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TITLE:

Horizontal Hippocampal Slices of the Mouse Brain

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SUMMARY:

This article aims to describe a systematic protocol to obtain horizontal hippocampal brain slices in mice. The objective of this methodology is to preserve the integrity of hippocampal fiber pathways, such as the perforant path and the mossy fiber tract to assess dentate gyrus related neurological processes.

ABSTRACT:

The hippocampus is a highly organized structure in the brain that is a part of the limbic system and is involved in memory formation and consolidation as well as the manifestation of severe brain disorders, including Alzheimer's disease and epilepsy. The hippocampus receives a high degree of intra- and inter-connectivity, securing a proper communication with internal and external brain structures. This connectivity is accomplished via different informational flows in the form of fiber pathways. Brain slices are a frequently used methodology when exploring neurophysiological functions of the hippocampus. Hippocampal brain slices can be used for several different applications, including electrophysiological recordings, light microscopic measurements as well as several molecular biological and histochemical techniques. Therefore, brain slices represent an ideal model system to assess protein functions, to investigate pathophysiological processes involved in neurological disorders as well as for drug discovery

purposes.

There exist several different ways of slice preparations. Brain slice preparations with a vibratome allow a better preservation of the tissue structure and guarantee a sufficient oxygen supply during slicing, which present advantages over the traditional use of a tissue chopper. Moreover, different cutting planes can be applied for vibratome brain slice preparations. Here, a detailed protocol for a successful preparation of vibratome-cut horizontal hippocampal slices of mouse brains is provided. In contrast to other slice preparations, horizontal slicing allows to keep the fibers of the hippocampal input path (perforant path) in a fully intact state within a slice, which facilitates the investigation of entorhinal-hippocampal interactions. Here, we provide a thorough protocol for the dissection, extraction, and acute horizontal slicing of the murine brain, and discuss challenges and potential pitfalls of this technique. Finally, we will show some examples for the use of brain slices in further applications.

INTRODUCTION:

The extensive exploration of the hippocampus started when Scoville and Milner reported the inability of a patient (H.M.) to form new, declarative memory after surgical removal of the hippocampus and nearby temporal lobe structures as a treatment for severe epilepsy[1]. From that moment on, the hippocampus has been studied extensively starting from general neuronal properties and functions up to the development of severe brain disorders, such as epilepsy and Alzheimer's disease[2–5]. The hippocampus is the part of the limbic system, consisting of a group of related brain structures involved in emotion and memory formation[6,7]. A dense network of several fiber pathways accomplishes a tight hippocampal connectivity to internal and external brain structures. These pathways include the medial and lateral perforant path (entorhinal cortex–dentate gyrus–CA3–CA1–subiculum)[8], the mossy fiber path (dentate gyrus to CA3)[9] and the Schaffer collateral/associational commissural pathway (CA3 to CA1)[10] (**Figure 1**). The hippocampus presents one of the most broadly explored brain areas so far because of its highly conserved laminar organization of the neuronal layer formation, and the possibility to obtain vital neuronal cultures and brain slices with relative ease[5].

[Place **Figure 1** here]

Brain slice protocols often result in the loss of connections from more distant brain areas to the area of interest[5]. Moreover, the capillaries are no longer functional[5] and the blood circulation is deprived[11]. Despite these limitations, brain slices are still primarily used for the investigation of neurophysiological functions of the hippocampus due to a number of advantages. First, the extraction of the hippocampus is fast[12] and does not require many materials. The only essential instruments include a dissection kit, a laboratory water bath, access to carbogen and a vibrating microtome (vibratome)[13]. Other assets of the brain slice technique are the circumvention of the blood-brain-barrier (BBB) and the wash-out of endogenously released molecules before the start of the experiment[5], which makes it possible to study the effect of drugs with relatively precise dosage control[14]. Furthermore, brain slices preserve the cyto-architecture and synaptic circuits within the hippocampus[15,16], where the neuroanatomy and the local environment with neuronal connectivity and complex neuron-glia interactions are preserved[4,11,17]. Additionally, hippocampal

fiber connections are predominantly unidirectional and hippocampal neurons have a high synaptic plasticity, which tremendously simplifies the collection and interpretation of high-quality electrophysiological recordings in order to understand neurological processes[18,19]. Importantly, brain slices present a valuable asset applicable in a wide range of different scientific techniques, spanning from molecular biological techniques over imaging recordings up to electrophysiological measurements[12,20–26].

As outlined above, hippocampal brain slices present a powerful experimental tool to study structural and functional features of the synaptic connectivity. This offers the opportunity to assess the effects of chemicals or mutations on neuronal excitability and plasicity[16].

Acute brain slice preparations are presenting a relatively sensitive technique and optimal slice quality is highly dependent on ideal experimental conditions, including the age of the animal, the method of euthanasia, the speed of dissection and slicing, the slicing solutions and parameters (e.g., slicing speed) as well as the conditions for slice recovery[4]. Therefore, a well-designed protocol is of uttermost importance and secures the reproducibility across different research units[13].

Here, we provide a detailed protocol for acute horizontal hippocampal slice preparations, with the aim to preserve the integrity of the hippocampal lateral and medial perforant path and the mossy fiber pathway, allowing the investigation of dentate gyrus related processes[9]. We will describe in detail the key steps to dissect, extract, and horizontally slice the murine brain, followed by representative results of calcium-microfluorimetric recordings and field excitatory postsynaptic recordings (fEPSPs) under baseline conditions and during LTP induction protocols in brain slices of wild type C57BL/6J mice.

PROTOCOL:

All animal experiments for this study were approved by the ethical review committee of the KU Leuven (Belgium) (P021/2012).

1. Preparation of high-sucrose slice solution and artificial cerebrospinal fluid (ACSF)

1.1. Prior to experimental day

1.1.1 Prepare 1 L of 10x slice pre-solution with laboratory grade Type 1 water containing (in mM): 25 KCl, 20 $CaCl_2$, 10 $MgSO_4$, 12.5 KH_2PO_4 (**Table 1**). In order to prevent calcium phosphate precipitation, slowly mix the chemicals in a beaker pre-filled with 800 mL H_2O while constantly stirring with a magnetic stir. Store the solution at 4 °C or room temperature (RT).

1.2. On the experimental day

1.2.1 Prepare 1 L of 1x ACSF with laboratory grade Type 1 water containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 Glucose (**Table 2**). Use a vapor pressure osmometer to validate the osmolarity between 305–315 mOsm (pH ~ 7.55–7.6).

NOTE: In order to prevent calcium phosphate precipitation, slowly mix all solid chemicals in a beaker pre-filled with 800 mL of H_2O while constantly stirring with a magnetic stirrer. Add $MgSO_4$ and $CaCl_2$ at the very end, slowly dripping in the necessary amount from 1 M stock solutions.

1.2.2 Continuously bubble 1x ACSF solution at RT with carbogen to set pH between 7.3–7.4.

NOTE: If the pH is slightly too high or too low, small adjustments in the carbogenation strength would be sufficient. If the pH is higher than 7.45 with carbogenation, adjust it by adding few drops of 1 M NaH_2PO_4 solution.

1.2.3 Prepare 250 mL (per brain) of 1x high-sucrose slice solution in a beaker containing 25 mL of 10x slice pre-solution (in mM): 252 Sucrose, 26 $NaHCO_3$, and 10 Glucose (**Table 3**). Verify that the osmolarity is between 320–325 mOsm (pH ~ 7.55–7.6).

1.2.4 Bubble the high-sucrose slice solution for 10–15 min with carbogen to control the pH between 7.3–7.4.

NOTE: If the pH is higher than 7.45 with carbogenation, adjust it by adding a few drops of 1 M KH_2PO_4 solution.

1.2.5 Store the high-sucrose slice solution for 20–30 min in the ultra freezer (-80 °C) until it is partially frozen.

2. Preparation of the workspace for the brain dissection

2.1. During the cool-down of the high sucrose slice solution, prepare the following.

2.1.1 Warm up the water bath to 32 °C.

2.1.2 Fill the recovery chamber (**Figure 2A**) with the carbogenated ACSF solution and place the chamber in the water bath. Continuously apply carbogen to the main ACSF bottle and the ACSF in the recovery chamber.

2.1.3 Usher the animal to the experimental room.

NOTE: The age, sex, and strain of the animal have to be determined by the individual experimenter and is dependent on the specific study question. However, the parameters of the animal should stay constant within one study in order to guarantee comparability between the different experimental days. This protocol was designed for the use of C57BL6/J male mice at the age of 2–6 weeks. If older animals will be used, slice and recovery solutions may have to be adapted accordingly[4,27] (e.g., $NMDG^+$-based solutions[23,28]) in order to preserve the brain health of the acute slices.

2.1.4 Prepare the anesthesia chamber.

2.1.5 Lay out tissue paper, plastic Pasteur pipette with wide opening (cut open), 90 mm culture dish filled with ice, a square of filter paper on top of the chilled culture dish, strong scissors for decapitation, dissection scissors, curved forceps, spatula, 35 mm culture dish, fine brush, blade, specimen plate (comes with vibratome), super glue, pipette tip, four straps of filter paper (~ 2 cm x 0.5 cm) (**Figure 2A**).

2.1.6 Set up the vibratome: First, program the vibratome for the correct settings (blade travel speed: 0.08 mm/s, cutting amplitude: 1.4 mm, cutting frequency: 85 Hz) and attach the carbogen line in the slice chamber. Next, place the slice chamber in the holder, fill the holder surrounding the slice chamber with ice and attach everything to the vibratome. Finally, place the razor blade in the vibratome blade holder (**Figure 2B**).

2.2. After freezing of the high-sucrose solution perform the following steps.

2.2.1 After 20–30 min, take the high-sucrose slice solution out of the -80 °C ultra-freezer and keep the beaker on ice.

2.2.2 Place the high-sucrose solution next to the vibratome on your bench, crush and mix the partially frozen solution with a spatula to get a nice slush and start bubbling with carbogen.

2.2.3 Take up some high-sucrose slice solution with the plastic Pasteur pipette to soak the squared filter paper on top of the chilled 90 mm culture dish.

2.2.4 Fill the 35 mm culture dish (or any equivalent small container that is suitable to store the whole brain) upto 75% with the high-sucrose slice solution (sufficient to cover the whole brain) and cool the culture dish on ice kept next to the beaker with the rest of the solution. Carbogenate the solution in the 35 mm culture dish.

3. Dissection and positioning of the murine brain

3.1 Anesthetize the animal with 5% isoflurane. Determine the proper depth of anesthesia by pinching the paw. No paw withdrawal reflex should occur.

3.2 Transfer the animal on tissue paper and decapitate it with strong scissors or a small animal guillotine.

3.3 Use dissection scissors to cut open the scalp.

3.4 Cut open the calvaria along the sagittal suture and remove it with the help of curved forceps until the whole brain, including the olfactory bulbs, is visible.

NOTE: Be careful to not damage the brain with the sharp edges of the calvaria or forceps.

3.5 Use a spatula to carefully scoop out the brain (olfactory bulbs should stay attached).

3.6 Transfer the brain in the chilled 35 mm culture dish and remove all hair or blood particles from the tissue by gently washing the brain with an ACSF-filled Pasteur pipette (blood has cytotoxic effects on brain tissue[29]).

3.7 Transfer the brain with the help of the spatula on the soaked filter paper on top of the chilled 90 mm culture dish (**Figure 2A**).

3.8 Use a blade to cut the brain in half, separating the two hemispheres, and place both hemispheres on the freshly cut medial side.

