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TITLE:

Investigating Long-term Synaptic Plasticity in Interlamellar Hippocampus CA1 by Electrophysiological Field Recording

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KEYWORDS:

Hippocampus, interlamellar CA1 hippocampus, long-term synaptic plasticity, in vitro extracellular field recording, in vivo extracellular field recording, CA1 pyramidal neuron, schizophrenia.

SUMMARY:

We used recording and stimulation electrodes in longitudinal hippocampal brain slices and longitudinally positioned recording and stimulation electrodes in the dorsal hippocampus in vivo to evoke extracellular postsynaptic potentials and demonstrate long-term synaptic plasticity along the longitudinal interlamellar CA1.

ABSTRACT:

The study of synaptic plasticity in the hippocampus has focused on the use of the CA3-CA1 lamellar network. Less attention has been given to the longitudinal interlamellar CA1-CA1 network. Recently however, an associational connection between CA1-CA1 pyramidal neurons has been shown. Therefore, there is the need to investigate whether the longitudinal interlamellar CA1-CA1 network of the hippocampus supports synaptic plasticity.

We designed a protocol to investigate the presence or absence of long-term synaptic plasticity in the interlamellar hippocampal CA1 network using electrophysiological field recordings *both* in vivo and in vitro. For in vivo extracellular field recordings, the recording and stimulation electrodes were placed in a septal-temporal axis of the dorsal hippocampus at a longitudinal angle, to evoke field excitatory postsynaptic potentials. For in vitro extracellular field recordings, hippocampal longitudinal slices were cut parallel to the septal-temporal plane. Recording and stimulation electrodes were placed in the stratum oriens (S.O) and the stratum radiatum (S.R) of the hippocampus along the longitudinal axis. This enabled us to investigate the directional and layer specificity of evoked excitatory postsynaptic potentials. Already established protocols were used to induce long-term potentiation (LTP) and long-term depression (LTD) both in vivo and in vitro. Our results demonstrated that the longitudinal interlamellar CA1 network supports N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) with no directional or layer specificity. The interlamellar network, however, in contrast to the transverse lamellar network, did not present with any significant long-term depression (LTD).

INTRODUCTION:

The hippocampus has been widely used in cognitive studies[1-3]. The hippocampal lamellar network in the transverse axis forms the tri-synaptic circuitry that is made up of the dentate gyrus, CA3, and CA1 regions. The lamellar network is considered to be a parallel and independent unit[4,5]. This lamellar viewpoint has influenced the use of transverse orientation and transverse slices for both in vivo and in vitro electrophysiological studies of the hippocampus. In light of emerging research, the lamellar hypothesis is being reevaluated[6] and attention is also being given to the interlamellar network of the hippocampus. With regards to the hippocampal interlamellar network, the CA3 region has long been investigated[7-10], however the longitudinal CA1 hippocampal region has received relatively little attention until recently. With regards to the CA1 interlamellar network, the short-term synaptic properties along the dorsoventral longitudinal hippocampal CA1 axis of rats have been shown to vary[11]. Also, clusters of hippocampal cells responding to the phase and the place were found to be arranged systematically along the longitudinal axis of the hippocampus in rats, undergoing a short term memory task[12]. Also, epileptic seizure activities were found to be synchronized along the whole hippocampus along the longitudinal axis[13].

Most studies of the longitudinal CA1 hippocampal region however, have utilized input from the CA3 to the CA1 regions[11,14,15]. Using a unique protocol to make longitudinal brain slices, our previous work demonstrated the associational connectivity of CA1 pyramidal neurons along the longitudinal axis and implicated its ability to process neuronal signaling effectively[16]. However,

there is a need to determine whether the CA1 pyramidal neurons along the longitudinal axis without transverse input can support long term synaptic plasticity. This finding can add another angle into investigations of neurological issues pertaining to the hippocampus.

The ability of neurons to adapt the efficacy of information transfer is known as synaptic plasticity. Synaptic plasticity is implicated as the underlying mechanism for cognitive processes such as learning and memory[17-20]. Long-term synaptic plasticity is demonstrated as either long-term potentiation (LTP), which represents the strengthening of neuronal response, or long-term depression (LTD), which represents the weakening of neuronal response. Long-term synaptic plasticity has been studied in the transverse axis of the hippocampus. However, this is the first study to demonstrate long-term synaptic plasticity in the hippocampal longitudinal axis of CA1 pyramidal neurons.

Building from a protocol used by Yang et al.[16], we designed the protocol to demonstrate LTP and LTD in the hippocampal longitudinal axis of CA1 pyramidal neurons. We used C57BL6 male mice with ages ranging between 5-9 weeks old for in vitro experiments and 6-12 weeks old for in vivo experiments. This detailed article shows how longitudinal hippocampal brain slices from mice were obtained for in vitro recordings and how in vivo recordings were recorded in the longitudinal axis. For in vitro recordings, we investigated directional specificity of longitudinal CA1 synaptic plasticity by targeting the septal and temporal end of the hippocampus. We also investigated layer specificity of the longitudinal CA1 synaptic plasticity by recording from the stratum oriens and stratum radiatum of the hippocampus. For in vivo recordings, we investigated the angles that best correspond to the longitudinal direction of the hippocampus.

