizing stimuli, from the rheobase current to the maximum tested currents (400 pA), at which trains of action potentials display both adaptation of inter-spike times and accommodation of action potential amplitude (**Figure 3C**). The use of a variable frequency sinusoidal wave (0.1 -20 Hz over 20 s) allows for characterization of the membrane resonance of the recorded neurons (**Figure 3D**). Finally, temporally controlled trains of action potential discharge over a range of frequencies allow for comparison of accommodation and recruitment of K^+ channels associated with the resulting hyperpolarization (**Figure 3E**). Following *post-hoc* confirmation of intact dendrites using streptavidin visualization of biocytin labelling performed during recordings, the spontaneous EPSC frequency measured from the continuous recording (**Figure 2B, upper**) allows for characterization of CA1 PC integration into the local network.

In summary, optimization of slice quality of the dorsal extent of the hippocampus allows for whole-cell recordings from multiple neurons per slice. This slice preparation facilitates the collection of large datasets of intrinsic excitability, the establishment of intra-animal variability measures, and the production of slices of sufficient quality to perform paired recordings from synaptically-coupled neurons.

Figure 1: Overview of experimental setup and dissection schematic. (**A**) Experimental tools for all aspects of slice preparation, labelled according to use. (**B**) Cartoon depicting the directions of cuts with bone snippers and the movement of the spatula (pink arrows) to remove the brain from the skull. (**C**) Overview of the cutting angle (dashed red line) to allow preservation of dorsal CA1. (**D**) Overview of the slicing chamber with the brain mounted, anterior aspect facing up.

Figure 2: **Identification of healthy neurons from the CA1 region of dorsal hippocampus.** (**A**) Micrograph of area CA1 from an acute dorsal hippocampal slice, produced from a near coronal brain slice. The patch pipette is shown in a whole-cell configuration from a healthy neuron in the slice (indicated with black arrow). A nearby highly contrasted neuron to be avoided for recording is indicated (red arrow). (**B**) Representative continuous recordings of spontaneous EPSCs performed at -70 mV voltage-clamp from a stable recording of a healthy CA1 PC (black), with spontaneous EPSCs identified (green circles) and an unstable/unhealthy cell recorded under the

same conditions (red). The holding current required to maintain -70 mV voltage-clamp is indicated. (**C**) Expanded view from the region of the trace in (**B**) indicated with a shaded box. Note the EPSC present in the top, stable trace (black) and the unstable, noisy trace (red).

Figure 3: **Cell identification and intrinsic electrophysiology, as measured by whole-cell patch-clamp recording from dorsal hippocampal CA1 PCs.** (**A**) Visualization of biocytin with fluorescent-conjugated streptavidin labelling, followed by confocal imaging, from a slice containing multiple (6) CA1 PCs recorded sequentially, confirming the cellular identity of neurons recorded. (**B**) Average response to a -10 pA, 500 ms small hyperpolarizing step to ascertain passive membrane properties. (**C**) Voltage response of an identified CA1 PC to hyper- to depolarizing current steps (-100 to +400 pA, 500 ms duration). Action potential discharge is shown at both the rheobase current (grey sweep) and maximal discharge at 400 pA. (**D**) Membrane response to a 100 pA sinusoidal wave, frequency modulated from 0.1 - 20 Hz. Note the larger voltage response at the lowest cycle rates. (**E**) Trains of action potentials generated in response to trains of 5 stimuli (2 nA, 2 ms duration) over a range of frequencies (indicated).

Table 1: List of solutions used in the preparation and recording of brain slices. Solutions are listed with their components reported as mM concentration. Specific notes prior to use are listed.

DISCUSSION:

Here, a protocol is described to produce high-quality brain slices from the dorsal extent of the CA1 of the hippocampus, allowing for recordings from multiple viable neurons within this region. The combinatorial approach of whole-cell recording from near-coronal slices followed by neuron visualization is critical to the confirmation of cell viability and identity.