3.9 Use a blade to remove the dorsal part (5%–10%) of the brain from each hemisphere with a parallel cut to the dorsal top (**Figure 2C**)[30] and place both hemispheres on the freshly cut side with the ventral part of the brain facing upwards.

3.10 Position a drop of super glue on the specimen plate and spread out properly with a pipette tip to accommodate both the hemispheres.

3.11 Use a filter paper strap to pick up one hemisphere with capillary forces by touching the ventral side with the filter paper strap, thereby not damaging the tissue.

3.12 Use another filter paper strap to carefully semi-dry the dorsal side of the brain before positioning the hemisphere with the dorsal side down on top of the glue on the specimen plate. Repeat the same procedure with the second hemisphere.

NOTE: The hemispheres should be positioned in horizontal alignment in a mirrored fashion on the vibratome plate, with the rostral sides pointing toward the outside and the caudal sides facing (but not touching) each other in the middle. The medial sides of both hemispheres should point toward the vibratome blade and the lateral sides toward the experimenter (**Figure 2D**).

3.13 Place the specimen plate in the slicing chamber and quickly, but carefully, cover it with ice-cold high-sucrose slice solution slush. As soon as the solution touches the glue, it will solidify and properly glue the hemispheres to the specimen plate.

3.14 Assure that the hemispheres are properly covered with high-sucrose slice solution and confirm that the solution is bubbled with carbogen.

NOTE: The entire dissection procedure should be performed as fast as possible. Please ensure that the brain does not stay without the supply of oxygen for a very long time. It should take only around 1–1.5 min from decapitation to brain submersion in the high-sucrose slice solution slush. This is the most critical requirement for acute brain slice preparations in order to warrant high quality of the slice.

4. Horizontal slicing of the brain

4.1 Position the vibratome blade in front of the medial side of the hemispheres and lower it to the same height as the ventral sides of the hemispheres that are now facing upwards. Lower the blade with the help of the vibratome control to 600 µm further in the dorsal direction and start slicing. The blade should hit the tissue (if not, reverse the blade and lower it a bit more). Slice until the first two slices are completely separated from the two hemispheres.

4.2 Reverse the blade and lower another 300 µm and slice again.

4.3 When hippocampus becomes visible (use the mouse[31] brain atlas for help, if necessary) (**Figure 2E**) collect the slices with the widened plastic Pasteur pipette. Collect the slices until the caudate putamen becomes visible next to the hippocampus. Typically, between 8–12 slices of 300 µm (4–6 per hemisphere) can be collected for the mouse brain.

4.4 Use the plastic Pasteur pipette to collect the slices and transfer them to the recovery chamber in the water bath (**Figure 2F**) (collect slices after each round of slicing to prevent them from floating around in the slice chamber).

NOTE**:** Work as fast as possible and ensure that the high-sucrose slice solution is ice-cold and carbogenated during the entire procedure. If necessary, refill the ice surrounding the slice chamber.

5. Recovery of brain slices for electrophysiological recordings

5.1 Leave the slices in the ACSF-filled recovery chamber in the 32 °C water bath for 1 h.

NOTE: Recovery chambers are also commercially available.

5.2 Take the recovery chamber out of the water bath.

5.3 Place the slices at RT for at least another 30 min for recovery before starting any further application.

6. fEPSP recordings in the medial perforant path (MPP) of the hippocampus

6.1 Pull recording pipettes from borosilicate glass capillaries with a horizontal pipette puller to receive pipettes of the size of ~2 MΩ when filled with ACSF solution and immersed in the ACSF-filled recording chamber.

6.2 Fill ACSF bath solution and ACSF solution containing the appropriate chemicals (e.g., Bicuculline) in a gravity-controlled multi-barrel perfusion system that is connected to the recording chamber. Continuously carbogenate all the solutions.

6.3 Turn on a peristaltic or vacuum pump that is connected to a suction tubing that ends opposite of the perfusion line in the recording chamber. Open the ACSF-filled barrel and start to establish a continuous flow (1–2 drops per second). Verify that the reference electrode is submerged in the ACSF solution.

6.4 Switch on the setup computer, the amplifier, the micromanipulator, the stimulator, the microscope light(s), the camera, and the monitor (if applicable).

6.5 Open the appropriate software for electrophysiological recordings (several different hardware and software providers for electrophysiological equipment exist).

6.6 Transfer one brain slice hemisphere from the recovery chamber to the recording chamber of the slice setup and position it in the correct orientation with the dentate gyrus granule cell layer and the molecular layer in the field of view. Make sure that the stimulation electrode can reach the MPP in the slice from the direction of the entorhinal cortex and that the recording electrode has access to the MPP from the exact opposite side from the direction of CA3.

6.7 Stabilize the slice in the recording chamber with a paper clip (**Figure 3A**) or a commercially available brain slice grid.

NOTE: It can be helpful to turn off the perfusion during slice transfer and positioning. However, this should not take too long in order to guarantee the quality of the brain slice.

6.8 Lower and position the recording and stimulation electrodes in the MPP in the lower third of the molecular layer close to the granule cell layer (**Figure 3B**), facing each other at a distance of approximately 100–150 µm. The stimulation electrode should minimally contact the surface, while the recording electrode should slightly infiltrate the upper slice layer.

6.9 Apply a low intensity stimulus (30–50 µA for 0.1 ms) to the brain slice in order to obtain the fEPSP signal. Adapt the position of the electrodes if necessary (e.g., low or atypical shaped fEPSP signal).

6.10 Start recording Input-Output curves: apply increasing stimulus intensities to the brain slice in intervals of 30 s.

6.11 Set the stimulus intensity to 50% of the maximal fEPSP response and start baseline fEPSP recordings applying the 50% stimulus intensity for 20–40 min in 30 s intervals to the brain slice.

6.12 If the baseline appears to be stable, proceed with additional recordings (e.g., LTP/LTD protocols and/or drug applications). (For LTP induction in the MPP, here a protocol consisting of four times 1 s 100 Hz pulses applied in an interval of 5 min was used. Bicuculline was applied 10 min before and during the conditioning phase.)

NOTE: All recordings should be performed in the current clamp mode with appropriate low-pass filtering (<5 kHz) and sampling rates (>10 kHz).

6.13 Extract the data in an appropriate file format and analyze fEPSP parameters, such as fiber volley, fEPSP amplitude, and fEPSP slope.

7. Calcium imaging recordings of brain slices

7.1 Transfer one brain slice hemisphere in a 12-well chamber and load it for 30 min to 1 h with an appropriate calcium dye. Continuously carbogenate the loading solution by inserting a carbogenation tube through a self-made hole (with a hot 18G needle) in the lid of the 12-well plate.

NOTE: This step is not necessary in case of brain slice preparations from genetically modified animals that endogenously express a fluorescent reporter such as GCaMP.

7.2 Prepare the recording setup as described in steps 6.2–6.5.

NOTE: No amplifier or micromanipulator are necessary for microfluorimetric imaging experiments. The use of a stimulator is experiment dependent. Specific software for fluorescence imaging experiments is essential.

7.3 Adjust the software to the correct imaging settings: this includes camera amplifier and binning setting, setting of the wavelength, exposure time, and timing protocol. Settings can vary depending on the exact experiments. (For the example traces, 1 x 1 binning, 2.4x pre-amplifier gain, and 300 cameras, exposure of 50 ms at 488 nm repeated every 500 ms for the duration of the experiment was used.)

7.4 After loading with the calcium dye, transfer the brain slice hemisphere from the 12-well to the recording chamber and position it on the microscope stage.

7.5 Stabilize the slice in the recording chamber with a paper clip (**Figure 3A**) or a commercially available brain slice grid similar to the one described in step 6.7.

7.6 Assure that the area of interest is in the correct microscopic field and in focus.

7.7 Use the fluorescence imaging software to select several regions of interest (ROIs) on your slice.

NOTE: It can be useful to select the whole field of view to follow the general fluctuations in brightness, due to photobleaching.

7.8 Start the recording and apply the experimental test-drugs at chosen time points.

NOTE: It is advisable to apply a high K^+ solution at the end of each experiment to verify the neuronal character of the cells and the slice quality.

7.9 Extract the data in an appropriate file format and analyze the fluorescence signal changes that occur in the ROIs over the entire measurement. ROIs can also be adapted during the off-line analysis.

NOTE: Other protocols that describe fEPSP recordings and calcium microfluorimetry in brain slices are available in literature[24,32–35].

REPRESENTATIVE RESULTS:

General overview of tools and critical steps needed for the protocol

Figure 2 presents all the necessary tools and critical steps for the preparation of horizontal acute hippocampal brain slices as described in this protocol. Generally, a limited number of key instruments are required, including a few dissection tools and a slice recovery chamber (**Figure 2A**), a laboratory water bath, and a vibratome (**Figure 2B**). **Figures 2C–E** visualize important steps and orientations of the brain and hemispheres during the slice preparation protocol. **Figure 2F** is an illustration of an expected result of horizontal brain slices.

fEPSP recordings in the medial perforant path

After the recovery period, the brain slices can be used for electrophysiological recordings of fEPSPs. Here, we used an upright microscope equipped with a multi-channel gravity-controlled perfusion system (**Figure 3A** and **Figure 3B**). A glass micropipette (~ 2 MΩ) was filled with ACSF solution and attached on top of a chloride-coated silver electrode that is mounted to an operational amplifier in circuit with a chlorinated bath electrode. fEPSPs were recorded and visualized with an amplifier and appropriate recording software by inserting the glass micropipette into the MPP of the hippocampus in the upper layer of the brain slice. fEPSPs were induced by stimulation with a 2-contact cluster microelectrode, applying different current intensities to the MPP upstream of the recording electrode. Note that this protocol is not intended to explain how to obtain MPP recordings, but simply uses recordings in the MPP as an example to demonstrate the success of the slice preparation protocol described here. If someone attempts to perform MPP recordings, certain controls (e.g., paired pulse recordings) might be necessary in order to ensure the proper recording site and distinguish the MPP from the LPP[8,36,37].

