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Preparation of Acute Slices from Dorsal Hippocampus for Whole-Cell Recording and Neuronal Reconstruction in the Dentate Gyrus of Adult Mice

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Citation

Baccini, G., Brandt, S., Wulff, P. Preparation of Acute Slices from Dorsal Hippocampus for Whole-Cell Recording and Neuronal Reconstruction in the Dentate Gyrus of Adult Mice. *J. Vis. Exp.* (170), e61980, doi:10.3791/61980 (2021).

Date Published

April 3, 2021

DOI

10.3791/61980

URL

jove.com/video/61980

Abstract

Although the general architecture of the hippocampus is similar along its longitudinal axis, recent studies have revealed prominent differences in molecular, anatomical and functional criteria suggesting a division into different sub-circuits along its rostro-caudal extent. Owing to differential connectivity and function the most fundamental distinction is made between the dorsal and the ventral hippocampus, which are preferentially involved in spatial and emotional processing, respectively. Accordingly, in vivo work regarding spatial memory formation has focused on the dorsal hippocampus.

In contrast, electro-physiological in vitro recordings have been preferentially performed on intermediate-ventral hippocampus, largely motivated by factors like slice viability and circuit integrity. To allow for direct correlation of in vivo data on spatial processing with in vitro data we have adapted previous sectioning methods to obtain highly viable transverse brain slices from the dorsal-intermediate hippocampus for long-term recordings of principal cells and interneurons in the dentate gyrus. As spatial behavior is routinely analyzed in adult mice, we have combined this transversal slicing procedure with the use of protective solutions to enhance viability of brain tissue from mature animals. We use this approach for mice of about 3 months of age. The method offers a good alternative to the coronal preparation which is frequently used for in vitro studies on dorsal hippocampus. We compare these two preparations in terms of quality of recordings and preservation of morphological features of recorded neurons.

Introduction

The hippocampus has been studied extensively for its pivotal role in different aspects of learning and memory, spatial navigation as well as emotion. The basic circuitry of the hippocampus, commonly called the "trisynaptic circuit", is a lamellar network in the transverse axis, which is largely preserved along the longitudinal axis[1]. The contributions

of the hippocampus to various cognitive and emotional behaviors likely arises from the diverse connections that this basic circuitry makes along the dorsoventral axis with several other brain regions[2,3]. Beyond afferent and efferent connectivity, however, an increasing number of studies point towards further differences along the septo-temporal axis of the hippocampus. Such differences concern the internal architecture and connectivity as well as differences in gene expression patterns and neuronal morphology[4,5,6,7,8].

Considering the existence of such differences in the basic circuitry, it is reasonable to select the specific hippocampal sub-circuit to be investigated according to the questions that are addressed. If for example the question concerns neuronal mechanisms involved in spatial processing, the dorsal rather than the ventral hippocampus is of interest, although the two do not act independently in vivo due to intra-hippocampal longitudinal connectivity[9,10,11]. Along these lines not only the differences along the longitudinal axis have to be considered but also care is required to preserve local and long-range circuitry as well as possible. For the preservation of the fiber paths and connectivity the angle at which the brain will be sectioned is essential.

The first method reported in the literature to include the trysinaptic circuit first isolated the hippocampus from the brain and then made transverse slices (perpendicular to the longitudinal axes) using a tissue chopper[12] and a vibratome[13]. Later physiologists preferred to obtain slices from an entire brain block to preserve also the adjacent brain structures connected to the hippocampus. For these block preparations, different sectioning angles with respect to the hippocampus have been developed, such as a coronal slice preparation[14] or a horizontal slice preparation named HEC slice for preserving hippocampal-entorhinal cortex connections[15,16,17].

In the latter preparation the parietal lobe is cut with an angle of 0° or 12° with respect to the horizontal plane along the rostro-caudal axis to form the base of the block. Slices are then collected starting from the ventral surface of the brain, thus allowing mainly the harvest of the intermediate-ventral hippocampal region. This method has become the most popular choice for physiological studies and can be reliably performed following several published protocols[18,19,20].

However, if the research interest concerns specific aspects of spatial learning, the dorsal hippocampus may be the more suitable region of investigation and it would be useful to find a slicing procedure of similar quality for this hippocampal region. Few protocols, which focus on the very rostral pole, have been developed that can satisfy this demand [21,22].

In this protocol, instead, we describe an approach to obtain viable transverse slices from the dorsal-intermediate hippocampus which uses the sectioning angle previously described for horizontal preparations[18,19] (**Figure 1A&B**). We demonstrate the quality of this protocol by comparing electrophysiological recordings and morphological reconstructions in this preparation to those obtained in coronal slices. This protocol is particularly suited for combination with anatomical and behavioral experiments in adult mice (three months old in our case).

Protocol

All procedures involving experimental animals were in accordance with the German Animal Welfare Act and approved by the ethics committee of the university of kiel. Parvalbumin-Cre (Pvalb-IRES-Cre) mice[23] (Jackson laboratories, Repository number 008069) were maintained

as heterozygous colonies or crossed with Ai9 Cre reporter mice[24] (Jackson laboratories, Repository number 007909). Female and male mice between P40-P90 were used. Mice were maintained in a 12-h light-dark cycle under standard group housing conditions and were provided with food and water *ad libitum*.