This protocol reliably produces viable slices for 2 major reasons. Firstly, the modification to the cutting angle, as a deviation from true coronal, allows for greater preservation of somatodendritic axis and thus biologically relevant function of neurons. Given that the orientation of the somatodendritic axis of CA1 PCs in the most dorsal extent of the hippocampus is not in plane with a true coronal section[1], this modification allows for greater tissue preservation. Alternatively, it is possible to remove the hippocampus fully and use a tissue chopper to prepare brain slices, as previously described for acute slices[4,33] and for slice culture[34,35]. A drawback to this approach is the potential for damage to the hippocampus during its extraction and chopping (in inexperienced hands), which is avoided by keeping the brain intact. This approach more closely resembles that of earlier studies that maintain the hippocampal neurons in the transverse plane, with respect to the septal/temporal axis[21,22]. The second major factor that contributes to viable neurons in mature rats is the use of ice-cold sucrose-ACSF perfusion immediately prior to decapitation, dissection, and slice preparation. Given that the rat skull at ages beyond 3 months is typically thick and much harder to cut with traditional brain slice preparation tools (i.e., fine scissors, scalpels, and forceps), the duration of dissection using Rongeur's and bone cutters is by its very nature longer,

thus pre-cooling of the brain affords the researcher more time for dissection and slicing. The speed at which the brain can be cooled and sliced has long been understood to be advantageous to slice quality[36], especially when the ice-cold ACSF is perfused before the cardiovascular system has been isolated from the brain[30,31,37,38]. Nevertheless, it has been suggested recently, that more physiological temperatures may also be useful for studying some brain regions[39].

The slice quality produced by the combination of ice-cold sucrose-ACSF perfusion and the modified near-coronal cutting angle provides slice quality near comparable to that of horizontal slices prepared at the same developmental stage. Indeed, the optimization of this technique, using a sucrose-ACSF composition with similar ionic composition to that used for recording, allows for great consistency between conditions used within the experiment and slice preparation approaches used for neonatal rats. One major drawback of the use of submerged slice storage and recording conditions, as described here, is that the activity of neuronal networks in brain slices is significantly reduced compared to the in vivo setting. This is overcome by alternatively transferring the cut slices into an interface chamber flowing with recording ACSF at 35 °C for storage. This approach significantly improves the activity of local circuit, allowing measurement of neuronal oscillations and functionally relevant neuronal firing[40,41]. Other methods of slice production from older rodents, such as the use of NMDG recovery can similarly produce very high-quality slices[30,31], which are suitable for the same recordings described. The specific advantage of this approach here is that it allows for direct comparison between recordings performed in younger rodents, based on slice preparations described previously[21,22] due to the identical ionic basis of the solutions and slice recovery conditions. A combination of this approach with NMDG-based recovery could also yield high quality slices.

Overall, the brain slice preparation and recording protocol described here allows for a direct comparison of neuronal physiology and anatomy in a high-throughput manner, with other experimental modalities performed in the dorsal hippocampus, as performed in other brain areas, such as the neocortex. Indeed, there is an increasing number of studies that address the neurophysiological differences between the dorsal and the ventral hippocampus[27,28,38,42,43]. However, few studies perform recordings at an age comparable to that used for behavioral, anatomical, or in vivo electrophysiological studies. As such, the combination of improved slicing procedures and an appropriate choice of rodent age will allow for more realistic correlation of neuronal physiology to brain function. The above protocols have been performed on rats up to 1 year of age, but there is no reason to believe that this could not be performed on older rats given the appropriate permissions.

In summary, the protocol presented here provides a reliable method to produce brain slices from adult rats, thus allowing comparison of electrophysiological properties of neurons to in vivo and anatomical experiments.

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DISCLOSURES:

The author declares that they have no competing financial interests.