Figure 3C illustrates a negative (left panel, low quality slice) and positive (right panel, high quality slice) example of an fEPSP recording. The negative example trace shows a large fiber volley (FV) amplitude that is even higher than the actual fEPSP amplitude (≈0.5 mV). In contrast, the high-quality slice example (right panel) shows a small FV to fEPSP ratio and a high fEPSP amplitude (>0.5 mV). The fiber volley is the signal that occurs upon depolarization of the stimulated neuronal fibers and therefore precedes the postsynaptic potentiation (fEPSP). The relation of FV to fEPSP properties provides important information about the preservation of the axonal and

441 synaptic properties. High quality slices with intact nerve fibers should show a high fEPSP
442 amplitude to FV ratio. On the contrary, low quality slices with impaired conduction properties
443 will have a decreased fEPSP to FV ratio. Similarly, the viability of a brain slice can be analyzed by
444 plotting fEPSP slopes versus the fiber volley amplitudes (**Figure 3D**).
445
446 Moreover, Input-Output curves (fEPSP slope and FV amplitude over stimulus intensity) are
447 standardly used in order to determine the slice quality. Such curves are obtained by applying
448 increasing current stimuli to the brain slice and by monitoring the subsequent fEPSP responses.
449 Low quality brain slices show a reduced Input-Output curve due to suboptimal conduction
450 properties of poorly preserved brain tissue (**Figure 3E,F**). Furthermore, Input-Output curves are
451 necessary to define the ideal stimulation intensity range for the investigation of synaptic
452 processes. Ideally, the stimulus intensity should be set around 50% of the intensity for maximal
453 responses. At this chosen stimulus intensity, the fEPSP responses are highly sensitive for any
454 changes in synaptic plasticity, which offers the opportunity to investigate both long-term
455 potentiation (LTP) and long-term depression (LTD).
456
457 In order to study synaptic plasticity, the synaptic transmission of the brain slice (fEPSP slope) at
458 the chosen 50% stimulus intensity is monitored for a longer time period (usually between 20–40
459 min) before the conditioning phase. Viable brain slices will have stable baselines, while brain
460 slices with an unstable baseline cannot be used for further conditioning protocols in order to
461 study synaptic plasticity of the brain circuits (**Figure 3G**, upper panel). fEPSP baseline recordings
462 can also be useful in order to monitor drug effects on synaptic transmission itself (**Figure 3G**,
463 lower panel). The mean of the recorded fEPSP baseline signals is typically used to normalize an
464 fEPSP time course and is standardly set at 100%.
465
466 Synaptic plasticity can be studied by applying specific conditioning protocols to the brain slices.
467 These protocols depend on the investigated brain circuit and the mechanism of interest (e.g., LTP
468 or LTD). In order to induce LTP in the MPP of the dentate gyrus, a strong conditioning protocol is
469 necessary due to the strong GABAergic inhibition that is present at the MPP synapses[38]. It is
470 reported that the GABAergic inhibition is even more pronounced in brain slices prepared with
471 high-sucrose slicing solutions[39]. Here, we use a protocol consisting of four stimulations of 1 s long
472 100 Hz pulses applied in a 5 min interval while being treated with the $GABA_A$ receptor antagonist
473 Bicuculline (**Figure 3H**). The co-addition of NMDA and Bicuculline during the conditioning period
474 results in an increased LTP (**Figure 3H**). Low quality of the slice and unstable synaptic transmission
475 (fEPSP baseline) could result in altered or unsuccessful LTP and LTD induction. Therefore, it is of
476 high importance to work with high quality slice preparations and use rigorous exclusion criteria
477 (high fEPSP amplitude to fiber volley ratio (>3), small fEPSP slope (<0.5 mV/ms) or amplitude
478 (<0.5 mV) and unstable fEPSP baseline (change of more than 5%) for unviable slices when
479 investigating synaptic processes.
480
481 **Calcium microfluorimetric measurements in the granule cell layer of the dentate gyrus**
482
483 After recovery, a brain slice was incubated at room temperature with 2 µM of a calcium-sensitive
484 dye for 1 h in carbogenated ASCF, shielded from light. The slice was transferred into a recording

chamber (**Figure 3A**) on an upright fluorescence microscope equipped with a multichannel gravity-controlled perfusion system. Fluorescence emission images were acquired every 500 milliseconds after illumination at 488 nm (**Figure 4A,B**). Excitation was done with a Xenon lamp and a scanner mounted diffraction grating monochromator and image acquisition was performed with a computer-controlled CCD camera. During the measurements, the slice was treated with the NMDA receptor antagonist APV, which resulted in a decrease in the intracellular calcium concentration. Stimulation of the slice with an extracellular solution containing a high potassium concentration (50 mM) resulted in a massive influx of extracellular calcium due to the depolarization of the neurons and the opening of voltage-gated ion channels (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Cartoon illustrating the different hippocampal regions and main fiber pathways. Representation of the hippocampus. The different hippocampal regions are indicated by solid colored lines: entorhinal cortex (EC; black), dentate gyrus (DG; orange), *Cornu Ammonis* (CA) 3 (cyan), 2 (yellow), and 1 (magenta), and the subiculum (green). Fiber pathways are shown with a colored dotted line: the medial (MPP, red) and lateral perforant path (LPP, blue) (from the entorhinal cortex to the dentate gyrus, CA3, CA1, and subiculum), the mossy fiber pathway (MF, violet) (from the dentate gyrus to CA3) and the Schaffer collateral (SC, brown) (ipsilateral from CA3 to CA1)/associational commissural pathways (AC, light green) (contralateral from CA3 to CA1).

Table 1: 10 x slice pre-solution (1 L)

Table 2: 1x ACSF (1 L) (osmolarity between 305–315 mOsm).

Table 3: 1x high-sucrose slice solution (250 mL) (osmolarity between 320–325 mOsm)

Figure 2: Detailed information on the preparation of horizontal hippocampal brain slices. (**A**) Image of tools required for dissection and slicing of the rodent brain: (a) ±2 cm long and ±0.5 cm wide straps of filter paper (e.g., grade 413); (b) blade; (c) super glue; (d) pipette tip; (e) specimen plate (comes with vibratome); (f) 35 mm culture dish; (g) fine brush; (h) spatula; (i) curved forceps; (j) dissection scissors; (k) strong scissors (blade length above 10 cm); (l) plastic Pasteur pipette with wide opening (between 0.6 to 0.8 cm in diameter); (m) recovery chamber (self-made with 250 mL beaker, plastic ring, nylon mesh, piece of a 10 mL serological pipette); (n) 90 mm culture dish filled with ice and (o) square of filter paper on top of the chilled culture dish. (**B**) Picture of a vibratome with (a) holder of slice chamber filled with ice; (b) slice chamber; (c) carbogen line and (d) slicing razor blade. (**C**) Cartoon illustrating the orientation of the cut of the dorsal side of one hemisphere in order to prepare the brain for horizontal slicing (see step 3.9). (**D**) Isometric projection of the brain orientation on the specimen plate of the vibratome. (**E**) Cartoon illustrating a top view of the position of the two hemispheres on the specimen plate. (**F**) Cartoon showing the position of the hippocampus in a horizontal brain slice. The dentate gyrus (DG) and *Cornu Ammonis* (CA)–Subiculum (SB) regions of the hippocampus are indicated. (**G**) Picture of a recovery chamber with carbogenated ACSF containing ten freshly sliced horizontal

brain slices.

Figure 3: Electrophysiological recordings of hippocampal brain slices. (**A**) Image of a recording chamber with perfusion and suction, used under an upright microscope. A brain slice will be placed in the chamber and immobilized with a piece of a paper clip before the start of the recordings. (**B**) Bright field picture of a hippocampal brain slice under an upright microscope (10x objective). The dentate gyrus (DG) and CA3 region are indicated as well as the stimulation (bottom left) and recording (bottom right) electrodes, targeting the medial perforant path during fEPSP recordings. (**C**) Left: representation of a low-quality slice example of a fEPSP recording with a robust fiber volley and a small amplitude. Right: high quality slice example of a fEPSP recording. The gray line indicates the baseline level. The dotted lines point out the cut-off amplitude of 0.5 mV. (**D**) Plot of the fEPSP slope versus the FV amplitude for high quality (black; n=10) and low-quality brain slices (gray; n=4). Data represented as mean ± SEM. (**E**) Input-Output plot (fEPSP slope) for different stimulation intensities (µA) for high quality slices (black; n = 10) and low-quality slices (gray; n = 4)). (**F**) Same as in *(E)* but for the FV amplitudes versus the stimulus intensities. (**G**) Time course of three different baseline fEPSP recordings (slope of fEPSP in %; normalized to the mean fEPSP slope of the first 5 min). Upper panel represents a positive (black) and negative (red) example, where the latter has an unstable baseline due to omission of carbogen during the recording. Lower panel shows two stable baseline recordings in treated (after 20 min of stable baseline, AMPA receptors were blocked by application of the AMPA receptor antagonist DNQX (10 µM)) (blue) and untreated condition (black). (**H**) Time course of LTP recordings for different treatment conditions (indicated in lower panel). Black color for application of Bicuculline (20 µM) during conditioning and blue for co-application of Bicuculline (20 µM) and NMDA (10 µM) during conditioning. Arrows in upper panel indicate the time points where high frequency stimulation was applied (4 x 1s of 100Hz). Bar graph in lower panel represents the mean fEPSP slopes (%) for 50–60 min after LTP induction of the experiments shown in the upper panel (single representative recording for each condition).

Figure 4: Calcium-microfluorimetry of hippocampal brain slices. (**A** and **B**) Fluorescence image (excited at 488 nm) (**A**) and corresponding heat map (**B**) of a horizontal hippocampal brain slice of the mouse brain. The dentate gyrus (DG), CA3 region, and an example of a region of interest (ROI) are indicated in panel A. (**C**) Time course of calcium responses ($F_{488\ nm}$) from a ROI in the dentate gyrus of an acute hippocampal brain slice during treatment with the NMDA receptor antagonist APV (50 µM) and solution containing high extracellular potassium (K^+) (50 mM). The trace is normalized to the highest calcium response during high K^+ perfusion and is baseline corrected for photobleaching.

DISCUSSION:

Although commonly used among the neuroscience community, brain slice preparations are also faced with several disadvantages. For instance, input and output connections to the brain areas of interest are no longer connected in a brain slice. Moreover, once isolated, the tissue starts degrading slowly over time and this process could alter the physiological conditions of the brain slice. This topic in particular is very concerning because most brain slice recordings are taking several minutes to hours, which results in long experimental days with recordings performed on

tissue that was isolated up to 6–8 h before the start of the experiment. Furthermore, the cerebrospinal fluid and blood circulation get interrupted during slice preparations, which may lead to the lack of important endogenous compounds within a brain slice. And most obviously, the slicing procedure itself may cause mechanical tissue damage that might compromise the obtained results. However, the actual benefits of brain slice preparations are still outweighing their disadvantages, which is why they present a highly valued and employed technique in neuroscience research.

Acute hippocampal brain slices present a powerful and therefore widely used technique to investigate neuronal processes from a molecular level up to complex brain circuit studies. This is based on the ideal neuroanatomy of the hippocampus that can be easily preserved in a slice preparation[18]. Consequently, hippocampal brain slices are used in a wide variety of scientific research projects, including drug screenings[17], studies of neuronal and synaptic properties involved in cognitive functions[40,41], and investigations of pathological brain conditions[14,42,43]. However, a broad spectrum of different applications also causes a wide range of available slice preparation protocols that can differ in various parameters, such as dissection conditions and cutting plane orientation, among others. Therefore, the exact research question of a scientific project has to be determined in order to choose an appropriate slice preparation protocol.

The tissue chopper presents one of the oldest used techniques in order to prepare hippocampal brain slices[44,45]. The major advantages of this preparation method include the low cost of the chopper and the fast and easy usage[46]. However, tissue choppers cause mechanical stress that results in morphological alterations and cell death[47]. In comparison, the vibratome is a rather expensive machine and the time for slice preparation is significantly increased which might have an impact on the quality of the slice. However, the vibratome usually offers a more gentle manner of separating the slices from the tissue and allows to keep the brain nicely cooled and oxygenated over the entire isolation procedure, thereby improving slice properties[46]. Therefore, several groups are standardly employing this technique and have brought forward protocols for the preparation of acute hippocampal brain slices using the vibratome[16,30,48]. While some protocols provide only a few details for the slicing itself but rather focus on a specific application of such slice preparation[48], others provide detailed slice protocols that differ in cutting plane or other protocol details (e.g., agarose embedding or slice/recovery solutions) given in this article[27,30].