Using both in vivo and in vitro extracellular field recordings, we observed that the longitudinally connected CA1 pyramidal neurons presented with LTP, not LTD. The transverse orientation involving both CA3 and CA1 neurons, however, supports both LTP and LTD. The distinction in the synaptic capabilities between the transverse and the longitudinal orientation of the hippocampus could speculatively signify differences in their functional connectivity. Further experiments are needed to decipher the differences in their synaptic capabilities.

PROTOCOL:

All animals were treated in accordance with the guidelines and regulations from the Animal Care and Use of Laboratory of National Institute of Health. All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of City University of Hong Kong and Incheon National University.

1. In vivo field recording

1.1. Animal preparation

1.1.1) Inject urethane (0.06 g per 25 g weight) intraperitoneally to anesthetize mouse. Supplement with intramuscular injection of atropine (0.05 mg/kg). Keep the mouse in a dark

quiet spot until full anesthesia takes effect.

CAUTION: Urethane has carcinogenic potential. Handle it with care and wear protective clothing to avoid contact with exposed skin.

NOTE: Alternatively, isoflurane can be used as anesthesia.

1.1.2) Check for depth of anesthesia intermittently until a full surgical plane of anesthesia takes effect. Check for depth of anesthesia by performing toe pinch, ear pinch, tail pinch and corneal touch tests to observe the response of the mouse to physical stimuli.

NOTE: A reflex or voluntary movement should not be observed when the mouse is at a full surgical plane of anesthesia.

1.1.3) For the toe pinch test, extend either the hind or foreleg of the mouse and pinch firmly with a pair of blunt forceps or fingers. The mouse is not confirmed for full anesthesia if it withdraws the leg or shakes body, has an observable increased respiratory rate or makes vocal sounds.

1.1.4) For the ear pinch test, pinch the ends of the pinna with a pair of blunt forceps or fingers firmly. Full anesthesia is not confirmed if the mouse moves the whiskers forward, shakes its head, makes vocal sounds or has an observable increased respiratory rate.

1.1.5) For the tail pinch test, hold the tail of the mouse gently and firmly pinch with a blunt forcep or finger. No tail movement, vocal sound or observed increase in respiratory rate should be observed when fully anesthetized for surgical procedures.

1.1.6) For the corneal touch test, touch the cornea of the mouse gently, with a cotton wick. No eyelid movement, whisker movement or observable increase in respiratory rate should be observed when fully anesthetized for surgery.

1.1.7) Shave the hair on the neck and skull of the mouse.

1.1.8) Place the fully anesthetized mouse on a heating pad set to 37 °C and insert the rectal temperature probe in the rectum. This enables the heat produced by the heating pad to adjust in response to changes of the mouse's body temperature.

1.1.9) Apply eye gel to moisten the eyes of the mouse.

1.1.10) Pull the tongue out to the side of the lips gently using forceps and fix the two front teeth into the second or third teeth hole of the stereotactic instrument. Fix the skull of the mouse firmly using the eye-clamp.

NOTE: Alternatively, a stereotactic ear clamp can be used.

1.1.11) Observing under a microscope, separate the subcutaneous tissue and muscles at the end of the interparietal and occipital bone of the mouse with a scalpel to expose the cisterna magna. Blot the dura mater dry with a cotton swab.

1.1.12) Gently puncture the cisterna magna by making a shallow cut with a sharp pointed scalpel blade to drain the cerebrospinal fluid (CSF). Attach a cotton swab to keep draining the CSF.

1.2) Craniotomy

1.2.1) Holding the skin on the scalp with forceps, cut and remove the skin with a pair of surgical scissors. Cut enough skin to expose the bregma and lambda marks on the scalp. Keep the exposed region dry.

1.2.2) Adjust the clamped skull of the mouse to enable the bregma and lambda points to be aligned in a horizontal level of similar height. Avoid tilting of the head in either the anterior-posterior position or the medial lateral position.

1.2.3) Mark the points corresponding to the hippocampal region using the aid of a Vernier caliper. Use the mouse brain in a stereotactic coordinate book as a reference to help determine the exact coordinates.

NOTE: The location to mark for incision for the hippocampus are 1 mm x 3 mm on the midline referred to as anterior-posterior (AP) using the bregma as reference point, and 3 mm x 3 mm perpendicular to the midline (ML) to connect the points on the midline. The stereotactic coordinates are given as (AP: 1,3 and ML: 3,3).

1.2.4) Make an incision in the skull above the dorsal region of the hippocampus along the marked points using a scalpel or high-speed drill while observing under a microscope.

NOTE: A rectangular shaped opening with a size of 2 mm x 3 mm should be obtained after incision. Keep the exposed area clean to avoid injury to the brain.

1.2.5) Carefully take out the loose skull with forceps to expose the dura mater and use a syringe or dropper to gently apply physiological saline solution to keep the surface moist.

