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

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Long 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 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 behaviourally 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.
Short Abstract:
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.

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, including: 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 pia 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) which extend into the dentate gyrus hilus (formerly known as CA4), based on connectivity, cellular anatomy, and genetic properties. This structure is maintained along the dorso-ventral extent of the hippocampus, albeit with major variations in synaptic properties, anatomy, genetic diversity, and behavioral function. Of the CA regions, the CA1 subfield is composed largely of glutamatergic CA1 pyramidal cells (CA1 PCs), for which 3 subtypes have been defined and inhibitory interneurons which make up ~10% of neurons, but are highly diverse with over 30 subtypes defined. In addition to regional specific differences, normal aging has been shown to have dramatic effects on synaptic transmission, anatomy, and genetic profile. 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.

Our understanding of hippocampus function is based largely on dorsal manipulation due to the ease with which it is accessed surgically or anatomically for behavioural tasks, implantation of electrodes or imaging windows, or viral plasmid expression; combined with the fact that in many studies 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. Where the whole dorso-ventral extent has been assessed, this has relied on the use of a tissue-chopper to maintain the transverse extent, or been performed in young rats or mice. Furthermore, cooling of tissue prior to dissection of the brain, is known to preserve hippocampal structure in rats and neocortical neurons in mice. 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. I show that 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:**

**Ethics Statement:**

All animals were generated and maintained according to Home Office and Institutional guidelines (HO# P135148E). All rats were maintained on a 12 hr 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 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% O₂ / 5% CO₂).

1.2. Collect an adult rat from its home cage and allow ~30 minutes holding cage in procedure room to acclimatize to noise and light levels.

1.3. In the meantime, prepare the dissection tools (Figure 1A), perfusion area and injectable anesthetic (approx. 1 mL of 200mg/mL sodium pentobarbital) – final concentration 100mg/kg.

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

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

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

1.7. Weigh rat and make note of its weight.

1.8. Terminally anesthetize rat by injecting 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 rat in holding chamber and allowing 0.5-5 minutes for onset of terminal anesthesia.
1.9. Confirm cessation of reflexes – using a blunt probe (i.e. rounded forceps) test both corneal blink (touch the pupil) and hind-paw pinch (lift the leg and pinch the hind-paw) reflexes. Once reflexes have ceased, pin rat to the polystyrene or cork surgical board using hyperdermic needles.

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

1.11. Once full exchange of fluids and cooling of body has occurred (<5 minutes), remove 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 and 1B). 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. Make 2 bilateral cuts through the skull, perpendicular to the midline. Using the Rongeurs, open the skull along the midline. Extra care should be taken to remove pia mater using fine scissors or a hooked needle.

Scoop brain out of skull using a blunt spatula, severing the cranial and optic nerves with side to side compression. Place 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 perfused brain from semi-frozen (0 – 1 °C) sucrose-ACSF and place in glass petri dish lined with filter paper. Place 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 centre of the glue. Using a Pasteur pipette, run 1-2 mL of ice-cold sucrose-ACSF over the brain to set glue away from brain block.
2.5. Place the brain into slicing chamber and flood with semi-frozen sucrose ACSF, use a spoon or spatula to keep excess ice away from brain block, then carbogenate (Figure 1D).

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

2.7. Start slicing of brain. For trimming down to dorsal hippocampus use a speed of 0.1 - 0.2 mm/s, with horizontal blade movement of 1 - 1.5 mm and reciprocal oscillatory rate of ~90 Hz. When you start to slice 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, I 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, to include the hippocampus, with overlying cortex and transfer to storage chamber, pre-warmed to 35 °C. Slices should be placed in the chamber with the anterior surface facing up.

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

2.11. For submerged storage conditions, allow slices to recover at 35ºC 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 storage chamber to room temperature.

3. **Recording protocols for dorsal hippocampal neurons**

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

3.2. Recording ACSF should be carbogenated and pre-warmed in a water bath (35-40 ºC) and delivered to the recording chamber via perfusion tubing assisted by a peristaltic pump. Perfusion should be initiated 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. Slices should be positioned so that stratum pyramidale of CA1 runs perpendicular to the axis of the first recording pipette.