The protocol described here presents a straightforward method in order to prepare high quality acute horizontal hippocampal mouse brain slices from young animals. The protocol is particularly useful to preserve the perforant path (medial and lateral) that presents the hippocampal input pathway, which projects from the entorhinal cortex to the hippocampus[8,49,50]. Sagittal, coronal, as well as isolated hippocampus transverse slice preparations do not properly preserve the perforant path, which originates from mainly Layers II and V of the entorhinal cortex and projects to several areas within the hippocampus[18]. Due to the anatomical positioning of the entorhinal cortex in relation to the hippocampus, horizontal brain slices are a necessity in order to maintain fully intact perforant path fibers within the slice preparation[31]. Additionally, horizontal slicing ideally preserves the mossy fibers that project from the dentate gyrus to the CA3 neurons within the hippocampus[9,30,50]. Therefore, this preparation method is of high value for studies that

investigate hippocampal input pathways and DG-related processes. In addition, this protocol allows the investigation of the Schaffer collateral pathway[50]. However, sagittal and coronal brain slice preparations are more commonly used when investigating CA3 to CA1 fiber projections, presumably because of their slightly faster preparation time that can increase the chance of obtaining high quality slices. Nevertheless, horizontal hippocampal slice preparations present a powerful research tool since it allows the preservation and investigation of all hippocampal fiber pathways within one slice hemisphere. This can be particularly useful when circuit responses are studied, for example, in multi electrode assay recordings.

A major concern when preparing brain slices is the proper preservation of the brain tissue. This is accomplished by several critical steps in our protocol, including a fast dissection, the continuous and sufficient oxygenation and cooling of the tissue, and the protection of the brain tissue by use of the protective cutting method with a low-sodium, high-sucrose slicing solution[39,51]. Despite the fact that the protocol described here yields a success rate around 90%, potentially additional protective steps might be required when working with tissue derived from older or genetically diverse animals or when trying to preserve a specific cell population. Several methods were already reported to protect sensitive brain tissue preparations. These methods include the use of NMDG-based slicing solutions to reduce the sodium permeation[52], the use of high magnesium levels in the slicing solution in order to block NMDA receptor activity[53], and the prolonged use of protective solutions also during the recovery period[23]. All of these measures will result in a reduced excitotoxicity. Additionally, a trans-cardial perfusion with ice-cold protective ACSF solutions is often employed and necessary when working with older animals[27].

Acute hippocampal brain slices are ideally suited and extensively used for electrophysiological studies for reasons such as the high amplitude signals that can be obtained from a relatively thick (300–500 µm) acute brain slice, which guarantees a high signal to noise ratio[11]. Standardly used electrophysiological applications include extracellular field recordings and intracellular whole-cell recordings in a voltage- or current-clamp mode. In order to acquire high quality electrophysiological data, the slice health is of primary concern and can be guaranteed by strictly following the presented protocol. However, as slice preparations present a highly sensitive technique, a quality check should be routinely included before the start of each experiment. Several parameters can be used as quality check of the slice and are standardly assessed via Input-Output curves and baseline fEPSP or EPSC recordings[19]. Nevertheless, it should be noted that suboptimal electrophysiological properties can arise from experimental errors such as electrode positioning, orientation or even damage and do not solely represent the health of the prepared slice. Therefore, it is advisable to perform additional quality controls such as simple visualization and assessment of the cells under a 40x objective or a DAPI nucleus staining. Such quality checks can be used to confirm constant slice health over several slice preparation sessions.

Calcium microfluorimetry presents a less commonly used technique to study hippocampal brain slices. However, this technique is of additional value to the standard extracellular and intracellular electrode recordings, as it allows to visualize and quantify intracellular calcium fluxes, which are of high importance in neuronal and synaptic signaling. Changes in intracellular

calcium concentrations are important in neuronal and synaptic signaling and are involved in neurotransmitter vesicle release, postsynaptic potential generation, regulation of synaptic plasticity and axonal nerve conduction[54–56]. As an illustration of this technique (**Figure 4**), we made use of a commercially available calcium dye. Inarguably, treatment of tissue slices with calcium dyes can yield difficulties such as an increased experimental time frame as well as inefficient loading of lower situated neuronal cells. However, variations on this technique could be used to circumvent these technical challenges. For instance, it is possible to combine calcium measurements and patch clamp recordings in hippocampal slices. In this way, a calcium fluorescent dye could be loaded into a specific cell through the patch pipette, allowing the measurements of calcium dynamics in one specific cell of interest[57]. Alternatively, genetically engineered animals expressing the calcium indicator, GCaMP[58], either in the whole brain, or driven by a cell-specific promotor, could be used. Interestingly, brain tissue from GCaMP animals with a direct linker to a protein of interest could provide opportunities to determine the neuronal expression pattern or investigate the involvement in calcium sparks and waves.

Altogether, we provide the guidelines for the successful preparation of healthy and viable horizontal hippocampal brain slices from mice for electrophysiological and imaging recordings. This methodology is very useful to access neurological changes that occur in brain pathologies that are described in the dentate gyrus.

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The authors have nothing to disclose.

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Figure2

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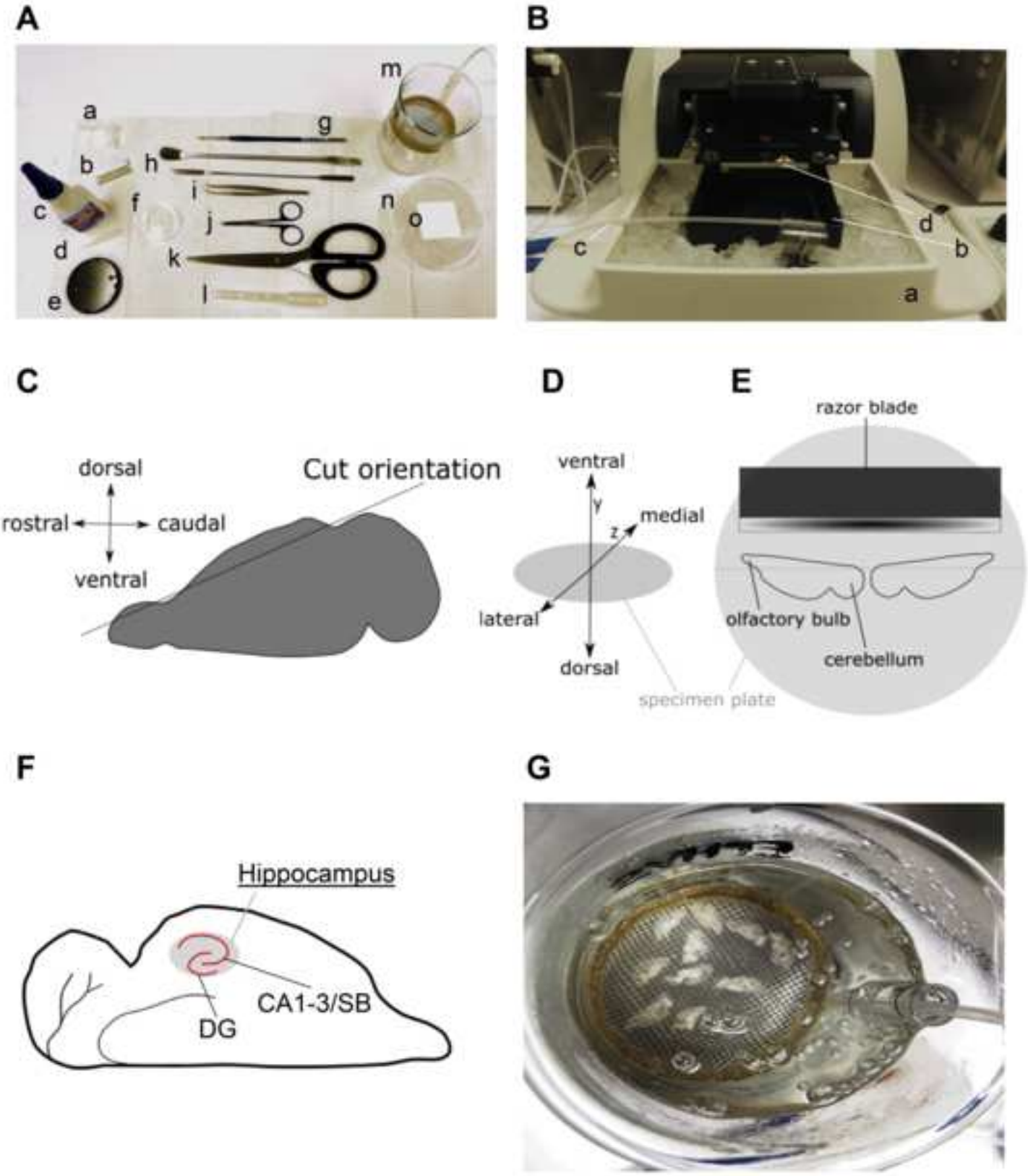

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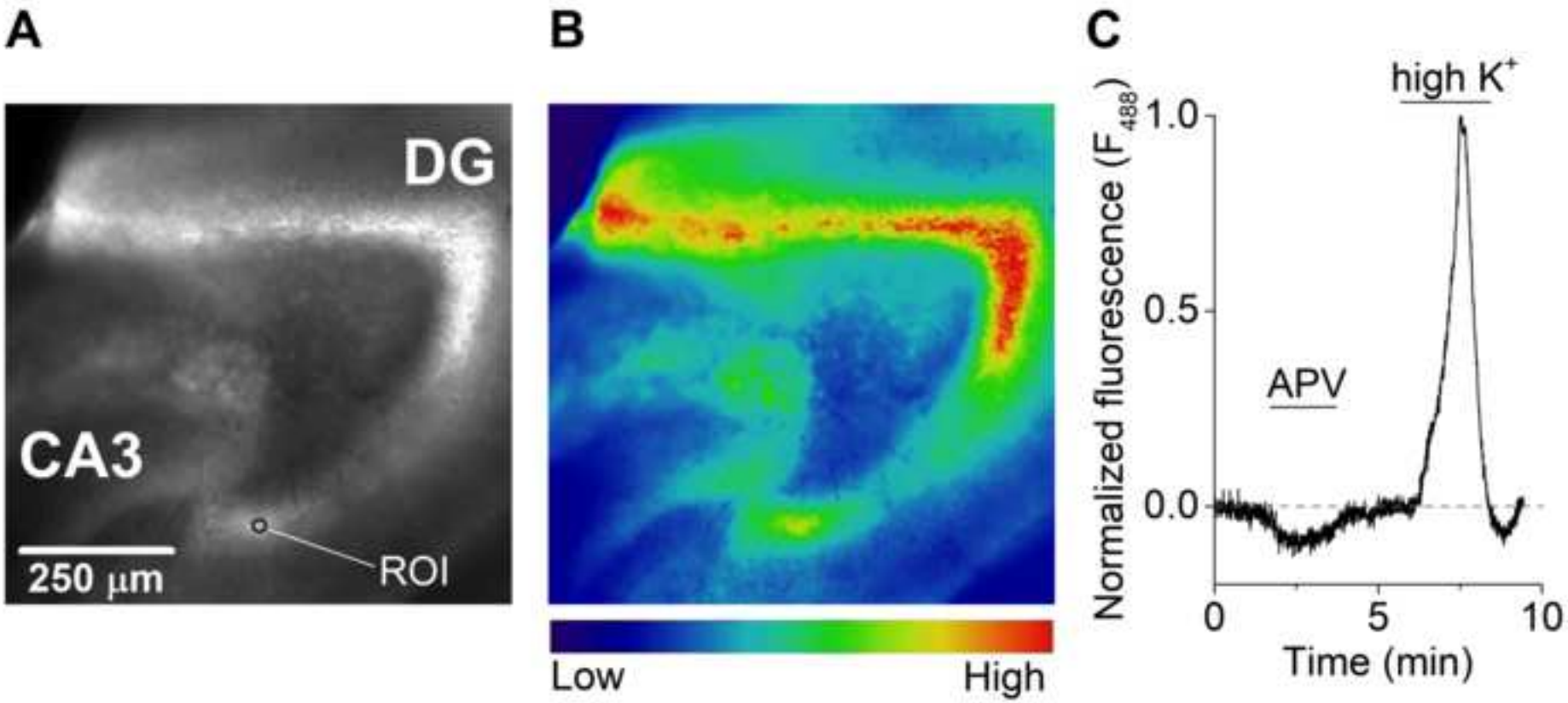