1.2.6) Remove the dura mater carefully with needle or sharp pointed tip forceps.

NOTE: Be careful not to cause any damage to the brain tissue during craniotomy. This will lead to the swelling of the brain and will affect the results.

1.2.7) Keep the exposed brain tissue moist by applying physiological saline or inert oil using a dropper or syringe.

1.3) In vivo recording

1.3.1) Fix and position the stimulation and recording electrode firmly in the stereotactic holder. Adjust the stereotactic instrument according to the position of the corresponding AP and ML coordinates for the stimulation and recording electrodes above the CA1 dorsal hippocampus.

NOTE: Use the mouse brain in stereotactic coordinates as a guide in locating the coordinates for the dorsal longitudinal CA1 hippocampal region. For example, a stereotactic coordinate for the CA1 hippocampal region will be (AP 1.5, ML 1.0) for the recording electrode and (AP 1.7, ML 1.5) for the stimulation electrode.

1.3.2) Locate the stimulating electrode lateral to the recording electrode in a longitudinal direction.

NOTE: Ensure that both stimulation and recording electrodes are clean before usage. This prevents the introduction of noise when recording.

1.3.3) For recordings, use multichannel electrodes. First, locate the stereotactic coordinates for the CA1 hippocampal region using the first channel. Position the remaining electrodes such that the angle of stimulation and recording electrodes are in the range of 30° to 60° in relation to the midline from bregma point.

NOTE: This angle corresponds to the longitudinal orientation of the CA1 hippocampal region.

1.3.4) As a control, locate the recording electrode above the CA1 region and the stimulation electrode above the CA3 region of the dorsal hippocampus. For example, using the mouse brain in stereotactic coordinates as a guide, a stereotactic coordinate for CA1-CA3 hippocampal region will be (AP 1.8, ML 1.0) for the recording electrode and (AP 1.5, ML 1.5) for the stimulation electrode.

NOTE: This step is an alternative control and should be done in a separate experiment.

1.3.5) Place the reference electrode at a distal part of the exposed brain region or under the skin of the mouse.

1.3.6) Turn on the recording system.

1.3.7) Open the software for recording and data acquisition.

NOTE: Different laboratories have their preferred software for recording and data acquisition.

1.3.8) Observing under a microscope, lower the recording and stimulation electrodes slowly using the micromanipulator until it just touches the surface of the brain. Mark the point as the

zero point to start calculating the accurate depth to the hippocampal region.

NOTE: The micromanipulator can be used to monitor the depth at which the electrode is inserted at any given time. Observe an increase in the impedance just when the electrode touches the brain surface.

1.3.9) Slowly lower the electrodes to the approximate depth corresponding to the chosen stereotactic coordinates for CA1 hippocampus.

NOTE: Use the mouse brain in stereotactic coordinates as a guide to obtain the approximate depth corresponding to the chosen stereotactic coordinates.

1.3.10) Give stimulation (100 µs duration, repeated at 30 s intervals) and adjust the electrode depth in steps of 50 µm or less until a stable evoked field excitatory postsynaptic potential (fEPSP) is observed.

NOTE: A stimulus of 20 µA is usually enough to evoke an observable response.

1.3.11) Ensure that a stable fEPSP has been evoked by varying the stimulus intensity. A notable increase in the slope or amplitude of the fEPSP should be observed with each increased stimulus intensity. This is termed as the input-output curve. Create an input-output (I-O) curve to detect maximum stimulus intensity at which there is no more increase in the slope of the evoked fEPSP (**Figure 6**).

NOTE: Discard data and change electrode position if the fiber volley disappears during experiment.

1.3.12) Use the input-output curve to set the baseline intensity to 40 - 50% of the maximum. Use the corresponding stimulus intensity for baseline recording.

1.3.13) Record the local field potential as a baseline for 20 - 30 min.

1.3.14) Use the same input-output curve to set the stimulus intensity for evoking high frequency stimulation (HFS) or tetanus to 75% of the maximum intensity. Alternatively, when inducing long-term depression, maintain the same stimulus intensity used for recording the baseline when evoking low frequency stimulation (LFS).

1.3.15) Apply a tetanic stimulation of 100 Hz pulses 4 times with a 10 s interval to induce LTP.

NOTE: Use this protocol only when working on a long-term potentiation experiment.

1.3.16) Apply low frequency stimulation of 5 Hz (900 stimuli during 3 min), 1 Hz LFS (900 stimuli during 15 min), or 1 Hz paired-pulse (50 ms paired-pulse interval, 900 pairs of stimuli during 15 min) to induce LTD according to already established protocols.

NOTE: Use these protocols only when working on a long-term depression experiment.

1.3.17) Record the local field potential for 1 h after HFS or LFS, alternatively.

1.3.18) Export data and analyze using the software.

1.3.19) Verify the position of the recording and stimulation electrode by giving a stimulation of 10 µA current for 30 s to lesion the recorded areas. Transcardially perfuse the mouse with 4% paraformaldehyde and harvest the brain for slicing and staining with Cresyl Violet according.