1. Preparation of solutions

NOTE: Prepare fresh solutions for every experimental day using ultrapure water (UPW) (resistivity at 25 °C 18.2 MΩcm). The solutions may be stored at 4 °C for a maximum of one day. Magnesium and calcium solutions can be stored separately as 1 M stock solutions. All the working solutions must be saturated with carbogen (95% O2:5% CO_2) for optimal oxygenation and pH maintenance before and during use.

1. Prepare the cutting solution (500 mL per mouse) (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 4 Na-pyruvate, 0.5 $CaCl_2{\cdot}2H_2O$, and 10 $MgSO_4{\cdot}7H_2O$. Titrate to pH 7.4 under a chemical hood with 4-5 mL of 16% hydrochloric acid before adding the divalent cations. This will avoid the precipitation of $MgSO_4$.

   NOTE: KCl and NaH_2PO_4 can be stored as 10x solutions at 4°C. We make two individual batches of cutting solution. One the day before the recording (stored in a freezer over night to produce crushed ice) and one on the day of recording.

2. Prepare the storage solution (250 mL per mouse) (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 4 Na-pyruvate, 2 $CaCl_2{\cdot}2H_2O$, and 2 $MgSO_4{\cdot}7H_2O$. Titrate to pH 7.3-7.4 with 1 N NaOH.

   NOTE: NaCl, KCl and NaH_2PO_4 can be stocked as 10x solution at 4 °C.

3. Prepare ACSF for recording (in mM): 122 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 12.5 glucose, 2 $CaCl_2{\cdot}2H_2O$, and 2 $MgSO_4{\cdot}7H_2O$, 2 Na-ascorbate, 4 Na-pyruvate. Titrate pH to 7.3-7.4 with 1 N NaOH.

   NOTE: NaCl, KCl and NaH_2PO_4 can be combined in a 10x stock solution at 4°C.

2. Preparation of the bench for slicing

1. Prepare on ice a beaker (150 mL) and two glass Petri dishes (10 cm diameter) and fill them with the fresh cutting solution. Keep the solution oxygenated with a carbogen bubbling device (a perforated tube or an air-stone connected to the carbogen system).

   NOTE: The solution in the beaker will be used for the perfusion while the Petri dishes will be used during the cutting procedure.

2. Equip the surgery bench with a stainless-steel blade, rounded tip tweezers, fine tip tweezers, big scissors, small scissors, a large metal spatula, a thin metal spatula, a brush, the vibratome plate and cyanoacrylate glue or the less toxic n-butyl-ester cyanoacrylate adhesive. Immerge a stainless-steel blade and a piece of filter paper in one of the glass Petri dishes on ice. This Petri will be used to cut the brain. Draw three parallel lines onto the bottom of a plastic Petri dish and position this dish close to the surgical instruments.

3. Prepare the set up for the trans-cardial perfusion according to published protocols[25].

4. Prepare the vibratome, mounting a new blade on the blade holder and set the parameters for cutting (angle of the blade from horizontal plane 17°, blade oscillation amplitude 1.5 mm, blade forward movement velocity at around 2 mm min^{-1}, Oscillation frequency 90 Hz, section thickness 350 µm). Fill the vibratome tray with ice-cold cutting solution and keep it under constant oxygenation.
   NOTE: We add the crushed ice made from the first batch of cutting solution to the cooled fresh cutting solution to keep the temperature down. Pay attention to keep the ice from the blade while slicing.

5. Prepare an incubation chamber filled with the cutting solution under constant oxygenation. Place the chamber in a pre-warmed water bath (35 °C).
   NOTE: An example for the incubation chamber can be found in Edwards and Konnerth (1992)[26].

6. Prepare a slice storage chamber filled with the storage solution and keep it at room temperature and under constant oxygenation. In case the tissue expresses a fluorescent protein or a light-activated opsin, it is recommendable to keep the chamber in the dark, for example, inside a box.
   NOTE: a storage chamber with multiple independent wells is useful to distinguish the level of the slices along the dorsoventral axis of the Hippocampus. A custom-made chamber can be built (**Figure 1D**). Take a piece of vial spacer grid from an 81x cryogenic vial storage box and glue the bottom on to a nylon net. Insert the upper one third of a 5mL pipette plastic tip into the center of this grid, so that it sticks out about 3 cm at the bottom. This plastic tip serves as the stand of the grid and later holds the air tubing for oxygenation. Insert the grid with its stand into a cylindric plastic box (e.g., packaging of syringe filters) so that it cannot tilt or wobble. This reservoir will hold 250 mL of storage solution.

3. Preparation of hippocampal slices

1. Anesthetize the mouse in a box chamber using isoflurane (about 0.5 mL) under the hood. Leave the mouse resting until breathing is slow and regular (about 2-3 minutes). Test for the absence of pain responses with toe pinches.