Figure1

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Compound	Concentration (mM)	Molecular weight (g/m	Amount (g)
KCl	25	74.55	1.86
$CaCl_2 * 2H_2O$	20	147.01	2.94
$MgSO_4 * 7H_2O$	10	246.48	2.46
KH_2PO_4	12.5	136.08	1.7

Compound	Concentration (mM)	Molecular weight (g/m	Amount (g)
NaCl	125	58.44	7.3
KCl	2.5	74.55	0.19
$CaCl_2 * 2H_2O$	2	from 1 M $CaCl_2$ solution	2 mL
$MgSO_4 * 7H_2O$	1	from 1 M $MgSO_4$ solution	1 mL
$NaH_2PO_4 * 2H_2O$	1.25	156.02	0.2
$NaHCO_3$	26	84.01	2.18
Glucose * H_2O	25	198.17	4.95

Table3 Click here to access/download;Table;Table3.xlsx

Compound	Concentration (mM)	Molecular weight (g/m	Amount (g)
10x slice presolution	N/A	N/A	25 mL
Sucrose	252	342.3	21.57
$NaHCO_3$	26	84.01	0.55
Glucose * H_2O	10	198.17	0.49

Name of Material/Equipment	Company	Catalog Number
Anesthesia chamber	home made - Generic	N/A
Anesthesia vaporizer	Dräger & MSS International	Isoflurane Vapor 19.3 & MSS
Barrels for the perfusion system	TERUMO	Hypodermic syringes without needle
Bicuculline methiodide	hellobio	HB0893
Borosilcate glass capillaries	Science Products	GB150F-8P
Calcium chlorid dihydrate	Merck	102382
Calcium Imaging software	Till Photonics	LiveAcquisition v2.3.0.18
Carbogen tank	Air Liquide	Alphagaz mix B50
Cluster microelectrode	FHC	CE2C55
Culture dish (35 mm)	Corning Life Sciences	353001
Culture dish (90 mm)	Thermo Fisher Scientific	101VR20
Curved forceps	Fine Science tools	11270-20
D-AP5	hellobio	HB0225
D-(+)-Glucose monohydrate	Sigma Aldrich	16301
Digital CMOS camera	HAMAMATSU	ORCA-spark C11440-36U
Dissection scissors	Fine Science tools	14058-09
DNQX	hellobio	HB0262
EMCCD camera	Andor	iXon TM + DU-897E-CSO-#BV
EPC10 USB Double Patch Clamp Amplifier	HEKA Elektronik	895278
Filter paper	VWR	516-0818
Fine brush	Raphael Kaerell	8204
18G needle	Henke Sass Wolf Fine-Ject	18G X 1 1/2" 4710012040
Isoflurane	Dechra Veterinary Products	Iso-Vet 1000mg/g
Loctite 406	Henkel Adhesive technologies	Loctite 406
Magnesium sulfate heptahydrate	Merck	105886
Micromanipulator	Luigs & Neumann	system
Microscope (for calcium imaging)	Olympus	BX51WI

Microscope (for ephys recordings)	Zeiss	Axio Examiner.A1
Microscope light source	CAIRN Research	dual OptoLed power supply
Monochromator	Till Photonics	Polychrome V
N-Methyl-D-aspartic acid (NMDA)	Sigma Aldrich	M3262
Oregon Green® 488 BAPTA-1	Invitrogen Molecular Probes	#06807
Osmometer	Wescor	5500 vapor pressure osmometer
Peristaltic pump	Thermo Fisher Scientific	Masterflex C/L 77120-62
pH meter	WTW	inoLab series pH 720
Pipette puller	Sutter Instrument	P-1000
Potassium chlorid	Chem-lab	CL00.1133
Potassium dihydrogen phosphate	Merck	104873
Razor blade to prepare hemispheres	SPI supplies	Safety Cartridge Dispenser - Pkg/10
Razor blade for vibratome	Ted Pella Inc	121-6
Recovery chamber	home made - Generic	N/A
Scissors	Any company	N/A
Silver electrode wire	Any company	
Sodium dihydrogen phosphate	Merck	106342
Sodium hydrogen carbonate	Alfa Aesar	14707
Sodium chlorid	Fisher Scientific	S/3160/60
Software for field recordings	HEKA Elektronik	PatchMaster
Spatula	Sigma Aldrich	S9147-12EA
Stimulator	A.M.P.I	ISO-FLEX
Sucrose	VWR International Ltd.	102745C
Tubing for carbogen, perfusion and suction lines 1	Warner Instruments	64-0167
Tubing for carbogen, perfusion and suction lines 2	Fisher Scientific	800/100/200 & 800/100/280
Vacuum pump	home made - Generic	N/A
8 valve multi-barrel perfusion system	home made	N/A

Magnetic valves (to control the perfusion lines)	NResearch Inc.	p/n 161P011
Vibratome	Leica	14912000001
Water bath	Memmert	WNB 7
Water purification system	Merck	Synergy millipore
12-well plates	Greiner Bio-One	CELLSTAR, 665180

Comments/Description
plexiglas
to vaporize isoflurane for rodent anesthetizatio
https://www.terumotmp.com/products/hypoderm
https://www.hellobio.com/bicuculline-methiodide.
https://science-products.com/en/shop/micropipett
https://www.merckmillipore.com/BE/en/product/C
Gasmixture CO2/O2: 5/95, purity 5
https://www.fh-co.com/product/cluster-microelect
https://ecatalog.corning.com/life-sciences/b2c/US/
https://www.thermofisher.com/order/catalog/proc
https://www.finescience.de/de-DE/Products/Force
https://www.hellobio.com/dap5.html
https://www.sigmaaldrich.com/catalog/product/sia
https://www.hamamatsu.com/eu/en/product/type
https://www.finescience.de/de-DE/Products/Scisso
https://www.hellobio.com/dnqx-disodium-salt.htm
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https://www.heka.com/sales/brochures_down/bro
grade 413
Size #1
https://www.henkesasswolf.de/cms/de/veterinaer
250 ml bottle
Super glue
https://www.merckmillipore.com/BE/en/product/M
https://www.luigs-neumann.org
https://www.olympus-lifescience.com/de/microscc

https://www.micro-shop.zeiss.com/de/de/system/
https://www.cairn-research.co.uk/product/optoled

https://www.sigmaaldrich.com/catalog/product/sig

10x50ug
to verify osmolarity of salt solutions
https://www.fishersci.be/shop/products/masterfle
https://www.geminibv.nl/wp-content/uploads/mar
https://www.sutter.com/MICROPIPETTE/p-1000.ht
https://www.chem-lab.be/#/en-gb/prod/1393528
https://www.merckmillipore.com/BE/en/product/F
GEM Scientific Single Edge Razor Blades
double edge breakable style razor blades (PTFE
to collect and store brain slices in (see details ir
Blade should be well sharpened and at least 15
for recording and reference electrodes
https://www.merckmillipore.com/BE/en/product/S
https://www.alfa.com/en/catalog/014707/
https://www.fishersci.co.uk/shop/products/sodium
https://www.heka.com/downloads/software/manu
https://www.sigmaaldrich.com/catalog/produc
http://www.ampi.co.il/isoflex.html
https://es.vwr-cmd.com/ex/downloads/magazine/l

Tygon tubing (TY-50) for standard valve system

Smiths Medical Portex Fine Bore LDPE Tubing

consists of barrels, tubing and a self-made auto

https://nresearch.com/
Semi-automatic vibrating blade microomei VT1
https://www.memmert.be/wp-content/uploads/20
to obtain highly purified water
http://www.greinerbioone.com/UserFiles/File/Cata

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axio+examiner-axio+examiner.a1-aufrechte+mikroskope/10185/
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gma/m3262?lang=en&region=BE

x-peristaltic-c-l-dual-channel-pump-2/p-8004229
nuals/wtw-720-ph-meter/wtw-inolab-ph-720-manual-eng.pdf
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otassium-dihydrogen-phosphate,MDA_CHEM-104873?ReferrerURL=https%3A%2F%2Fwww.google.com%2F

-coated stainless steel)
n manuscript)
cm long for easy decapitation

odium-dihydrogen-phosphate-dihydrate,MDA_CHEM-106342?ReferrerURL=https%3A%2F%2Fwww.google.com%2F

n-chloride-certified-ar-analysis-meets-analytical-specification-ph-eur/10428420
ual/m_patchmaster.pdf
t/sigma/s9147?lang=en&region=BE

upc_userguide_uk.pdf

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mated valve control (specifications of all purchased parts can be found in this Table)

200
)19/09/Memmert-Waterbath-WNB-7.en_.pdf

ılogue%202010_11/UK/3680_005-Kapitel1_UK.pdf

Dear Review editor,
Dear Dr. Vineeta Bajaj,

Please find enclosed the revised version of our methods manuscript entitled: “Horizontal Hippocampal Brain Slices of the mouse brain.” that we recently submitted for peer-revision in the *Journal of Visualized Experiments.* We aim to contribute with this manuscript to the methods collection: “Preparation of acute hippocampal slices” by guest editor Dr. Felix Leroy.

Several editorial and reviewer comments were provided after the first revision of our work and we addressed all of the comments as detailed in the point-by-point response listed below. We believe that we could greatly improve the quality of our manuscript after carefully revising our prior submission.

We hope that you will find our revised manuscript suitable for publication in *JoVE.*

Yours sincerely,

Katharina Held

Editorial Comments:

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

We carefully proofread our manuscript and included several changes in the revised manuscript version.

• **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. Examples:
1) 2.1.3: mention animal strain, age, sex.
2) 3.1: how do you ensure depth of anesthesia?

We adapted points 2.1.3 and 3.1 in order to provide some more detail. We checked all other protocol points and believe to have provided enough details to easily replicate the here presented technique.

• **Protocol Numbering:**
1) All steps should be lined up at the left margin with no indentations.
2) Please add a one-line space after each protocol step.

In the revised manuscript, all steps are lined up at the left margin without indentations with a space after each protocol step.

• **Protocol Highlight:** 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.

A total of 2.5 pages was marked in yellow, excluding notes.

• **References:**
1) Please spell out journal names.

The reference list was adapted accordingly in the revised version of the manuscript.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MilliQ, Fine Science, Loctite 406,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have removed all commercial sounding language from the manuscript text. This information is now solely available in the Table of Materials and Reagents.

• 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]."

All here presented Figures were specifically recorded for the purpose to publish in this JoVE article. No material was published elsewhere before.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the protocol submitted by Van Hoeymissen et al. for consideration at JoVE titled "Horizontal Hippocampal Brain Slices of Mouse and Rat for secondary applications" the authors provide a step-by-step protocol for the production of horizontal brain slices from the rodent brain. This protocol is from a lab that has produced a number of key studies using this approach, including recent

examples, i.e. Held et al 2020. This protocol complements a number of previous protocols both in JoVE and elsewhere to describe the production of brain slices containing the hippocampus. I have a number of concerns that should be addressed by the authors to improve the understanding and usefulness of this protocol in relation to its intended audience:

Major Concerns:
1) I feel that the title of this protocol is somewhat misleading/confusing. I am unclear what a secondary application is in the production of brain slices. Presumably all experiments performed on brain slices as produced by the description here are the primary application? In the abstract, the authors identify a list of applications, with the first listed application being extracellular field recordings. This protocol contains representative results which describe extracellular field recordings in detail. This would not imply a "secondary application". Furthermore, the title mentions that this protocol is for mouse and rat brain slices. The provided protocol text only details murine techniques, and minimally discusses that this could be used in rats as well. Perhaps say this is primarily for mouse.