1.3.20) Euthanize mouse by cervical dislocation or injection of lethal anesthetic dosage after experiment.

2. In vitro field recording

2.1) Preparing oxygenated slicing and artificial cerebrospinal fluid (ACSF) solutions

2.1.1) For 2 L of slicing solution, add approximately 1 L of double distilled water in a volumetric flask and stir vigorously on a stirrer plate.

2.1.2) Add the following slicing solution components (in mM): 87.0 NaCl, 2.5 KCl, 1.3 NaH_2PO_4, 25.0 $NaHCO_3$, 25.0 glucose, 75.0 sucrose, 7.0 $MgCl_2.6H_2O$ and 0.5 $CaCl_2{\cdot}2H_2O$ (**Table 1**).

NOTE: Alternatively, $MgCl_2{\cdot}6H_2O$ and $CaCl_2{\cdot}2H_2O$ can be excluded in this stage and added later in a ready to use volume.

2.1.3) Top up to 2 L with double distilled water while stirring vigorously.

2.1.4) For 2 L of ACSF, add approximately 1 L of double distilled water in a volumetric flask and stir vigorously on a stirrer plate.

2.1.5) Add the following ACSF solution components (in mM): 125.0 NaCl, 2.5 KCl, 1.3 NaH_2PO_4, 25.0 $NaHCO_3$, 25 glucose, 1.0 $MgCl_2{\cdot}6H_2O$ and 2.0 $CaCl_2{\cdot}2H_2O$ (**Table 1**).

NOTE: Alternatively, $MgCl_2.6H_2O$ and $CaCl_2{\cdot}2H_2O$ can be excluded in this stage and added later in a ready to use volume.

2.1.6) Top up to 2 L with double distilled water while stirring vigorously.

2.2) Setup and brain slicing

2.2.1) Pour 400 mL of already prepared slicing solution into a separate flask and oxygenate (95% O_2/5% CO_2) for approximately 20 min.

2.2.2) Store the rest of the 1.6 L solution in a 4 °C refrigerator. Keep solution up to 1 week after which it must be discarded if not used to avoid fungal growth.

2.2.3) Pour 200 mL of the oxygenated slicing solution in the flask, cover with parafilm and transfer to a -80 °C freezer for approximately 20 min to make a slush.

2.2.4) Pour the remaining 200 mL of slicing solution in a brain slice holding chamber and keep in a 32 °C water bath with continuous bubbling.

2.2.5) Prepare the bench for slicing. Place paper towels down and surgical tools on top of bench. Arrange the surgical tools in order of use to facilitate a fast and efficient process (**Figure 7**).

2.2.6) Take out the chilled slicing solution from the freezer and pour approximately 50 mL in a beaker. Pour approximately 10 mL in a Petri dish containing filter paper to moisten it. Place them on ice by the dissection area.

2.2.7) Pour the rest of the slushed slicing solution into the slicing chamber and fix it on the vibratome.

2.2.8) Anesthetize the mouse with isoflurane using guidelines and regulations on Animal Care and use of laboratory of National Institute of Health, and the approved methods of Institutional Animal Use and Care Committee of City University of Hong Kong and Incheon National University.

2.2.9) Decapitate the anesthetized mouse with a pair of scissors and place the head on tissue paper. Cut the skin covering the skull of the mouse and cut through the cutaneous muscles with scissors. Cut the skull plates along the midline to the occipital bone using surgical scissors.

2.2.10) Open the skull with blunt forceps to expose the brain. Gently scoop out the brain with a spatula and place in the chilled slicing solution in the beaker. Wait about 30 s.

2.2.11) Take out the brain using the spoon and carefully place it on the previously moistened filter paper in the Petri dish.

2.2.12) Separate the two brain hemispheres along the midline with a scalpel blade. Isolate the hippocampus by gently detaching it from the cortex with a spatula and place it carefully on the moistened filter paper. Cut out the septal and temporal end of the isolated hippocampus using the scalpel.

2.2.13) Pick up the isolated hippocampus using a blunt spatula and brush. Gently dab the spatula on a tissue paper to remove excess water.

2.2.14) Apply a small amount of glue to the slicing plate of the vibratome.

2.2.15) For a longitudinal CA1 hippocampal slice, attach the CA3 region of the hippocampus to the slicing plate with the glue. Quickly but carefully place it in the slicing chamber of the vibratome containing the chilled oxygenated slicing solution.

2.2.16) For the transverse slice that will serve as a control, attach the ventral end of the hippocampus to the slicing plate of the vibratome. Quickly but carefully place it in the slicing chamber containing the chilled oxygenated dissection solution.

NOTE: This step is an alternative to the step above and should be performed separately.

2.2.17) Position the blade angle to 90°. Set the vibratome parameters to a speed of 0.05 mm/s, an amplitude of 1.20 mm and a slice thickness of 400 µm.

2.2.18) Slice the attached hippocampus with the vibratome.