2. Perform trans-cardial perfusion using 25 mL of carbonated cutting solution from the beaker on ice following published protocols[25,27]. This step is recommended to cool down the brain rapidly to quickly slow neuronal metabolism.

3. Decapitate the animal using the big scissors and place the head into the cooled Petri dish on ice containing the cutting solution.

4. Open the skin with the fine scissors to expose the skull. Make small lateral incisions into the base of the skull on both sides of the foramen magnum. Then cut along the sagittal suture starting at the foramen magnum until reaching the naso-frontal suture above the olfactory bulb. Pull up the scissor during the sagittal cut to avoid damage of the underlying brain tissue.
   NOTE: Keeping the head submerged in the cutting solution helps to clear it from blood and keeps the temperature down.

5. Use the rounded tip tweezer to pull up the parietal bones to expose the brain. Pay attention to get hold of and remove the meninges too. If left, they can damage the brain during extraction.

6. Use the small spatula to gently scoop out the brain into the second Petri dish.

7. Cut the brain in halves along the longitudinal fissure using the pre-chilled blade.

8. Use the large spatula to lift one hemisphere out of the cutting solution and onto the plastic Petri dish. With the hemisphere lying on its medial surface, align the parietal cortex with one of the parallel lines to have a reference for the second cut as shown in **Figure 1B**.

9. Perform the second cut at the ventral part of the hemisphere positioning the blade in parallel to the lines to obtain the surface, which is later used for gluing the brain onto the specimen holder (see next step), as shown in **Figure 1B**. Return the hemisphere into the oxygenated cutting solution of the glass petri dish and perform the same procedure on the second hemisphere.

10. Glue the hemispheres with the freshly cut ventral side onto the vibratome-specimen holder using the adhesive. Put a few drops of cutting solution onto the hemispheres to solidify the glue and move the specimen holder into the vibratome tray. This way the slicing will proceed from the dorsal to the ventral hippocampus.

11. Remove the initial one or two non-transversal slices (**Figure 1C,ii-iii**) until the region of interest becomes visible and then start collecting the slices.
    NOTE: Non-transversal but usable, healthy slices can be obtained from the dorsal hippocampus at the beginning of the slicing procedure (**Figure 1C,ii-iii**). The collection of each slice requires about 3-4 min. By starting the cutting from the dorsal instead of ventral hippocampus we save about 10 minutes, which improves the viability of dorsal slices.

12. With a plastic pipette (cut away the narrow end of the tip) transfer each slice into the incubation chamber (containing cutting solution at 35 °C) and let it rest for 12 min. The brief recovery period in warm cutting solution greatly reduces initial neuronal swelling, as reported in Ting et al. (2014)[28]. Then using a brush transfer the slice into the storage chamber (containing storage solution at RT) and let it rest until the start of the experiment.

4. Whole-cell recording and biocytin filling

NOTE: The description of whole-cell patch-clamp recording is reduced here only to key steps that help to obtain good biocytin filling and is generally applicable to neurons in ACSF. For details regarding the procedures of electrophysiological recordings, several other protocols can be consulted[29,30].

1. Choose an appropriate intracellular solution according to the parameters to be recorded. As an example, for current clamp measurements, a potassium gluconate-based solution can be used (in mM): 135 K-Gluconate, 3 KCl, 10 HEPES, 0.2 EGTA, 10 phosphocreatine-Na_2, 4 MgATP, 0.3 Na_2-GTP. Adjust the pH to 7.3 with 1 M KOH. The osmolality should be around 285-290 mOsmol/kg once 3-5 mg/mL biocytin is added.
    NOTE: To monitor the shape of the neuron during the recording, 0.3-0.5 µL/mL of 1 mM Alexa Fluor Hydrazide can be added to the intracellular solution. In this case, remember for biocytin revelation, to match the Alexa dye color with the fluorescent probe coupled to streptavidin.

2. Prepare patch-clamp electrodes of ≤1 µm tip diameter and 3-5 MΩ resistance from thick-walled borosilicate capillaries.

3. Start the perfusion system of the patch-clamp setup with a speed of 2.5-3 mL/min at RT or 35-37 °C and secure a slice in the chamber with a U-shaped anchor.

4. Identify the brain region of interest and choose a healthy neuron with the soma at least 30-50 µm below the surface of the slice.

   NOTE: The soma of a healthy neuron has a smooth surface, and the membrane borders are slightly contrasted. The soma of unhealthy neuron appears shrunk and highly contrasted or swollen and semi-transparent.

5. Load a glass pipette with the intracellular solution until the tip of the AgCl electrode is covered. Move the pipette into the solution of the recording chamber, applying a light positive pressure to keep clear the tip of the glass electrode.

   NOTE: If the solution contains an Alexa dye, the pressure can be controlled visually adjusting the amount of fluorescent solution released from the tip. Minimal pressure would avoid the staining of the tissue around the cell.