We thank the reviewer for his/her comments and adapted the title accordingly. We used the here provided protocol in the past in mice as well as rats. However, we agree with the referee's comment and will focus on one species.

2) I believe the authors make a bit of a faux pas in their summary: "apparent hippocampal slice protocols including a detailed systematic description are scarce", and introduction (line 117-118). There are indeed a great many protocols for describing slice preparation from mice and rats which provide a systematic approach both with recent advances - Booker et al., 2014 (JoVE); Ting et al., 2014 (JoVE); Hajos and Mody, 2009; Bischofberger et al., 2006, and with a traditional approach - Schwartzkroin, 1975, Newman et al., 1992. Several of these seminal studies are referenced by the authors, but a thorough review of these examples are absent.

The authors should carefully describe how their protocol differs from those previously produced for JoVE, or other protocol publications - especially in the context of horizontal slice preparation from the hippocampus - which have been extensively detailed in Booker et al., 2014. Without this clarification it does not signify a substantial advance of technique from these earlier studies.

We thank the reviewer for this comment. We did exclude the mentioned sentence from our Abstract and instead included some sentences highlighting the advantages of horizontal slice preparations over others (line 46 - 53). Furthermore, we included a new paragraph in the discussion of the revised manuscript, where we put our manuscript in perspective to other reported slice protocols (lines 569 - 581) and extended the paragraph in the discussion where we mention the advantages of horizontal brain slices in particular (lines 583 - 601).
However, we would also like to point out, that the protocol of Booker et al. 2014 that was mentioned by the reviewer is not providing a detailed description of the horizontal slicing process itself, but rather focuses on the patch clamp recording of parvalbumin-expressing interneurons. The details of each individual slice step and used materials are lacking in our opinion in the protocol as well as in the supporting video. Further, Booker at al., employ a different cutting angle for their preparations which was reported to be of particular importance for proper mossy fiber preservation (Bischofsberger et al., 2006).

3) The text contains a number of grammatical inaccuracies and unusual sentence structures which should be addressed. A careful proofread of the manuscript text would greatly benefit the protocol. A number have been highlighted in the minor comments section.

We thank the reviewer for his/her attentive reading and have made several adaptations within the manuscript in order to improve the language.

4) It is unclear to me why the authors discuss the pros and cons of organotypic slice cultures in the introduction. It is somewhat distracting and unrelated to the protocol presented. This should be toned down or removed entirely.

We can agree with the reviewer's comment. In the revised version of the manuscript we excluded organotypic cultures from the introduction.

5) I have a concern about the storage and preparation of ACSF solutions, from a number of the protocols referenced (Ting et al., 2014; Bischofberger et al., 2006) and those not mentioned (Booker et al., 2014), these studies, as well as a great many other published physiological reports suggest using solutions of osmolarity 290-310 mOsm (within the physiological range). The authors state that they use a slicing solution with osmolarity of 205-215 mOsm (which is entirely non-physiological) - however the ACSF recipe they provide has a theoretical range of ~300 mOsm. Can the authors please comment?

We thank the reviewer for noticing that rather dramatic mistake. These were typo's in our previous manuscript version. We adapted the osmolarity to an isotonic solution ~300 mOsm.

Furthermore, the statement that 1x physiological solutions can be kept for up to 1 week is rather at odds with the majority of labs, which make their 1x solutions on the day of slicing and only keep for 1 day, perhaps 2 days under exceptional circumstances - see method details outlined in Bischofberger et al., 2006.

We do agree with the comment of the reviewer concerning the storage of the ACSF solution. In fact, we standardly prepare our ACSF solution at the day of experimentation in order to guarantee high quality slices. Only in very rare cases, we do prepare it the evening before. We know from peers that performed recordings without any issues when storing the ACSF at 4°C for up to one week and similar suggestions are even published in other JoVE brain slice protocols (Ting et al., 2018). However, as we intend to provide a protocol that guarantees consistent high-quality slice preparations, we adapted this part in our manuscript as suggested by the reviewer and as it is standardly performed in our lab (Step 1.2.1).

6) The authors discuss how "Only a tight control of all these key factors is allowing the preparation of continuous high-quality brain slices, ultimately rendering stable and comparable results". It is not clear how the authors control for these factors in their protocol either. Either tone this down or include quantification or qualification of quality controls, beyond only a fEPSP with a large fibre volley.

We did provide more detailed information concerning some of the key factors mentioned in the sentence before (lines 98 - 101). These details are now included in the relevant steps of the protocol and should suffice as a guideline for setting up the experiments. Furthermore, we mention other quality controls in our revised manuscript version such as the addition of high potassium in calcium-microfluorimetric experiments (step 7.8), microscopic cell assessment or a DAPI nucleus staining (lines 629 - 631).

7) It is unclear what the methods are for fEPSP recording or calcium imaging, as depicted in the representative results. I appreciate that this protocol focuses on slice preparation, but much of the

method detail is limited to the results text itself. I would suggest adding a short section to the methods describing one or both/either protocol.

We included two short sections with the necessary steps for fEPSP and calcium recordings (steps 6 and 7) and referred to other manuscripts providing detailed protocols (lines 398 - 399).

The authors then provide qualitative detail about what constitutes a good slice or a "bad" slice. However, the examples given and detailed could equally arise due to errors in slice handling in terms of the fEPSP recording, solution preparation, or other errors from the specific experiments outlined, and not simple due to slice quality. None of the measures used rule out these other factors to poor recordings, as such they do not confirm slice health. These other sources of error need to be specifically addressed or an independent confirmation of slice health i.e. condensed nuclei from DAPI labelling or a representative chart showing a reduced input-Output curve from an unhealthy slice. The discussion (Line 424 onwards) should also be modified to reflect this.

The reviewer is correct that other technical or experimental problems could also have an impact on the quality of the measurements and could be interpreted as bad slice quality. However, for electrophysiological recordings Input-Output curves and stable baseline recordings are the best measure to judge the slice quality. Moreover, a high-quality slice might appear unhealthy due to these other issues, but a low-quality slice will never look like a 'healthy' one. Therefore, in our opinion, these measures still present a valid quality control during electrophysiological experiments. Nevertheless, as requested by the reviewer, we did discuss this pitfall in the revised manuscript (lines 626 - 631) and also proposed to build in a quality check in form of a DAPI staining. Additionally, Input-Output curves for 4 representative low-quality slices are included in Figure 3D-F.

Regarding the presented input-output curve, it would be more appropriate to show both the input-output curves for both the afferent fibre-volley and then the fEPSP plotted against fibre-volley, which better reflects the amplitude of fEPSPs produced in response to the intra-slice control.

We agree with the comment of the reviewer and have adapted the Input-Output curves in the revised version of the manuscript (Figure 3D-F).

8) Given that the authors show LTD in the absence of Bicuculline or NMDA, but LTP in the presence of either drug, can the authors comment in the results and/or discussion that the protocol they are employing is not an LTP induction protocol per se, but rather a mechanism to induce heterosynaptic activation of synapses, which can lead to bi-directional plasticity. Again, given the absence of any method detail, this panel is confusing and at odds with the LTP narrative being put forward.

Independent of a hetero- or homosynaptic mechanism, the here employed protocol (4 HFS of 100Hz with 5 min ISI in presence of Bicuculline) is clearly inducing LTP as it results in a long-lasting increase in the fEPSP signal (up to 60 min after conditioning), which is the definition of Long Term Potentiation. Also note, that this long-lasting increase is in the absence of Bicuculline and NMDA itself, as the drugs are only applied during the induction protocol and omitted directly after the last HFS pulse. It is commonly known that LTP induction in the MPP is requiring a stronger induction protocol than in other regions of the hippocampus (e.g. CA1) and the use of Bicuculline is therefore often recommended in order to facilitate the LTP induction. For terms of simplicity, we did remove the red time course in Figure 3H and only focus on the LTP examples itself now. The exact induction protocol is explained in more detail in the Figure legend of the revised manuscript.

9) If detailed protocol methods are not provided, remove Calcium Fluorimetry representative results and discussion. As many JoVE videos have been produced on calcium-imaging techniques, going into

more detail than presented here (i.e. Ma et al., 2011; Laclar et al., 2012; Irwin and Allen 2013; Leinweber et al., 2014, amongst other protocols published elsewhere) it is unclear how useful further methodology would be, trying to cover many aspects of their usefulness.

We have included a more detailed description of the calcium microfluorimetric experiments in the protocol section (step 7) of the revised manuscript and referred to relevant JoVE videos that provide further details (lines 398 - 399).

Minor Concerns:
Line 29: "Highly preserve the integrity" -subjective, remove "highly"
Line 30: "Paths" should not be pluralised in "Perforant Paths"
Line 36: "The hippocampus includes a high degree" - includes should be replaced with "receives"
Line 39: "Brain slices are the number one ex vivo choice when exploring neurophysiological functions of the hippocampus" - that slice preparations are the "number one…choice" is highly arguable and dependent on the experimental aims/method.
Line 64: This sentence has a very confusing syntax and tense - "A dense network of informational flows in the form of fiber pathways is accomplishing a tight hippocampal connection to internal and external brain structures." Consider revising.
Line 69: "neatly" - subjective and implies a level of design. Consider replacing with an alternative such as "highly conserved laminar organisation"
Line 84: "secure" should be replaced with "preserve"
Line 137: MilliQ water - MilliQ water comes in many grades. Is it possible for the authors to comment on the quality of water - Grade 1, 2, or 3. Ultrapure double deionised or just single distilled.
Line 151-152: From experience ACSF does not emerge from the freezer as a semi-slushy solution, does this require shaking/homogenisation? Please elaborate
Line 183: What makes this an ultra-freezer? Is it different in some way from a standard -80°C freezer?
Line 189: Point 2.2.4 - does this specifically have to be a 35 mm culture dish? Could it be any small beaker/container on ice? A 35 mm dish maybe not appropriate for a larger brain - i.e. a 6 week old rat, which this protocol states it would be suitable for.
Line 196: Point 3.2 unusually structured and the meaning is unclear
Line 214: Please clarify why is meant by diffusional forces - I am not sure this strictly applies to this procedure. A more simple terminology might be better.
Line 238: The use of the word "further downwards" is somewhat unusual given the previous use of anatomical directions. Indeed, the "downward" direction is actually in the dorsal direction, if I have understood that the brain slicing method is very similar to Bischofberger et al., (2006) or Booker et al., (2014). Indeed, the vibratome used (Leica VT1200S) actually brings the brain to the blade, as such terminology is not correct in either case. Perhaps consider replacing with "more dorsally in the brain", for example.

We thank the reviewer for pointing out these errors. We did revise all of the upper comments in our revised manuscript version.

Line 417: "hippocampal input pathways and DG-related processes such as epilepsy", presumably the authors mean experimental epilepsy? Please revise.

We removed the example of epilepsy from the sentence.

Reviewer #2:
Manuscript Summary:

The manuscript describes a clear protocol on how to produce healthy brain slices for physiological recordings. Some of the critical steps, which remain under the radar in regular material & method sections of papers, are very well described. The authors show some of the applications that can be performed on hippocampal slices and I appreciate that it is indicated clearly how healthy slices can be distinguished from bad slices.