NOTE: A good longitudinal CA1 hippocampal brain slice will have one layer of CA1 and 2 layers of dentate gyrus. A maximum of 2 good hippocampal slices can be obtained. Alternatively, a transverse hippocampal brain slice will have the dentate gyrus, CA3 and CA1 regions intact.

2.2.19) Transfer the longitudinal hippocampal brain slices from the vibratome with a pipette and incubate it in the brain slice holding chamber in the water bath.

2.2.20) Incubate slices in the water bath for 20 min at a temperature of 32 °C and bring out to room temperature for 30 min of recovery.

NOTE: Alternatively, transfer the brain slice when out of the water bath and gently place it in the recording chamber with flowing oxygenated ACSF at a temperature of 32 °C for 30 min of recovery.

2.2.21) While incubating in the water bath, pour 400 mL of ACSF solution into a separate flask and oxygenate (95% O_2/5% CO_2) for approximately 20 - 30 min before transferring the brain slice into the recording chamber. Continuously superfuse the ACSF into the recording chamber at a speed of 2 mL/min.

2.2.22) Turn on the temperature controller to heat the flowing ACSF to 32 °C.

2.2.23) Store the rest of the 1.6 L solution in a 4 °C refrigerator. Keep the solution up to one week after which it must be discarded if not used to avoid fungal growth.

2.3) In vitro recording

2.3.1) Set up a recording rig by turning on all needed hardware.

2.3.2) Transfer the brain slice from the slice holding chamber into the recording chamber. Adjust the position of the brain slice with blunt forceps and hold it in place with a harp. Allow the ACSF to run for at least 20 min. This enables the brain slice to be stable before recording.

2.3.3) Fill the recording pipette with ACSF as an internal solution.

2.3.4) Check the recording pipette resistance with the designated software. The recording pipette resistance should be within the range of 3-5 MΩ.

2.3.5) Turn on the software for data acquisition. This software should have similar features that enable data acquisition in a similar way as the in vivo recordings shown earlier.

2.3.6) Fix the recording and the stimulation electrodes firmly in the stereotactic instrument holder. Place the reference electrode in the ACSF in the recording chamber.

2.3.7) For longitudinal slices, position the stimulation and recording electrode in the stratum oriens (S.O.). Keep the distance between electrodes to about 300 to 500 µm. Place the stimulation electrode on either the septal or temporal side of the brain slice and record from the same layer (**Figure 8**).

2.3.8) Alternatively, position the stimulation and recording electrode in the stratum radiatum (S.R.). Place the stimulation electrode on either the septal or temporal side of the brain slice.

2.3.9) For transverse slices as a control, position the stimulating electrode on the CA3 (Schaffer collateral pathway) region, and the recording electrode on the CA1 region (**Figure 9**).

NOTE: This is an alternative control step and should be performed in a separate experiment.

2.3.10) Turn on the isolated stimulus generator and give stimulation (100 µs duration, repeated at 30 s intervals). Adjust the recording electrode depth and/or position till a stable evoked excitatory postsynaptic field potential is observed.

2.3.11) Ensure that a stable fEPSP has been evoked by varying the stimulus intensity. A notable change in the slope of the fEPSP should be observed with each change of stimulus intensity. This is termed as the input-output curve. Create an input-output (I-O) curve to detect maximum stimulus intensity at which there is no more increase in the slope of the FEPSP (**Figure 6**).

NOTE: Discard data and change electrode position if the fiber volley disappears during experiment.

2.3.12) Use the input-output curve to set the stimulus intensity for baseline recording and for evoking high frequency stimulation (HFS) or tetanus to 30-40% of the maximum evoked fEPSP.

2.3.13) Alternatively, for experiments regarding LTD, use the input-output curve to set the baseline intensity for baseline recording and for evoking low frequency stimulation (LFS) to 70% of the maximum evoked fEPSP.

2.3.14) Record the local field potential as the baseline for 20 - 30 min.

2.3.15) Apply HFS of 100 Hz pulses twice with a 30 s interval to induce LTP.

2.3.16) Alternatively, for LTD experiments, apply low frequency stimulation of 5 Hz (900 stimuli during 3 min) or 1 Hz LFS (900 stimuli during 15 min) or 1 Hz paired-pulse (50 ms paired-pulse interval, 900 pairs of stimuli during 15 min) to induce LTD according to already established protocol.

2.3.15 Record the local field potential for 1 h after HFS or LFS.

2.3.16 Export data and analyze.

REPRESENTATIVE RESULTS:

We explored long-term synaptic plasticity of longitudinal CA1 pyramidal neurons of the hippocampus using extracellular field recordings both in vivo and in vitro. LTP and LTD are facets of long-term synaptic plasticity that have been demonstrated in the transverse axis of the hippocampus to be unidirectional.