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Figure 1

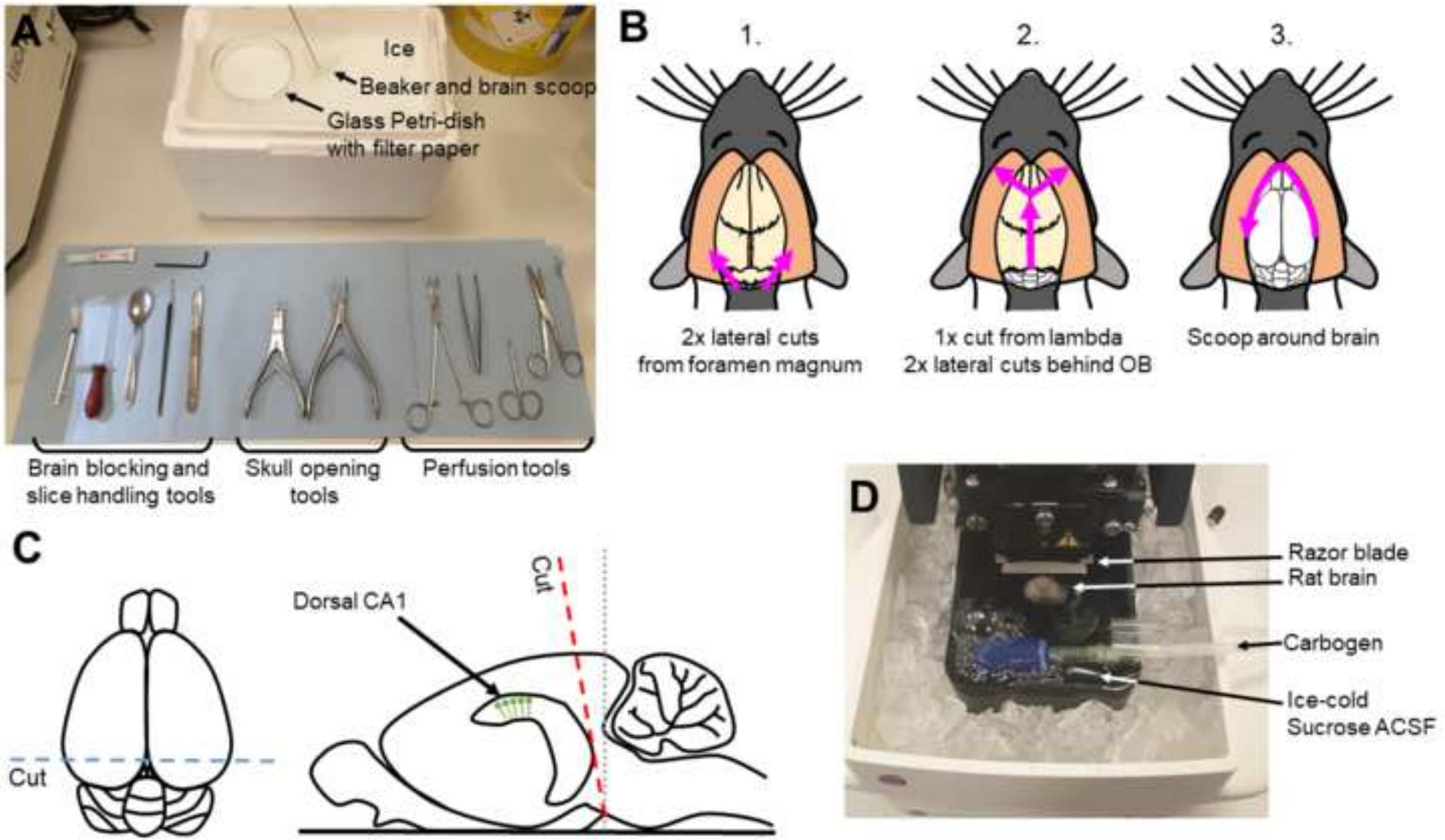

Figure 2

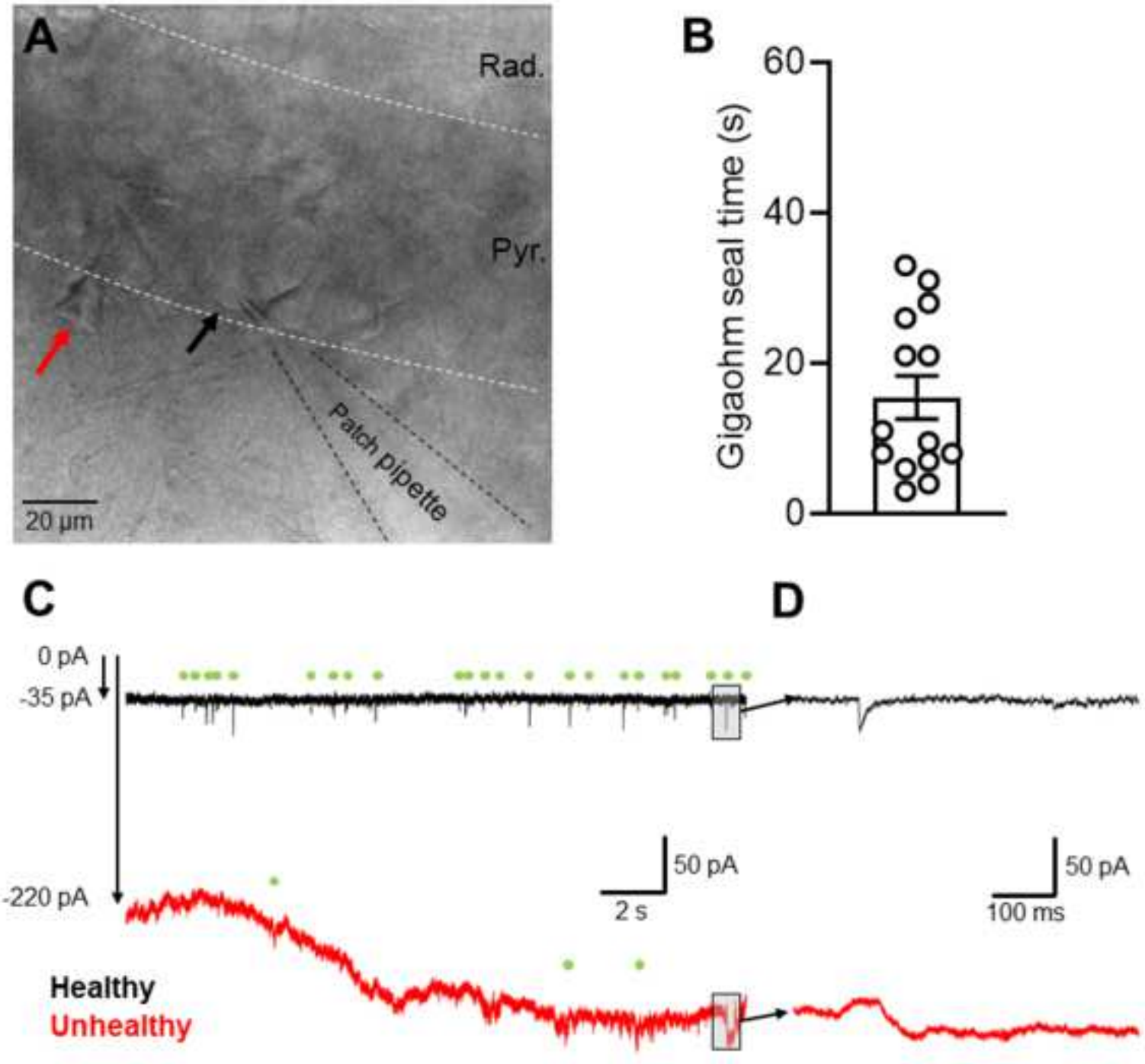

Figure 3

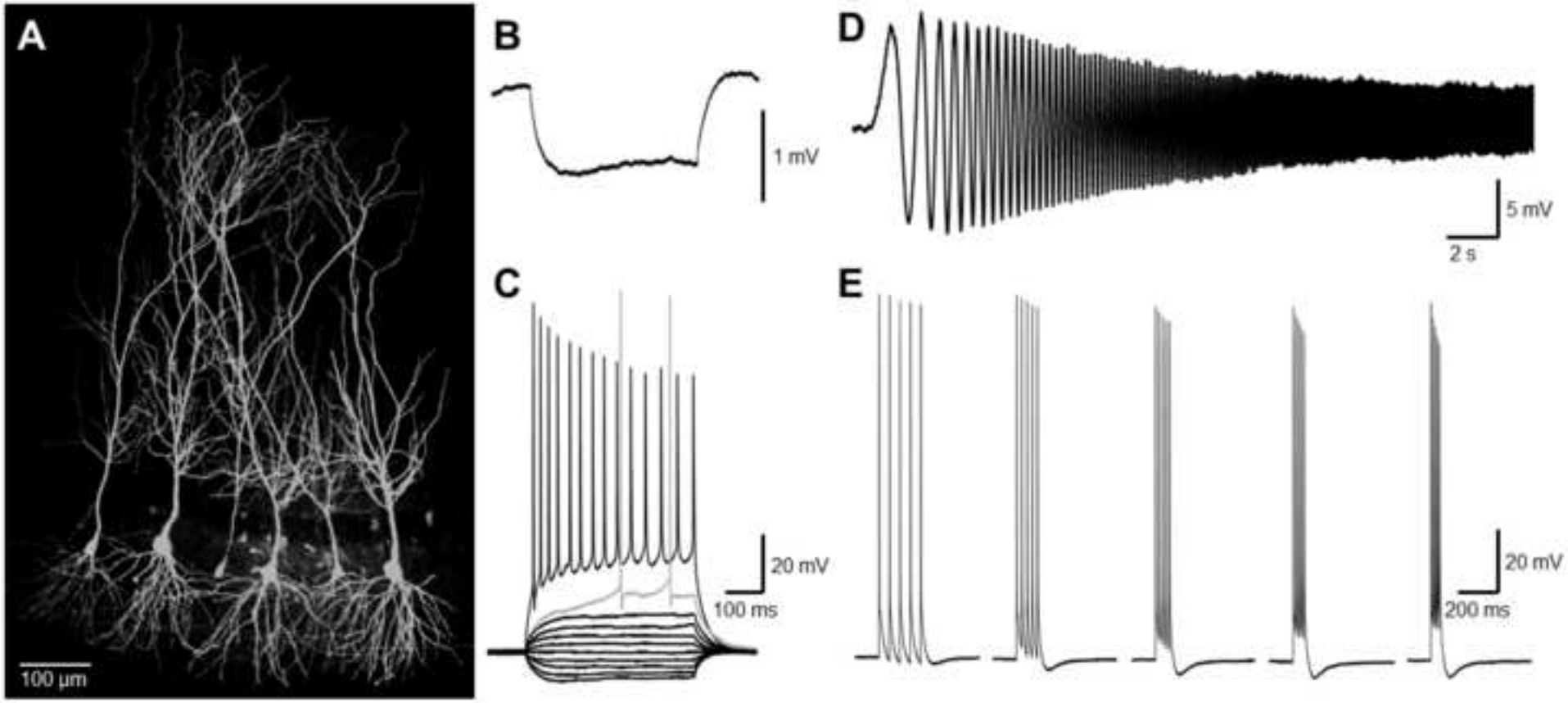

Table 1

Solution
Sucrose-ACSF
Recording-ACSF
Intracellular Solution (K-gluconate)

Composition (in mM)
87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose, 7 $MgCl_2$, 0.5 $CaCl_2$
125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, 2 $CaCl_2$
142 K-gluconate, 4 KCl, 0.5 EGTA, 10 HEPES, 2 $MgCl_2$, 2 Na_2-ATP, 0.3 Na_2-GTP, 1 Na_2-Phosphocreatine, 2.7 Biocytin (Osm ≈ 300 mOsm)