3.4. Restart 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, as described at 6-8 mL.min⁻¹ are optimal for maintaining network activity in slice 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). Good slice quality is assumed 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, for example based on an intracellular [Cl⁻] of 24 mM, to allow comparison of data to other researchers produced previously.

3.7. Perform whole-cell patch-clamp recordings as previously described 22. Exclude cells from analysis if membrane potential (V_M) on break-through is more depolarized than -50 mV, series resistance is >30 MΩ; or 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. For the purpose of examining 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 resting membrane potential, no bias current applied:
Small (-10 pA, 500ms) current step repeated 30 times.

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

3.8.3. Sinusoidal wave of 100 pA peak-to-peak amplitude, with variable frequency from 0.1 to 20 Hz. Repeated 3 times.

3.8.4. 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 -70 mV voltage-clamp: 5 minutes of spontaneous excitatory postsynaptic currents (EPSC) 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 >1GΩ, as measured by a -5 mV test pulse, the holding potential is raised to -40 mV and the pipette retracted 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, placed in 24-well plate, the ACSF replaced with 4% paraformaldehyde (in 0.1 M phosphate buffer) and left overnight.

3.12 The next day, replace the PFA with 0.1 M PB and stored until histological processing. Visualize cells with fluorescent-conjugated streptavidin as previously described.

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 slice produced is assessed visually under IR-DIC optics, and healthy cells identified as having large, ovoid-shaped cell bodies located through 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, as well as rarely in the depth 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. Healthy neurons in mature rats, when the
membrane is ruptured, 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 and 2D), given low electrical noise (<10 pA peak-to-peak) when filtered at 10 kHz. Furthermore, stable cell recordings of hyperpolarized neurons require typically <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 modification of 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 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 afterhyperpolarisation (Figure 3E). Following post-hoc confirmation of intact dendrites, using streptavidin visualization of biocytin labelling performed during recordings, the spontaneous EPSC frequency measured from 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, and establishment of intra-animal variability measures, as well as producing slices of sufficient quality to perform paired recordings from synaptically-coupled neurons.

**Figure Legends:**

**Figure 1:** Overview of experimental set up and dissection schematic. A laid out experimental tools for all aspects of slice preparation, labelled according to use. B cartoon depicting the directions of cuts with bone snippers and movement of spatula (pink arrows) to remove brain from skull. C overview of cutting angle (dashed red line) to allow preservation of dorsal CA1. D overview of slicing chamber with 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 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 at 100 pA sinusoidal wave, frequency modulated from 0.1 - 20 Hz. Note the larger voltage response at 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:
In the present protocol, I describe a protocol for the production of high quality brain
slices from the dorsal extent of 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.

In my hands this protocol produces viable slices reliably 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 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 feasible 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, which aim to 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 been long 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.

In my hands, the slice quality produced by the combination of ice-cold sucrose-ACSF
perfusion and the modified near-coronal cutting angle provide slice quality near
comparable to that of horizontal slices prepared at the same developmental stage.
Indeed, the optimization of this technique using sucrose-ACSF composition with similar
ionic composition to that used for recording, allows for great consistency between
conditions used within 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 my approach here is that it
allows for direct comparison between recordings performed in younger rodents, based on slice preparations described by myself \(^{22}\) and others \(^{21}\); due to the identical ionic basis of the solutions and slice recovery conditions. 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 dorsal and ventral hippocampus \(^{27,28,38,42,43}\). However, few studies perform recordings at an age comparable to that used for behavioural, anatomical, or \textit{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. I have performed the above protocols on rats up to 1 year of age, but there is no reason to believe that this could not be performed on still older rats; given the appropriate permissions.

In summary, the protocol presented here provides a reliable method for the production of brain slices from adult rats thus allowing comparison of electrophysiological properties of neurons to \textit{in vivo} and anatomical experiments, as well as .

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