We showed here that using longitudinal hippocampal brain slices, there is LTP in the CA1 longitudinal axis of the hippocampus. We prepared longitudinal slices of the hippocampus along the septotemporal axis, which is perpendicular to the transverse slices (**Figure 1**). Using recordings from the CA1 region of the hippocampus, we showed the presence of LTP that was not direction specific. There were no statistically significant differences in the recordings from the septal or temporal (**Figure 2**) side of the longitudinal hippocampal brain slice. We also showed the presence of LTP that was not layer specific; thus, recordings from both stratum radiatum and stratum oriens (**Figure 2**) showed successfully induced LTP in the longitudinal brain slice. We used D-AP5, an NMDAR antagonist to demonstrate that the LTP induced was dependent on NMDA receptors (**Figure 3**). What happens in vitro does not necessarily reflect in vivo conditions, so we investigated LTP in vivo. **Figure 4a** shows a schematic diagram of the stimulation and recording electrode positioned in the dorsal hippocampus along the longitudinal axis of CA1 region in vivo. The position of the electrodes used for the recording and stimulation was verified by lesion marks and crystal violet staining (**Figure 4a**). We demonstrated the presence of LTP in vivo in the longitudinal CA1 region (**Figure 4b**).

Using already established protocols for inducing LTD, we failed to successfully induce LTD both in vivo and in vitro (**Figure 5**).

Figure Legends:

Figure 1. A schematic drawing of transverse and longitudinal hippocampal brain slices. This figure is adapted and modified from Sun et al. 2018[21].

Figure 2. LTP in longitudinal slices. Synaptic responses at S.R. (**a**) or S.O. (**b**) in longitudinal slices are potentiated right after tetanus stimulation with both temporal and septal inputs (S.R./temporal (n = 12, c), S.R./septal (n = 12, c), S.O./temporal (n = 10, d), S.O./septal (n = 9, d). The n stands for the number of slices. Error bars represent SE. This figure is adapted and modified from Sun et al. 2018[21].

Figure 3. NMDAR-dependent LTP in longitudinal slices. (**a**,**b**) LTP induction in temporal and septal direction is blocked by 50 μM D-AP5 (temporal, n = 6, a) (septal, n = 5, b). (**c**,**d**) LTP induction in temporal and septal direction is also blocked by D-AP5. The n stands for slices. Error bars represent SE. This figure is adapted and modified from Sun et al. 2018[21].

Figure 4. In vivo LTP in the interlamellar network. (**a**) A schematic drawing of recording and stimulation electrodes in anesthetized animals. The loci of recording (on the septal side of CA1) and stimulating electrodes (on the temporal side of CA1) were identified by lesion marks. (**b**) LTP is induced in the interlamellar connection by 100 Hz high frequency stimulation (HFS) (n = 10 mice). Color traces: before (black) and after (red) HFS. Error bars represent SE. This figure is adapted and modified from Sun et al. 2018[21].

Figure 5. Absence of in vivo and in vitro LTD in Interlamellar CA1 network. (**a**) 1 Hz-pp LFS does not induce in vivo LTD. (**b**) 1 Hz pp-LTP, (**c**) 5 Hz LFS, and (**d**) 1 Hz LFS do not produce LTD on either the temporal or septal sides of longitudinal brain slice. while LTD is induced by 1 Hz pp-LFS in transverse slices: temporal (n = 8), septal (n = 11) and transverse (n = 6) with 1 Hz pp-LFS; temporal (n = 3) and septal (n = 3) with 5 Hz LFS; temporal (n = 3) and septal (n = 3) with 1 Hz LFS. The n stands for slices. Error bars represent SE. This figure is adapted and modified from Sun et al. 2018[21].

Figure 6. **Input-output curve presenting fEPSP slope in response to increasing stimulus input in hippocampal brain slice.**

Figure 7. Surgical tools used for hippocampal isolation during in vitro brain slicing.

Figure 8. A longitudinal brain slice ready for recording. Stimulation electrode and recording pipette are inserted in the stratum radiatum.

Figure 9. **A transverse hippocampal brain slice ready for recording.** Stimulation electrode is inserted at Schaffer collateral CA3 region and recording pippette is inserted at CA1 region.

Table 1: Concentrations of compounds in brain slice and artificial cerebrospinal fluid solutions.

DISCUSSION:

The protocol demonstrates the method to induce long-term synaptic plasticity in vivo as well as from brain slices in the longitudinal CA1-CA1 axis of the hippocampus in vitro. The steps outlined give enough details for an experimenter to investigate LTP and LTD in a longitudinal hippocampal CA1-CA1 connection. Practice is needed to hone the skills required to successfully record field excitatory potentials.

In addition to needing practice, there are several critical steps that are essential to obtaining good results. First, it was shown previously that the angle to which the brain slices were made could either truncate or preserve the longitudinal projections of the pyramidal neurons in the CA1-CA1 region of the hippocampus[16]. The longitudinal pyramidal neurons project from the transverse neurons at an angle that is nearly perpendicular. As the CA1 neurons propagate in diverse angles within the hippocampus, the longitudinal connection between them lays out along the dorsoventral axis of the hippocampus. Thus, for in vitro recording, the experimenter must keep this in mind to accurately target the CA1-CA1 hippocampal neurons along the longitudinal axis by cutting the isolated hippocampus tissue along the dorsal-ventral axis. Also, for in vivo recordings, the angle at which the stimulation and the recording electrodes are positioned determines whether the results obtained are representative of the longitudinal axis or a mixture of both the transverse and longitudinal axis. Further investigations utilizing CRISPR-Cas9 can be done to confirm whether the evoked response is solely from the CA1 region since it could be a mixture of responses from both the CA1 and the CA3 regions.