6. Approach the cell from an oblique angle and establish contact within the first one third of the soma surface when forming the giga-seal. Release the pressure, slowly move the holding potential to-65 mV and apply a gentle suction by mouth to facilitate the giga-seal formation.

7. To establish the whole-cell configuration, apply strong and brief suction to break the membrane. Discard the recording if the neuron has a membrane potential more depolarized than-50 mV, if the holding current is increasing beyond 100 pA or if the access resistance increases beyond 30 MΩ.

8. After the recording (for sufficient filling of somata and dendrites at least 15 min are recommended; for filling of complex axons up to one hour maybe required[31]) carefully remove the electrode. To this end set the holding potential to 0 mV and try to re-form a giga-seal by slowly retracting the pipette in the direction opposite from the approach, such as to obtain an outside-out patch configuration.

   NOTE: The presence of the Alexa dye in the intracellular solutions helps the visually guided removal of the pipette from the neuronal surface. If the nucleus of the cell is not in contact with the pipette tip, a quick method to reform the seal is pulling the pipette out of the tissue diagonally and upward at high speed. If the nucleus or part of the membrane resides within the tip, there is a danger of breaking the plasma membrane. In this case, a slight positive pressure application and lateral movement can help.

9. Note the position of the cell and the orientation of the slice. After fixation the exact orientation of the slice and the integrity of the filled cell can be checked under a fluorescent microscope.

5. Immunostaining, image acquisition and morphological reconstruction

1. After recording, transfer the slices into a 24-well plate to fix it in paraformaldehyde (PFA) 4% in 0.1 M phosphate-buffered saline (PBS). Incubate for 60 min at RT or overnight at 4 °C, according to the sensitivity of the primary antibodies used. Slices can be stored in 0.1 M PBS and 0.02% NaN_3.

   CAUTION: PFA is toxic. Use under the hood.

2. Perform immunostaining and biocytin revelation within one week of recording according to established protocols[32,33].

   NOTE: We use a procedure that avoids re-sectioning of the slice to preserve the integrity of dendritic and axonal processes. However, this procedure requires

longer incubation times as antibodies need to penetrate into the tissue. We suggest testing the time for optimal penetration of antibodies into the tissue before performing the experiment.

3. Acquire images using a confocal microscope with Z-stack and tile-scanning function.

4. Perform morphological reconstruction using Fiji imageJ[34].

   NOTE: Several image file formats can be imported into the program using the *Bio-format* plugin. We recommend the use of the Simple Neurite Tracer (STN) plugin[35] for the reconstruction of the dendrites and the axon. A simple and clear tutorial is available at https://imagej.net/SNT and http://snyderlab.com/2016/05/25/tracing-neurons-using-fiji-imagej/. The file containing the tracing data(.traces), if saved un-compressed, can be converted into a text file and opened with Matlab or other software for text data processing.

Representative Results

In this protocol we describe how to prepare acute hippocampal slices from the dorsal-intermediate part of the hippocampus (**Figure 1A**). The protocol is particularly suitable for experiments that investigate mechanisms involved in spatial learning and can be combined with behavioral work or viral labeling or manipulation strategies in the dorsal hippocampus[35]. Applying the sectioning procedure described here to the animals injected with Cre-dependent GFP expressing adeno-associated virus (AAV-FLEX-GFP) into the dorsal hippocampus of Pvalb-IRES-Cre mice at different Bregma coordinates AP-1.94 mm, ML ± 0.5-2 mm, depth-1.25-2.25 mm to target different regions of the hippocampal formation[36] we were able to obtain at least three transversal slices containing the infected regions (**Figure 1A** light green coloration on the 3D model of hippocampus). In addition, several non-transversal but healthy slices can be obtained from the more rostral parts of the dorsal hippocampus (**Figure 1C**).

To demonstrate the quality and viability of our slices we have recorded basic electro-physiological and morphological parameters of granule cells and tdTomato-labeled Parvalbumin-positive (PV+) interneurons in the dentate gyrus of Pvalb-IRES-Cre;Ai9 transgenic mice (7-12 weeks of age) and compared these to recordings from coronal slices of the same region obtained with a standard protocol.

Upon visual inspection under the infra-red differential-interference contrast (IR-DIC) microscope, we already noticed clear differences between our transversal and the coronal slice. Whereas neurons of the principal cell layer in coronal slices often appeared coarse and displayed strongly contrasted outlines, neurons in the transversal slice mostly showed smooth surfaces and only lightly contrasted borders, indicative of better cellular vitality (**Figure 2A**). The reason for these differences in cell viability between coronal and transversal slices may lie in the orientation of the sectioning plane with respect to the fiber tracts. As these are not in parallel in the coronal sections, axons and dendrites will be severed. In line with this assumption, we found that within the slices the surface planes of granule cell layer and hilus showed greater discontinuity in the coronal than the transversal slice (step size in surface planes: 41.40 ± 3.28 µm vs 25.60 ± 2.94 µm, Mean ± SEM, Unpaired t-test P = 0.023), suggesting a larger degree of tissue disconnection in the coronal slice (**Figure 2B**). This means that suitable cells for patch-clamp recordings will only be found at deeper planes of the granule cell layer for coronal slices, which in turn may reduce the throughput of patch-