One major comment is that is not clear/obvious what the advantage is of the described horizontal hippocampal slices above sagital slices in full brains or even 'chopped isolated hipocampi'. This should be better explained.

We thank the reviewer for this very relevant comment. We extended our manuscript by adding some more detailed explanation of the advantages of horizontal brain slices over other slicing techniques to the Abstract (lines 46 - 53) as well as the Discussion (lines 569 - 601) sections.

Minor Concerns:
Title:
the description "for secondary application" is very unclear and doesn't add anything to the message. This part can be deleted or should be explained more specific. What is a primary application by the way?

We thank the reviewer for his/her comment and adapted the title accordingly.

Abstract:
Some illogical sentences are present, probably due to size restriction:
"electrophysiological recordings, such as field potentials, whole-cell patch clamp and calcium fluorometry" --> calcium fluorometry is not an electrophysiological technique. In addition calcium fluorometry is not the only light microscopic technique that is used in hippocampal slices: e.g. potential measurements, measurements of pH, other ions synaptic release,… not to forget optogenetic techniques. --> I suggest to rephrase to electrophysiological recordings, light microscopic measurements as well as molecular biological and histochemical techniques.
Although the manuscript describes horizontal brain slices, no notice of the advantages/difference of sagittal versus horizontal brain slices is even mentioned. In general, the abstract should focus more on the need for, and advantages of horizontal brain slices, rather than discussing the use of brain slices in general, as these are mostly referring to sagittal brain slices. Advantages compared to using isolated hippocampus cut with a tissue chopper should also be highlighted.

We adapted the Abstract accordingly in lines 42 and lines 46 - 53.

Line 75: Brain slice protocols are laborious, time-consuming and often result in the loss of connections…. --> it is mostly not the brain slicing that is laborious, but the subsequent experiment and analysis that is performed on the cells of the slices. Any primary cell culture would take more time than brain slicing. Only the loss of connections is an issue in my opinion.

The reviewer is absolutely right with this comment, so we deleted this part in our revised manuscript version.

102: "organotypic hippocampal slices, acute hippocampal slices are usually prepared from adult rodent brains" I agree that organotypic brain slices are usually prepared from ebryos, but it is not true that acute slices are mostly from adult. The age of the mice to prepare acute brain slices is dependent on the research question (developmental versus adult). Rephrase or delete the sentence.

In our revised manuscript, we deleted the complete section discussing organotypic slices as was suggested by another reviewer and with it also the statement addressed by the reviewer in this comment.

112: optimal slice quality is highly dependent on ideal experimental conditions, including the age of the animal, the method of euthanasia, the speed of dissection and slicing, the slicing solutions and parameters (e.g. slicing speed) as well as the conditions for slice recovery --> Although not specifically mentioned as a topic of this work, trans-cardiac perfusion of the animal with ice cold PBS before the dissection, is in some cases essential (especially for old rats) to produce high quality brain slices. This might be mentioned somewhere is the manuscript.

We thank the referee for this comment and have included this additional information in the revised manuscript (lines 614 - 615).

166: If older animals are used, slice and recovery solutions may have to be adapted accordingly4 (e.g. NMDG+-based solutions) in order to preserve the brain health of the acute slices. --> in the same line of the previous comment, it might be worthwhile to add a reference for perfusing animals and for adding additional Mg2+ to block NMDA receptors and excitotoxicity).

We added a paragraph in the discussion where we mention several measures that could be taken for slice health preservation. Appropriate references are provided in the same paragraph (lines 603 - 615).

3.11 Use a filter paper strap to pick up one hemisphere with diffusional forces, thereby not damaging the tissue. --> it is not clear to me how this step prepares for the next steps, because, if I didn't' miss it, it is not written here which side should be facing the bottom (=glue) on the specimen plate. In fact, this only becomes clear in point 4.1.

We thank the reviewer for this comment. We revised this part in step 3.1.1 and 3.1.2 in order to make the orientation better comprehensible.

The left part of figure 2D shows this orientation but the right part of Fig 2D is a top view of the positioning, which does not fit the orientation of the left part of the figure. Use a separate panel number for both figures or any other way to make this more clear at first sight.
In addition, please mention why the dorsal side should be 'dried' in the next step (probably to glue it on the specimen plate?).

We adapted the Figure 2 and Figure 2 legend accordingly.

230 Important: the entire dissection procedure should be performed as fast as possible ◊ indeed a critical step, so please indicate a maximal time window in which the brains should be dissected and submersion in the slush. (Max 5 minutes, but can be faster?)

We added a time frame to the protocol in order to guarantee slice quality (lines 258 - 260).

Results:
Indicate the pipette resistance used for field potential measurements.

The pipette resistance of ~ 2 MΩ was included in the revised version of the manuscript (step 6.1 and line 416).

e.g. line 276: Is a solution heater used in line with the gravity controlled perfusion system? Or another heating system? Is this not essential for normal synaptic facilitation?

We thank the reviewer for this critical question. We did not use a solution heater in line with the gravity-controlled system, as it is practically very challenging to accomplish a **constant heating** of all tubes of a **multi-barrel system**. However, this system allows us to perfuse several different drugs during one slice recording. Although we agree that it would be more physiological to heat the slices during the recordings, it is clearly not essential, as we are able to obtain LTP in several hippocampal fiber pathways even at RT. The temperature might be seen as a limitation of our specific recordings that are represented here, but as this protocol is focusing on describing the brain slice procedure rather than the implementations thereafter, and simply shows the fEPSP recordings as an example result, we don't consider this comment as relevant to address for the here submitted manuscript.

Figure 3: terms 'positive and negative' examples are weird. Is 'example of a good quality slice' and 'example of a bad quality slice' not better?

We did adapt the Figure legend accordingly.

Reviewer #3:
Manuscript Summary:
This paper describes a protocol for obtaining rodent hippocampal brain slices that preserve the integrity of the perforant path - hippocampus and mossy fibers - hippocampal CA3 circuitry for use in electrophysiology and fluorometry experiments (and other suggested secondary applications). The protocol is written in a clear and objective language and described in sufficient detail for an independent user to follow it and obtain good quality slices for the described applications. There are however some details that need reviewing/clarification and are listed below.

Concerns:
1) First of all, although the manuscript is written in a clear/objective language, the use of English and phrasing needs reviewing in several instances as there is a continuous mixing of verb tenses (past, present, future, infinitive) throughout. For example, in lines 63-65. "A dense network of informational flows in the form of fiber pathways is accomplishing a tight hippocampal connection to internal and external brain structures" (should read, for example, "(…) which accomplishes"). Another example, in lines 82-84: "Second, the circumvention of the blood-brain-barrier (BBB) and the wash-out of endogenously released molecules before the start of the experiment are making it possible to study the effect of compounds and drugs with relatively precise dosage control." (should read, for example "make it possible"). These are just two examples from the introduction, but several other similar issues occur throughout the manuscript, especially in the introduction and discussion. I would advise the authors to review the use of English thoroughly.

We thank the reviewer for this constructive criticism. We carefully reviewed the use of English grammar in our manuscript.

2) Regarding the introduction, the paragraph on the comparison between brain slices and organotypic cultures seems unnecessary and not pertaining to the objective of the paper. I agree that some of advantages listed on that paragraph are important advantages of brain slices, but could be included on the second paragraph of the introduction, where other advantages of brain slices are listed/discussed.

We do agree with this reviewer's comment and adapted the Introduction accordingly. The paragraph of organotypic slice cultures was erased and general advantages of brain slices were

integrated in the paragraph before (lines 76 - 96).

3) On line 105/106, it reads "which results in higher amplitude signals obtained by extracellular recordings, thereby decreasing the signal to noise ratio". It should be "increasing the signal to noise ratio".

We thank the reviewer for his/her attentive reading of the manuscript. We adapted this statement (now in line 619).

Regarding the protocol itself:
4) On 1.1.2 (line 140) and 1.2.2 (line148) the target osmolarity of the solutions are described as 205-215 and 220-230 mOsm respectively. However, osmolarity for brain slices' solutions is usually described at 300 mOsm and solutions are typically expected to be in the range of 300 +/-10 mOsm. Can you please double-check your osmolarity values?

We thank the reviewer for noticing that rather dramatic mistake. This was a typo in our previous manuscript version. We adapted the osmolarities to 305-325 mOsm.

5) On 1.2.1 (line 146), the instruction starts with "warm up (…) to RT". This phrasing seems to imply that the solution should be actively warmed up. Could the sentence be rephrased? For example "Remove from 4º C and allow solution to reach RT".

This comment does not apply anymore to our revised manuscript version, as we changed the timeline of the preparation. We now propose to prepare ACSF fresh at the day of experimentation (step 1.2.1).

6) Also on 1.2.1 (line 146) it is stated that the pH of the carbogenated solution should be 7.3 to 7.4. Does this mean that the pH solution is not measured after preparation and before carbogenation? Considering that the pH drops approx. 0.1 in the carbogenated solution, it is usual to measure the pH before carbogenation, considering the pH drop (i.e. setting the pH before carbogenation to 7.4-7.5). This facilitates adjusting the pH to the correct range, when necessary.

We do agree that an extra pH check directly after solution preparation can be performed in order to notice potential mistakes that occurred during the preparation of the solution. However, it seems to us that the pH check is of actual relevance before the start of the experiments in order to guarantee the proper conditions. By thoroughly following the here described protocol the pH should be in a good range before the carbogenation. Therefore, a prior pH check can be useful, but is not necessarily required. We added the expected pH before carbogenation to steps 1.2.1 and 1.2.3 to provide the reader with this extra information.

7) In line with previous comment, the protocol should also describe how to proceed to adjust the pH when it is not at the target value.

We inserted a note in steps 1.2.2 and 1.2.4, where potentially necessary pH adjustments are discussed.

8) Still regarding the pH, the protocol does not describe what is the target pH level of the cutting solution (and if it is measured at all) - this pH should also be checked and adjusted.

We thank the reviewer for his/her attentive reading. Indeed, we missed to report the pH check for the slicing solution. We included this in step 1.2.4.

9) Regarding tables 1 and 3, pertaining to protocol steps 1.1.1 and 1.2.2, the authors should review the listed concentrations. In table one, since it's a 10x solution, shouldn't the concentration be 10x higher than listed (for example, KCl 25 mM instead of KCL 2.5mM), since it will be diluted in aprox. 1:10 in step 1.2.2, for a final concentration of 2.5 mM?

We thank the reviewer for that comment. We adapted the concentrations in Table 1 accordingly in order to facilitate the protocol.

10) Still regarding tables 1 and 3, to facilitate reproducibility, the exact molecular weight of the reagents used should be listed (as they can vary according to each manufacturers' specific formulation). The weighed mass of each reagent for each of the solutions should also be listed.

The exact molecular weight of the products used and the weighed amounts were added to Table 1-3.

11) On 1.2.2 (line 148), the use of a plastic beaker is suggested. Glass labware should always be preferred to plastic labware whenever possible.

The reviewer is right with his/her comment. Therefore, we erased the plastic out of the revised manuscript (step 1.2.3). We do usually use a plastic beaker because of the storage in the -80 freezer which bares the risk to break the glass. As this is not a crucial point of the protocol, we leave it open for the experimenter to decide which beaker to use.

12) On 3.6 (line 203) the use of a fine brush to clean the extracted brain is recommended. I would advise against this method of cleaning as it can cause inadvertent damage. The brain can be cleaned by gently rinsing it with aCSF poured by a pipette.