Notes
Chill before use
Pre-warm before use
pH to 7.35 with 10 M KOH

Table of Materials

Name of Material/Equipment	Company	Catalog Number
Acquisition software	Molecular Devices	pClamp 10
Adult rats	Various	n/a
Amplifier	Molecular Devices	Axopatch 700B
Analysis software	Freeware	Stimfit
Bone Scissors	FST	16152-12
Capillary Glass	Harvard Apparatus	30-0060
Carbogen	BOC	Various
CCD camera	Scientifica	SciCamPro
Chemicals/Reagents	Sigma Aldrich	Various
Cyanoacrylate (i.e. RS Pro 3)	RS Components	918-6872
Digitizer	Molecular Devices	Digidata 1550B
Dissection pins/needles	Various	Various
Electrophysiology system	Scientifica	SliceScope
Fine iris scissors	FST	14568-12
Glass Petri dish	Fisher Scientific	12911408
Hypodermic needles	BD Healthcare	Various
Isofluorane 100% W/W (i.e.IsoFlo)	Zoetis	50019100
Mayo-type scissors	FST	14110-17
Micromanipulators	Scientifica	Microstar
Paintbrush	Art store	n/a
Pasteur pipette	Fisher Scientific	11546963
Peristaltic pump	Watson Marlow	12466260
Pipette puller	Sutter Instruments	P-97
Plastic syringes (1, 2, 5 mL)	BD Healthcare	Various
Rongeur bone tool	FST	16021-14
Slice holding chamber	Homemade	