clamp recordings. Indeed, the average time to seal formation in our transversal slice was more rapid than for coronal slices (granule cells: 12.64± 1.50 s, n=11 in coronal vs. 8.40 ± 0.75 s, n=14 in transversal slices, Mean ± SEM, P= 0.0335 Mann-Whitney test; PV+ interneurons: 31.11 ± 2.60 s, n=9 in coronal vs. 22.00 ± 2.18, n=7 in transversal slices, Mean ± SEM, P= 0.0283 Mann-Whitney test) (**Figure 2C**). As a proxy for cell integrity and health we then recorded the resting membrane potentials (RMP) of granule cells and PV+ interneurons, which were significantly more depolarized in both granule cells and PV+ interneurons in coronal vs. transversal slices (granule cells:-62.55 ± 3.54 mV, n=11 in coronal vs.-71.06 ± 2.31 mV, n=14 in transversal slices, Mean ± SEM, P=0.0455 Mann-Whitney test; PV+ interneurons:-52.75 ± 1.66 mV, n=7 in coronal vs.-59.36 ± 2.25 mV, n=6 in transversal slices Mean ± SEM,, P= 0.0271 Mann-Whitney test) (**Figure 2D**). These data suggest a higher number of healthy neurons in the transversal vs. the coronal slice preparation. Indeed, introduction of a cut-off for the acceptable RMP (-55 mV for granule cells;-45 mV for PV+ interneurons) resulted in a higher percentage of excluded cells in coronal than in transversal slices (39.67 ± 8.37 %, n=3 experimental Sessions vs. 23.00 ± 3.85 %, n=4 experimental Sessions) (**Figure 2E**). Moreover, reconstruction of neuronal morphology from recorded granule cells indicated that as expected chances were much better to retrieve a complete axonal arborization for granule cells in the transversal slice (**Figure 3A,B**). In addition, the morphological reconstruction of PV+ interneurons in transversal slices allowed the depiction of extensive axonal and dendritic arborizations including the visualization of small details such as dendritic spines[35] (**Figure 3C**).