We thank the reviewer for this helpful tip. The manuscript was adapted accordingly.

13) On 3.9 (line 209), the instruction is confusing. I understand that it is hard to explain the cut through words, but could the authors try to make this instruction clearer?

We tried to express the orientation of the cut in more clear words in step 3.9.

14) Regarding the recovery/holding chamber (step 5), the chamber is listed on the table of materials as "self-made" and the materials need to build one are listed on the legend of Figure 2. However, can you provide a reference for a commercial alternative? (for example, the one listed on the manuscript's reference 23).

In the revised manuscript version, we do point out the commercial availability of recovery chambers (step 5.1), but do not name any because of the JoVE journal restrictions that don’t allow to use commercial language.

15) Regarding the discussion: I missed some words on the comparison of this protocol for the preparation of brain slices with other brain slices' protocols currently on JOVE. Although novelty is not an objective of a protocol/methods paper, it should be discussed in comparison with other available methods (especially on the same journal/platform).

We included a short paragraph in the discussion of the revised manuscript, where we put our manuscript in perspective to other reported slice protocols (lines 569 - 581).

Reviewer #4:

Manuscript Summary:

The manuscript by Van Hoeymissen et al. describes a method to obtain horizontal slices of live, healthy murine hippocampus that preserves performant path connections for use in electrophysiology, calcium imaging or any number of secondary experiments. The authors' goal is to present the protocol clearly and in detail to ensure consistent production of healthy slices. The authors give examples of electrophysiological markers to assess slice health and present examples of calcium imaging and electrophysiology experiments. This is a well written manuscript, but has several shortcomings that limit its usefulness over other protocols.

Major Concerns:

1. There is no discussion of the reasons why the proposed protocol results in healthy slices compared to other protocols, nor why horizontal slices are necessary to obtain perforant path synapses.

We never intended to claim that our protocol is superior in providing healthy brain slices in comparison to other protocols. We simply want to communicate a detailed slice protocol that yields a high success rate in our hands. The major advantage that we see in using that protocol is indeed the preservation of the perforant path next to all other hippocampal pathways, which creates the opportunity to study multiple circuits by use of a single slicing technique and even allows multiple-circuit studies within one slice. We explain the advantages and differences to other slice protocols in some more detail in our revised manuscript (lines 569 - 601).

2. No data is provided for quantifying the purported success rates of obtaining healthy slices; therefore, it is impossible to determine if this method is consistent and should be followed. This should also identify which steps are absolutely critical to the health of the slice.

We thank the reviewer for his/her comment. We inserted a new paragraph (lines 603 - 615) discussing the critical steps for healthy slice preparations and state the success rate obtained in our lab. Further, we also point out that other measures might be necessary in order to obtain high quality slices in different test conditions.

3. One of the stated goals is give precise details that are lacking in other descriptions of slicing so that there is no question on how to do this. However, the authors remain vague at critical points. For example, at the end of the dissection and slicing description on lines 230 and 250 it is noted that it is important to work fast. This is one of the most critical aspects of preparing healthy slices! The authors should be more explicit. At what points do you need to be fast? How fast? Right from the start, the mouse needs to be decapitated and brain removed and placed in the sucrose-acsf slush within 30-45 seconds.

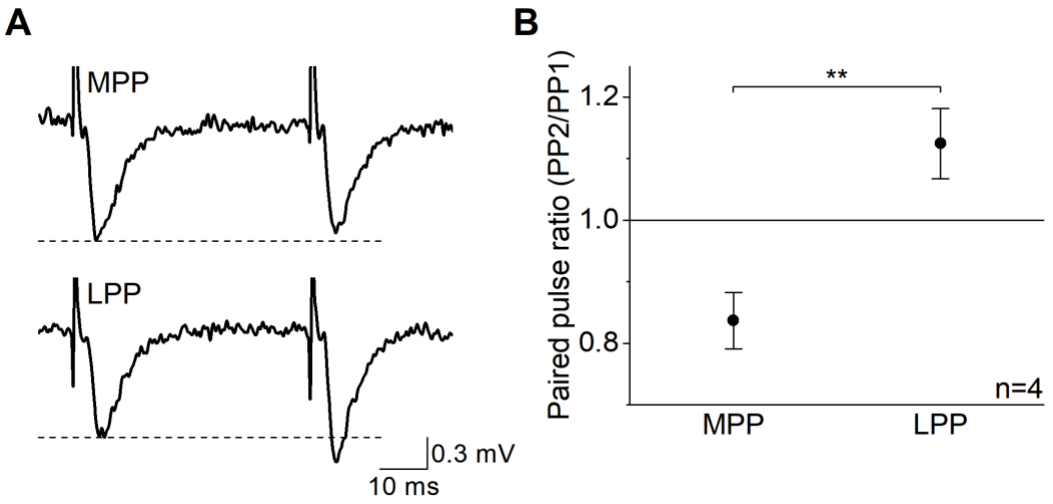
We thank the reviewer for noticing this inaccuracy. We added a time frame to the protocol in order to guarantee slice health (lines 256 - 260).

4. Experimental example: The authors example of an input-output curve isn't the best, stimulation intensities start high (almost at the 50% intensity) and the error bars suggest wide variability between slices. One should be provided that has a wider span of intensities and fit with a Boltzmann equation and calculate V50, not dose-response function.

We do agree with the reviewer's comment and provide better quality Input-Output curves in the revised manuscript (Figures 3D-F), now showing a wider range of stimulation intensities. We removed the dose-response function fit.

How do the authors know they are recording from MPP? To assess the health of the slice/synapse the authors also need to test paired pulse ratio. The MPP to DG synapse should have paired pulse depression whereas the LPP to DG synapse should exhibit facilitation.

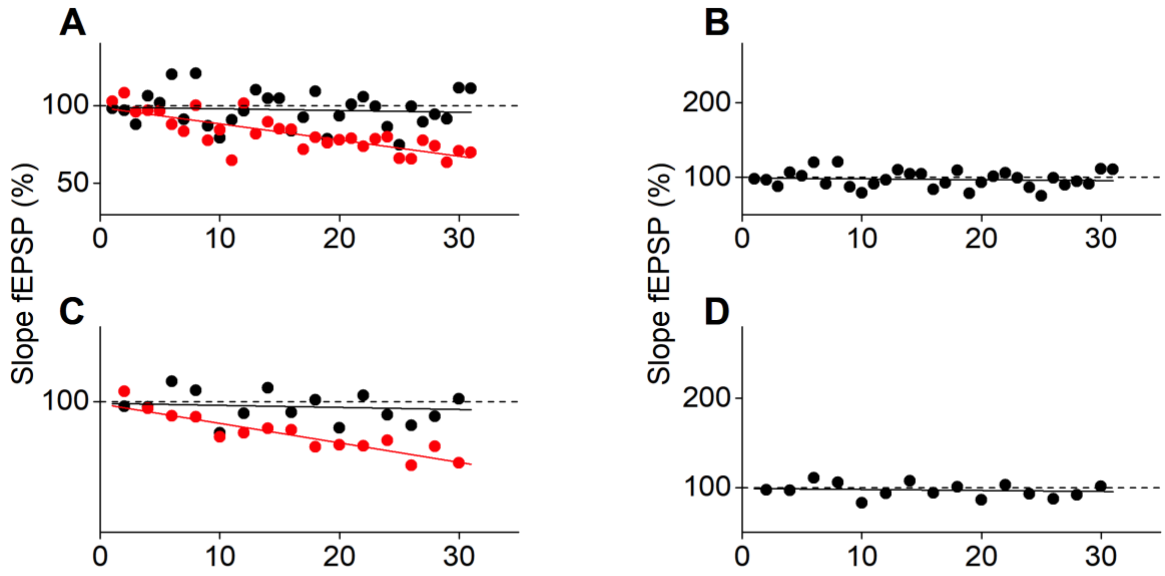
We do not fully agree with the reviewer statement that a paired-pulse protocol would give us any additional information concerning the **slice health**. The reviewer is absolutely right that a paired-pulse protocol can be useful in order to verify the proper electrode positioning, differentiating the MPP from the LPP. Indeed, we occasionally do use this protocol in our recordings in order to verify the right pathway. See below the results from paired-pulse recordings in MPP and LPP within the same slice: A) Paired-pulse traces (with 50 ms ISI) recorded in the MPP and LPP of four individual slices, B) Statistics of the paired-pulse ratios showing PPD for recordings in the MPP and PPF for recordings in the LPP. We could monitor PPD in 100% of cases when recording in the MPP (a total of n=10). However, it should be also noted that several manuscripts report already that PPD is not always restricted to MPP and PPF not to LPP and even more useful test protocols were brought forward (Petersen et al., Neuroscience, 2013). Therefore, the best way to guarantee the investigation of the right perforant path is the careful positioning of small diameter electrodes. As the scope of this manuscript was not to provide a protocol for MPP recordings but solely focuses on the brain slice preparation procedure itself, we do prefer to not show and discuss any such recordings in the here presented manuscript. In order to anyway address the reviewer's comment, we did insert a few sentences pointing out the importance of a proper control for MPP recordings (lines 422 -426).



Also, be precise on the definitions of a healthy slice: How small is too small for the fEPSP to fiber volley ratio? How small is too small for the fEPSP slope/amplitude? How much variation in the fEPSP is acceptable/unacceptable? The example in 3E is quite variable.

We thank the reviewer for this notification. We inserted some specifications in the results part (lines 466 - 469). This also includes a cut-off baseline alteration value that will hopefully clarify why we consider the presented baseline to be stable. We do agree with the reviewer that the baseline example in Figure 3E shows some variations. Variations are in our hands not untypical and may be caused for example by perfusion level differences. Nevertheless, we consider the recording shown in 3E stable, because the overall change of the baseline is below 5% and therefore in an acceptable

range. The variations may also just seem more pronounced because of the representation of a single example recording which yields more variations than seen when the mean of several recordings is presented. Furthermore, the scale of the graphs might make the data look more variable. Find below a Figure showing the same data as presented in the manuscript (Panel A) compared to the same data presented on a broader Y-axis range (Panel B) that would be typically applied for LTP recordings. Furthermore, Panels C and D show the same recording represented with mean fEPSP values of every 2 min instead of every 1 min. The Y-axes are similar as seen in Panels A and B above. These representations show an acceptable stability of the baseline recording.



Minor Concerns:

1. The sentence starting on line 63 and ending on line 65 does not make sense.
2. The sentence starting on line 103 indicates that several reasons will be presented to illustrate the importance of hippocampal slices. The following sentences only provide two reasons. Two is not several.
3. Protocol, line 141 and 150. The osmolarities are quite low. What type of osmometer should be used?
4. Protocol, The protocol lacks a description to prevent calcium phosphate from precipitating out of solution.
5. Protocol, line 137. The 10x presolution does not appear to be 10x.
6. line 196. some IACUCs require a small animal guillotine rather than scissors to decapitate.
7. The description of the dissection and orientation of the brain and Figure 2c-f is not clear. It almost looks like these are saggital sections. Why do the authors not block the brain, cutting off the prefrontal cortex and cerebellum? Are the hemisopheres glued dorsal side down or ventral side down?

All minor comments above were addressed accordingly in the revised manuscript version.

8. Describe why stimulation intensity is set at 50%.

This explanation is provided in the Results section (lines 444 - 447).

9. Describe why it is difficult to induce LTP at the MPP synapse.

We inserted a short explanation why LTP induction in the MPP is difficult to achieve (lines 458 - 461).