Secondly, for in vitro experiments, the experimenter must ensure that the brain slicing solution, ACSF, work bench and all equipment or instruments that come in contact with the brain slice are free from contaminants. Any form of contamination will lead to the deterioration of the integrity or death of the brain slice. Maintaining a clean electrode surface will ensure good and stable recordings for both in vitro and in vivo experiments.

We have shown that the longitudinal hippocampal CA1 network exhibits NMDAR-dependent LTPs, but not LTDs. The trisynaptic circuit, however, presents with both LTP and LTD[22,23]. This implies that the longitudinal CA1 network and the tri-synaptic circuitry have unique features. Our protocol makes use of only electrophysiological recordings and therefore is limited in finding the difference between these two networks.

The search for a cure for brain diseases such as schizophrenia continues. Decline or deformity of CA1 hippocampal subregions have been linked with some schizophrenic symptoms[24,25]. The application of our protocol, though basic, has brought to light a unique synaptic capability of the CA1 longitudinal hippocampal subregion. This knowledge is useful in designing experiments that can further investigate this debilitating brain disease along the longitudinal CA1 axis of the hippocampus.

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DISCLOSURES:

We have nothing to disclose.

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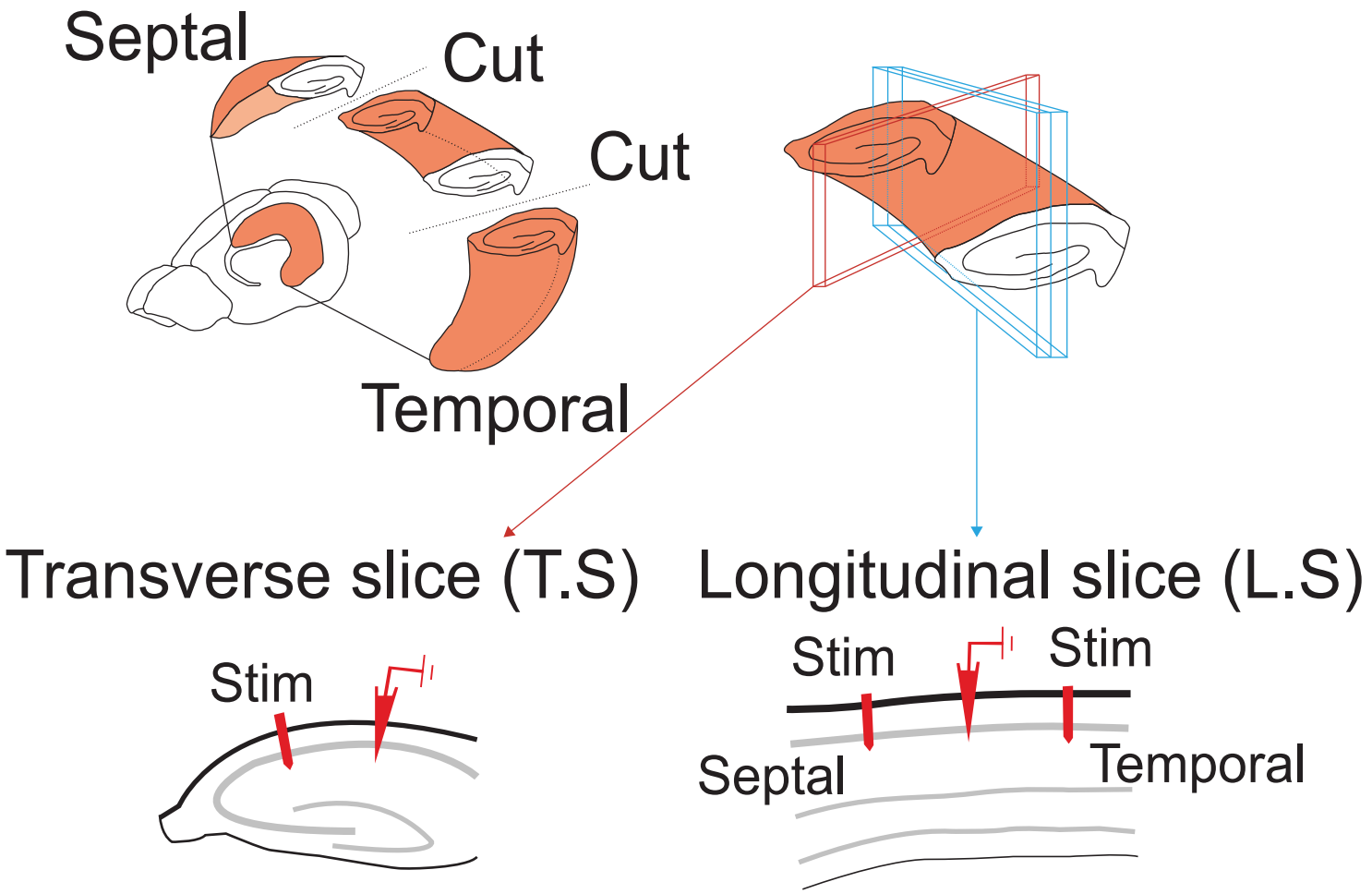