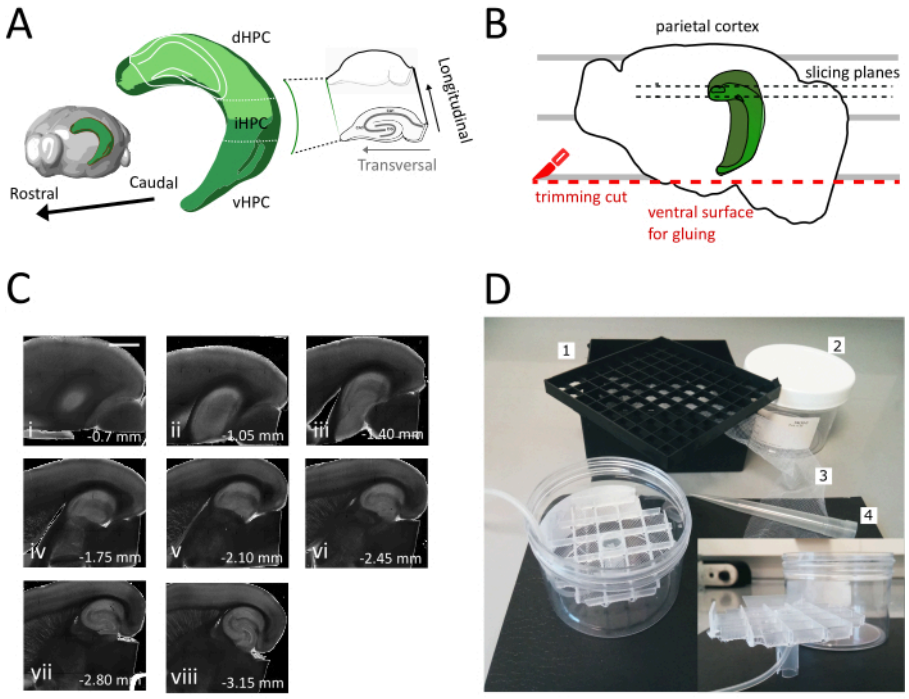

Figure 1: Illustration of the sectioning procedure to obtain slices from dorsal-intermediate hippocampus. (**A**) Three-dimensional representation of the hippocampal formation showing its spatial orientation in the brain (modified from Brain Explorer, Allen Institute)[37]. Dorsal, intermediate and ventral divisions of the hippocampus (dHPC, iHPC, vHPC) are indicated, according to Dong et al. (2009)[7]. The part of dorsal-intermediate hippocampus that will be sliced is indicated in light green. The inset on the right shows the orientation of the reference axes. (**B**) Cartoon of a brain hemisphere depicting the alignment of the parietal cortex with the parallel lines on the Petri dish. The red dotted line indicates where to perform the trimming cut (point 3.9 in the protocol) to create the surface for gluing the hemisphere onto the specimen holder. The black dotted lines indicate where slices are collected. (**C**) Bright field image series of hippocampal slices obtained following this procedure. From the pial surface, dorsal to ventral: (i) 0.70 mm, (ii) 1.05 mm, (iii) 1.40 mm, (iv) 1.75 mm, (v) 2.10 mm, (vi) 2.45 mm (vii) 2.80 mm, (viii) 3.15 mm. Scale bar= 1 mm. (**D**) Photo of the storage chamber and the material needed for its assembly. 1. Vial spacer grid from an 81x cryogenic vial storage box, 2. Cylindric plastic box. 3. Nylon net, 4. Pipette tip. Inset. Lateral view of the grid and tube-holder to insert into the cylindric box. Please click here to view a larger version of this figure.