Slice weight/harp	Harvard Apparatus	SHD-22L/15
Sodium Pentobarbital (i.e. Pentoject)	Animalcare Ltd	10347/4014
Spatula	Bochem	3213
Syringe filters	Fisher Scientific	10482012
Vibtratome	Leica	1491200S001
Water Bath	Fisher Scientific	15167015

Comments/Description

Any strain of adult rat (60 days and older)

https://github.com/neurodroid/stimfit
Littauer style

Borosilicate glass pipettes with filament 1.5 mm outer diameter, 0.86 mm inner diameter.

95% O_2/5% CO_2

https://www.scientifica.uk.com/products/
All laboratory reagents procured from Sigma Aldrich.
Avoid gel based cyanoacrylate formulations

Use 16 gauge needles (above)
https://www.scientifica.uk.com/products/ scientifica-slicescope
With Tungsten-Carbide tips

16, 18, and 22 gauge

Blunt tips
https://www.scientifica.uk.com/products/scientifica-microstar-micromanipulator
A fine bristled paintbrush, procured from a local art shop.
A glass Pasteur pipette, but cut so that the blunt end is used to transfer pipette.
Single channel peristaltic pump

Other models and methods of pipette production would work equally well.

Alternatives would be suitable.

200 mg/mL; other formulations of pentobarbital would be suitable

Available from Fisher Scientific

Corning brand syringe filters, 0.22 µm pore diameter.

VT1200S model with Vibrocheck

5 Litre water bath, for example: Grant Instruments™JBA5

scientifica-scicam-pro

Response to Editors and Reviewers:

Editorial comments:
NOTE: Please read this entire email before making edits to your manuscript. Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

The manuscript has been proofread thoroughly.

• Please agree to the UK-specific Article License agreement and double-check the access type.

Done.

• **Abstracts:** Please add a Short Abstract/summary (10-50 words) that clearly states the goal of the protocol.

A short abstract has been added.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in the imperative: 3.5, 3.6, 3.7, Lines 249-261, etc

These examples and others have been corrected.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 1.7: How much pentobarbital?
This has been added.

2) 1.9: Mention all tools used.
These have been mentioned.

3) 1.11: What is the temperature of semi-frozen ACSF?
This has been added.

4) 2.9: how much is trimmed?
This has been added.

• **Protocol Numbering:**
1) Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations.
This has been formatted accordingly

2) Add a one-line space between each protocol step.
This is done

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

The filmable elements have been highlighted in green.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

I feel that the discussion is focussed, in fact several of the reviewers commended the thorough discussion. If there are specific concerns, please highlight these for me.

• **Figures:** Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

These have been removed accordingly.

• **Tables:** Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

This has been removed accordingly

• **References:**

1) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, (YEAR).]

I have downloaded and applied the JoVE Endnote style. I trust this has brought the references into the correct format.

• **Table of Materials:**
1) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
2) Sort the list alphabetically.

These have been done accordingly.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Booker describes a modified perfusion protocol to obtain high-quality brain slices from adult rodents. Usually, transcardial perfusion protocols are used for fixation of tissue. However, here a sucrose-based artificial cerebrospinal fluid (sucrose-aCSF) is being used for perfusion. The protocol will be of interest to other scientists.

I thank the reviewer for their positive appraisal of my protocol.

Major Concerns:

NMDG-aCSF is considered the optimal solution for making adult brain slices (References 30 and 31). The author may want to discuss differences between a sucrose- and a NMDG-based solution in more detail.

The reviewer makes a valid point, the introduction of NMDG-based slice recovery is an exciting new approach that addresses many issues covered by this protocol. Despite this, many recordings performed in younger rodents (<1 month) are highly successful without the use of NMDG-based recovery. This protocol therefore allows for the generation of high-quality, reliable electrophysiological recordings from slices of mature rats, as produced in solutions of identical ionic composition to recordings from younger rodents. A comment to this effect has been added to the discussion.