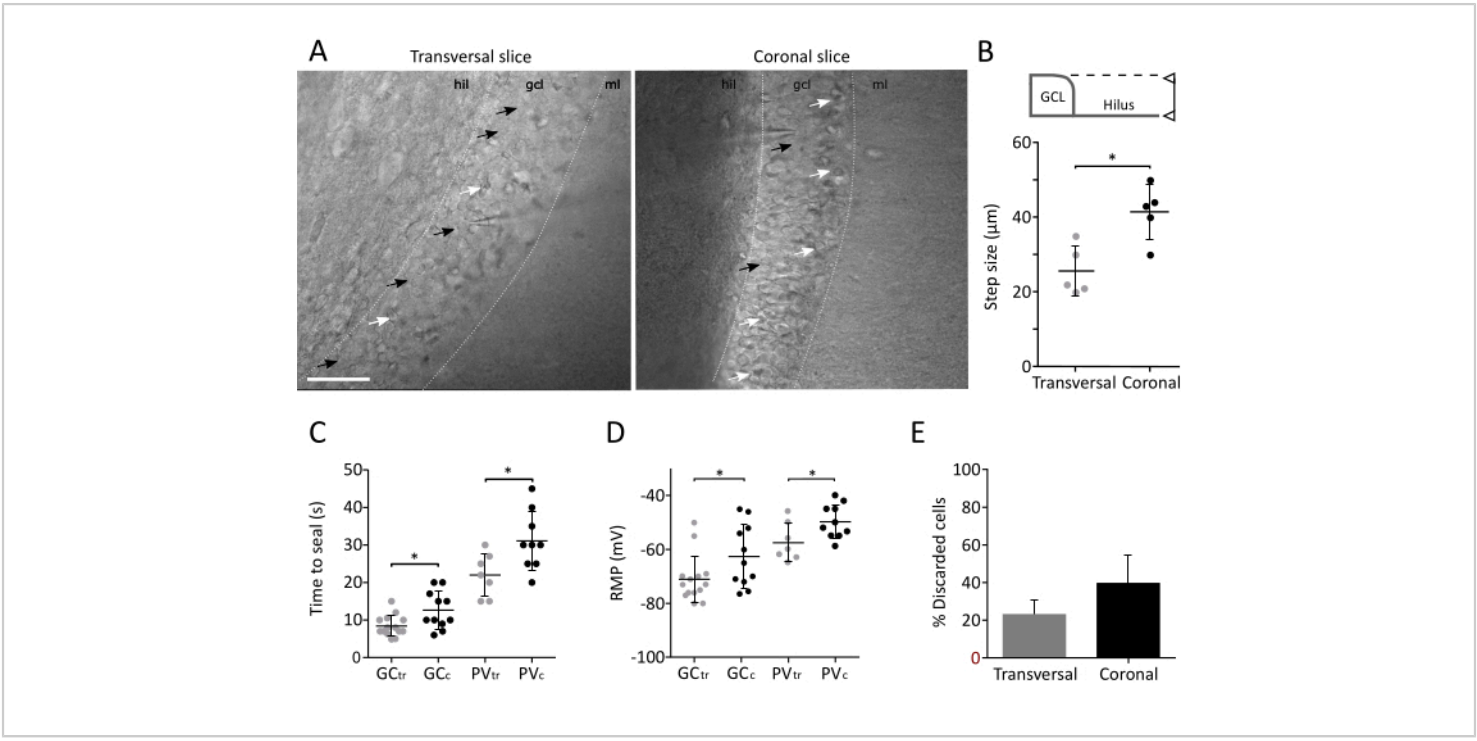

Figure 2: The transversal slice shows enhanced slice viability compared to the coronal slice. (**A**) DIC-IR micrographs showing healthy (black arrows) and unhealthy (white arrows) examples of neuronal somata in transversal and coronal slices. Hil=hilus, gcl=granule cell layer, ml= molecular layer. Scale bar = 50 µm. (**B**) Both sectioning procedures produce a step between the surface of the granule cell layer and the hilus (indicated by arrow heads). The height of the step is indicative of the extent of tissue disconnection and is significantly lower in transversal sections than in coronal (n=5 transversal and n=5 coronal slices, Mean±SEM, P=0.0238 Mann-Whitney test). (**C**) Time of giga-ohm seal formation in granule cells (n=14 cells in transversal, n=11 cells coronal; Mean±SEM, P= 0.0355, Mann-Whitney test) and PV+ INs (n=7 cells in transversal, n=9 cells in coronal slices, Mean±SEM, P= 0.0283 Mann-Whitney test) slices. (**D**) Resting membrane potential (RMP) of cells patched (respectively, n= 14 and n=11 granule cells. Mean±SEM, P=0.0455 Mann-Whitney test. n=7 PV+ INs and n=10 PV + INs, P= 0.0271 Mann-Whitney test). (**E**) Percentage of discarded cells within an experimental session, (n=3 sessions with coronal slice, n=4 with transversal slice, Mean±SEM). Please click here to view a larger version of this figure.

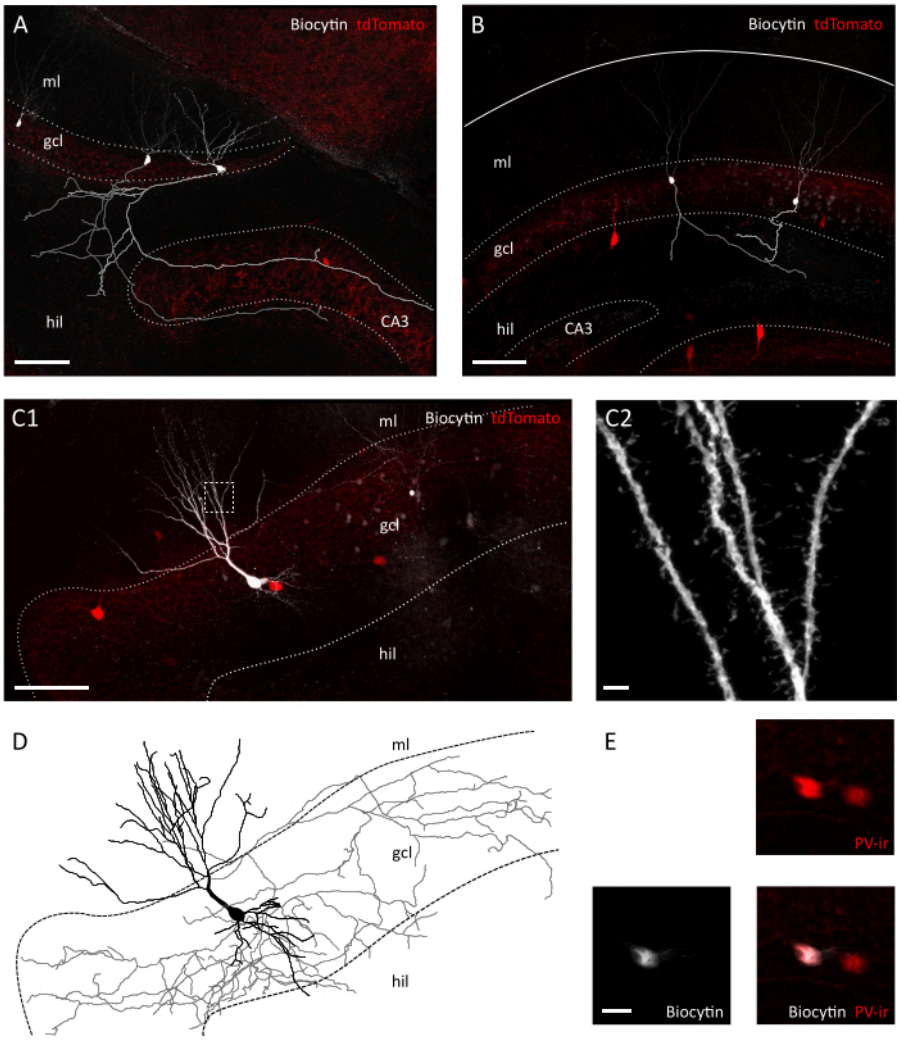

Figure 3: Morphological preservation of granule cells and interneurons in the transversal slice. Confocal images showing biocytin filled granule cells in a transversal slice (**A**) and in a coronal slices (**B**) of Pvalb-IRES-Cre;Ai9 transgenic mice. The respective axons have been reconstructed in grey and light-grey. Note the difference in axon length and complexity between the preparations. Scale bar=100 µm. (**C1**) Confocal image showing a biocityn-filled tdTomato-positive interneuron. Hil=hilus, gcl=granule cell layers, ml= molecular layer. Scale bar=50 µm. (**C2**) Magnification of the boxed area in C1, showing dendritic spines. Scale bar=2 µm (**D**) Morphological reconstruction of axons and dendrites of the biocytin filled interneuron in C1 (axon in grey, soma and dendrites in black). (**E**) Close up of the somata of the cells depicted in C1 showing colocalization of biocytin and Parvalbumin-immunoreactivity (PVir). Scale bar=20 µm. Please click here to view a larger version of this figure.

Discussion

The dorsal hippocampus has been extensively studied for its role in spatial learning and navigation mainly through behavioral experiments, anatomical tracing, and region-specific manipulations. To combine slice-electro-physiological inquires with these techniques, we have assembled a protocol that uses a similar angle of sectioning as the modified horizontal slicing for the intermediate-ventral region of the hippocampus, but uses an inverted slicing order to obtain early slices from the dorsal-intermediate region. This approach reduces the time required to slice and collect the dorsal region of hippocampus, thus enhancing slices viability.

Using this method, we are able to routinely retrieve about three slices per hemisphere of the dorsal hippocampal region between 1.4 mm-2.4 mm from the pial surface, as shown in the **Figure 1C**. Although it is not possible with this procedure to obtain transversal slices from the very septal pole of the hippocampus, it is possible to collect about two additional viable non-transversal slices per hemisphere from the septal pole (**Figure 1C ii,iii**). If the septal pole of the hippocampus is the primary research focus, other protocols, which permit collection of transversal slices, especially from the very septal pole of the hippocampus, may be better suited[21,32]. Behavioral experiments on spatial navigation and learning are preferably carried out in mature mice with fully developed neuronal connectivity. Consequently, we have optimized our slicing procedure for the application to the brains of adult animals (shown here for three months old mice), which are more sensitive to stress than the resilient juvenile preparation. To this end we have combined several strategies which reduce the hypoxic stress the brain is exposed to in the time between extraction and the placement of the slices into the oxygenated ACSF. The protective cutting solution is a NMDG-based ACSF[25,27,28] with low Na+ and Ca^{2+} but high Mg^{2+} to reduce excitotoxic damage and cell swelling due to activation of NMDA receptors. In addition, HEPES provides stable buffering and compounds such as ascorbate and pyruvate reduce oxidative stress. The trans-cardial perfusion with the chilled and oxygenated protective cutting solution takes advantage of the extremely dense capillary network supplying the brain to rapidly and homogeneously reduce metabolic demand and glutamate-induced excitotoxicity in the brain tissue. Subsequently nearly all steps following decapitation are performed within the cooled and oxygenated solutions to keep metabolism and oxygen deprivation to a minimum during the entire procedure. Other strategies for reducing brain damage during slicing exist and may be equally valid[38]. To demonstrate the quality of our preparation, we compare it to a coronal slice preparation, which is commonly used to record from the dorsal hippocampus. Even though coronal slices can be used to obtain good patch-clamp recording in the dentate gyrus, the number of unhealthy and disconnected neurons is higher than in the transversal slice. In addition, the integrity of the axonal and dendritic arborizations is better preserved in the transversal slice. As a matter of fact the integrity of granule cell axons (**Figure 3A**), which run orthogonal to the longitudinal axis of the hippocampus serves as an indicator of a transversal slicing plane[1].

For the filling of patched neurons, we suggest an electrode resistance between 3 and 5 MΩ. A diameter of the tip of about 1 µm, allows accomplishment of a good seal resistance during recording and good re-sealing upon electrode retraction. The most crucial detail is to avoid the suction of parts of the soma or nucleus into the pipette. For this reason, we suggest including an Alexa dye in the intracellular solution when possible. The dye allows monitoring the cell shape during recording and re-sealing. Moreover, it permits to assess the integrity of the patched cell after fixation, which can save

immunohistochemistry time, in cases of unsuccessful fillings. Because of Alexa dyes are quenched with long fixation time, we suggest short fixation if possible.

For subsequent immunostaining, we use a protocol that does not require the re-sectioning of the slice. We suggest making the staining within one week after fixation. The longer the slices remain in the fridge, the higher the chance of tissue degradation. If a long storage cannot be avoided, we suggest increasing the NaN3 concentration in the PBS to 0.05% and to refresh it weekly. Immunostaining of the entire slice means that the incubation times with primary and secondary antibodies increase. Usually, for revealing biocytin, one overnight incubation at 4 °C is enough, but if combined with the staining for other proteins, the whole staining procedure can last much longer. Permeabilization-blocking and anti-body incubation need to be optimized individually. Usually, for the primary antibody, two days are sufficient while one day can be enough for the secondary. We recommend increasing the duration of the washing steps together with longer antibody incubations to avoid the increase of background.

In this protocol we have presented a slicing method to obtain transversal or nearly transversal hippocampal slices preserving neuronal viability of adult tissue and a practical approach to recover the morphology and the neurochemical identity of the patched neurons. This method can be easily performed to match electro-physiological results with anatomical and behavioral studies focusing on the intermediate-dorsal part of the hippocampus.

Disclosures

The authors have nothing to disclose.

Acknowledgments

We thank Kerstin Kronenbitter and Didier Gremelle, for technical assistance. We thank Umberto Morelli for assistance with graphic software and Mathias Hoppe for videography, and video-editing. Work in our lab was supported by the Deutsche Forschungsgemeinschaft (DFG) FOR2143, SFB 1461 (Project-ID 434434223) and GRK2154, the Medical Research Council grant G1100546/2 and Kiel University.

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