Minor Concerns:

- Point 3.3 states that the flow rate of recording ACSF should be between 6-8 mL/min. It might be worth mentioning that a lower rate of 2-3 mL/min is often used, too.

A note mentioning that lower flow rates are also possible has been added.

- Line 282: "... major drawback to this approach is the potential for mechanical damage ...". One could argue that someone with green fingers might be able to remove the hippocampus without damaging it. Please rephrase.

This has been rephrased.

Reviewer #2:

Manuscript Summary:

Among other factors, the preservation of neuronal health and the relative preservation of brain circuits are the major technical barriers in obtaining reliable and rigorous data. Among the many brain regions that are commonly studied, the hippocampus present many challenges, such as its layered structure, which requires slicing the brain with a specific angle in order to preserve neuronal inputs from afferents, and the large apical dendrites, whose the cut damages neuronal health and function significantly. Here, the author describes a brain slice technique that significantly enhances the quality of recordings in the CA1 field of the hippocampus. This method is very welcome.

I thank the reviewer for their positive appraisal of my protocol text.

Major Concerns:

I have no major concerns.

Minor Concerns:
1. In the introduction, line 59: please acknowledge the existence of CA4.
An acknowledgement has been added.

2. Line 65: "to" is missing after "In addition".
This has been added.

3. In protocol:
Point 1.7: while I am more familiar with mice, 5 minutes for onset of anesthesia seems long. Aren't neurons damaged after 5 minutes of anesthesia with sodium pentobarbital?
In my experience, individual rats respond differently to the same dose of Sodium Pentobarbital. I have added the distinction that 5 minutes is the upper limit. Often it is much less time (0.5 minutes) for reflexes to cease.

Point 2.2: Indicate the direction of the 10˚ angle. Although it seems obvious for the experienced physiologist, it may not for the novice. It would be very helpful if the author could provide a picture (real rat brain not necessary) or schematic for this step.

A schematic of the slicing angle, along with the slicing chamber and preparation of tools etc has been added as a new figure 1.

Point 2.6: similar to point 2.2, the description of this step could be ameliorated. A schematic would be very helpful for the visualization.

As above.

Point 2.8: Please mention why slices do not need to be cut beyond the 3rd ventricle.

This was to provide a landmark after which the dorsal hippocampus is no longer being sliced. I have clarified that this is the reason in the text.

Point 3.3: Why such a high flow rate? We typically use 2 mL/min, even in the hippocampus. If there is a clear rationale for using 6-8 mL/min compared to 2 mL/min, please explain.

The high flow rate maintains better the biologically relevant activity patterns of incoming synaptic events as well as intrinsic excitability observed *in vivo* (Hajos *et al.*, 2009). I consistently use higher flow rates, which lead to higher levels of spontaneous activity within slices. A note has been added, with reference, defining this aspect.

Point 3.4: I think the term "ovoid-shaped neurons" would be more accurate than round.

That is a very fair and correct point. Now changed accordingly.

Point 3.6: Vrest of pyramidal neurons in the hippocampus is typically between -80 and -70 mV (although the author indicates "-60 mV to -70 mV" in line 309), or at least -65 mV. -50 mV as a cut-off for Vrest seems very depolarized. Please justify.

I use -50 mV consistently as a cut-off to allow comparison of data produced over a variety of ages and between cell types, where Vrest is not as hyperpolarised as in mature CA1 neurons. A range of membrane potentials measured has been added to clarify the expected range.

Point 3.8: "When an increase in series resistance is observed". Please specify by how much?
This has been added.

4. Line 286: please replace "round" with "ovoid-shaped neurons".
Changed.

5. Line 295: "hyperpolarized resting membrane potentials": please give a range.

With respect to the above comment, a range has been added.

6. Line 298: Remove the "s" at the end of "stables".
Removed

7. Line 320: please elaborate on how you confirm intact dendrites post hoc.
This has been added.

Reviewer #3:
Manuscript Summary:
This is the text portion of a JoVE article describing a procedure to obtain brain slices suitable for electrophysiological experiments from the dorsal region of the adult rodent hippocampus. This procedure includes precooling the brain prior to slicing via transcardial perfusion with ice cold sucrose/ACSF and then cutting at a slight angle to better preserve the dendritic arbor of pyramidal neurons in the dorsal pole of this brain region. In general, the author has provided a sufficiently detailed description of the methodology. I do however, have several issues that can be addressed to improve the impact of this paper.

I thank the reviewer for their thorough and positive appraisal of my protocol text. I appreciate their concerns and have made a number of changes to the protocol to accommodate these issues.

General Concerns:
1. While I recognize that this is a methods paper, it would be informative if the author could provide some data demonstrating the superiority of his protocol over other brain slice procedures. I have no doubt that this procedure improves slice viability and the dendritic arbor of pyramidal neurons but it would be informative to know the extent of the improvement. There are certainly papers published on adult rodents that did not employ these methods (e.g. Mol Cell Neurosci 86: 50-57, 2018; J Physiol 576.2 351-377, 2006)

Indeed, many methods have been used within the published literature with alternative approaches. In my experience, the method described here allows for reliable patch recordings from dorsal hippocampal neurons. Due to the limited laboratory access available to myself currently, due to the ongoing COVID-19 restrictions of work, I was not able to collect parallel experiments performed under other conditions. However to address the reviewers

comments, I have now included a scatter plot in Figure 2 displaying the time to Gigaohm seal formation time from 3 separate experiments allowing comparison to the method cited below (Point 3), which shows that seal formation time is comparable with that of this other improved method.

2. This concern is amplified by the slice image in Fig 1, in which there is at least one unhealthy neuron proximal to a viable neuron in the field of view.

From my experience, it is uncommon to find a field of view within the upper 100 µm of acute slices that does not contain a single apparently unhealthy neuron, even in slices from younger rodents. I selectively chose this image to highlight which neuron would be better for whole-cell recording purposes.

3. The author provides a decent discussion of the likely reasons for the improvement in slice viability obtained with his protocol. However, he may want to briefly discuss how this method may be integrated with the NMDG ACSF recovery method, another recent advance in brain slice protocols that has been adopted by many laboratories. In fact, there is even a recent JoVE article on this method which also greatly improves slice viability and facilitates patch-clamp recordings in slices from older animals (https://www.jove.com/video/53825/preparation-acute-brain-slices-using-an-optimized-n-methyl-d).

This protocol is aimed at producing slices in adult rats under very similar conditions to those produced in young rats, where slice quality is far less of an issue and NMDG-recovery is not required. Discussion of alternative methods, in particular the use of NMDG-recovery has been added.

4. Finally, the only important methodological detail that I could not find was the thickness of the slices that were used in these experiments. If there is an optimal range, this should be included.

The range of slice thicknesses that can be used has been added as a note to the protocol.

Reviewer #4:

Manuscript Summary:

This protocol describes the preparation of acute slice from the dorsal hippocampus of the adult rat and its suitability for whole-cell recordings from multiple CA1 pyramidal cells per slice. The goal is to suggest an appropriate preparation to allow a direct correlation of in vitro electrophysiological data to anatomical and behavioural studies of dorsal hippocampal function.

The first key factor in this protocol is the use of semi-coronal dissection to follow the orientation of dendrites and axons of principal cells in CA1, thus preserving the integrity of neurons and their connections. The second is the use of transcardial perfusion of sucrose-ACSF before sectioning, that allows the cooling and cleaning of the brain from the blood. As the brain of adult animals is susceptible to the stress produced by the slicing process, I consider the perfusion important for reducing excitotoxic damage and enhance the quality of the slices.

Overall, the procedure is well explained, and the figures correctly represent the results. The viability of the preparation is confirmed by the representative results that show the feature of healthy neurons under IR-DIC optics, the expected electrophysiological properties and a

preserved morphology after recording. The discussion addresses all the significant points and reports the relevant literature on comparable methodology, giving the reader a good reason for choosing this method.

I thank the reviewer for their positive appraisal of my experimental approach.

Minor Concerns:
Given that the main point of this protocol is the procedure to dissect the brain, the only suggestion would be to add a simplified representation depicting this procedure if not already shown in the video.

A schematic has been added as part of new figure 1.