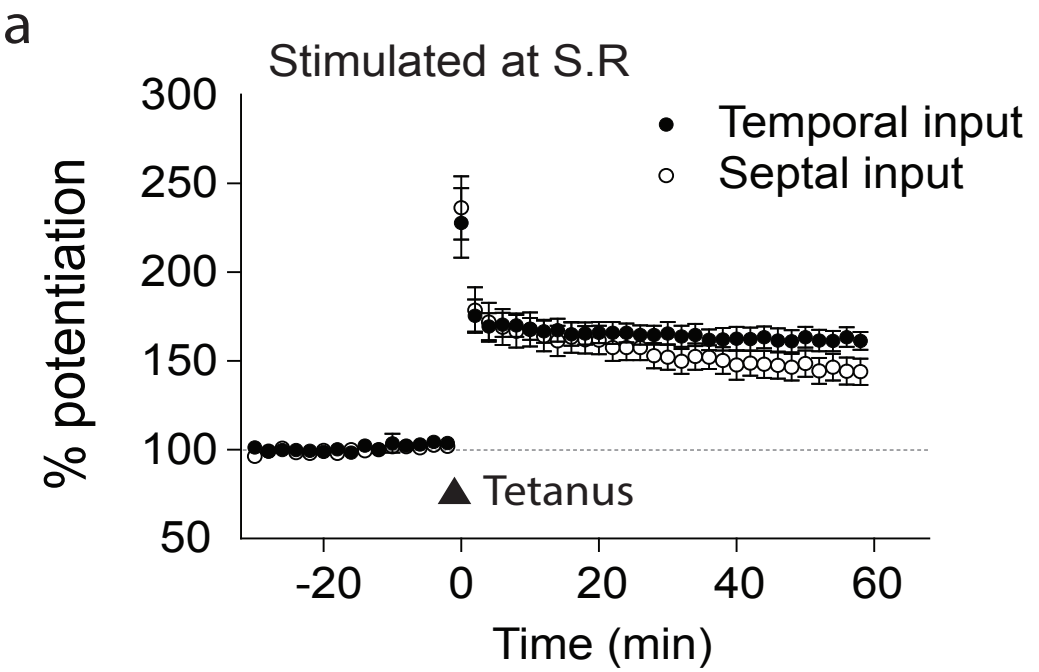

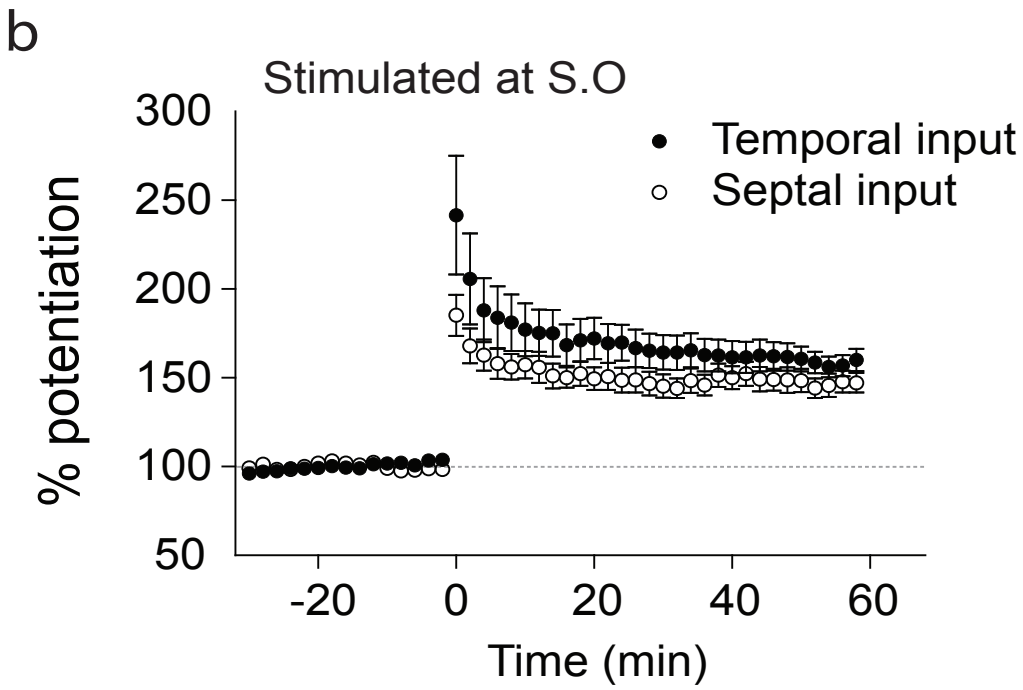

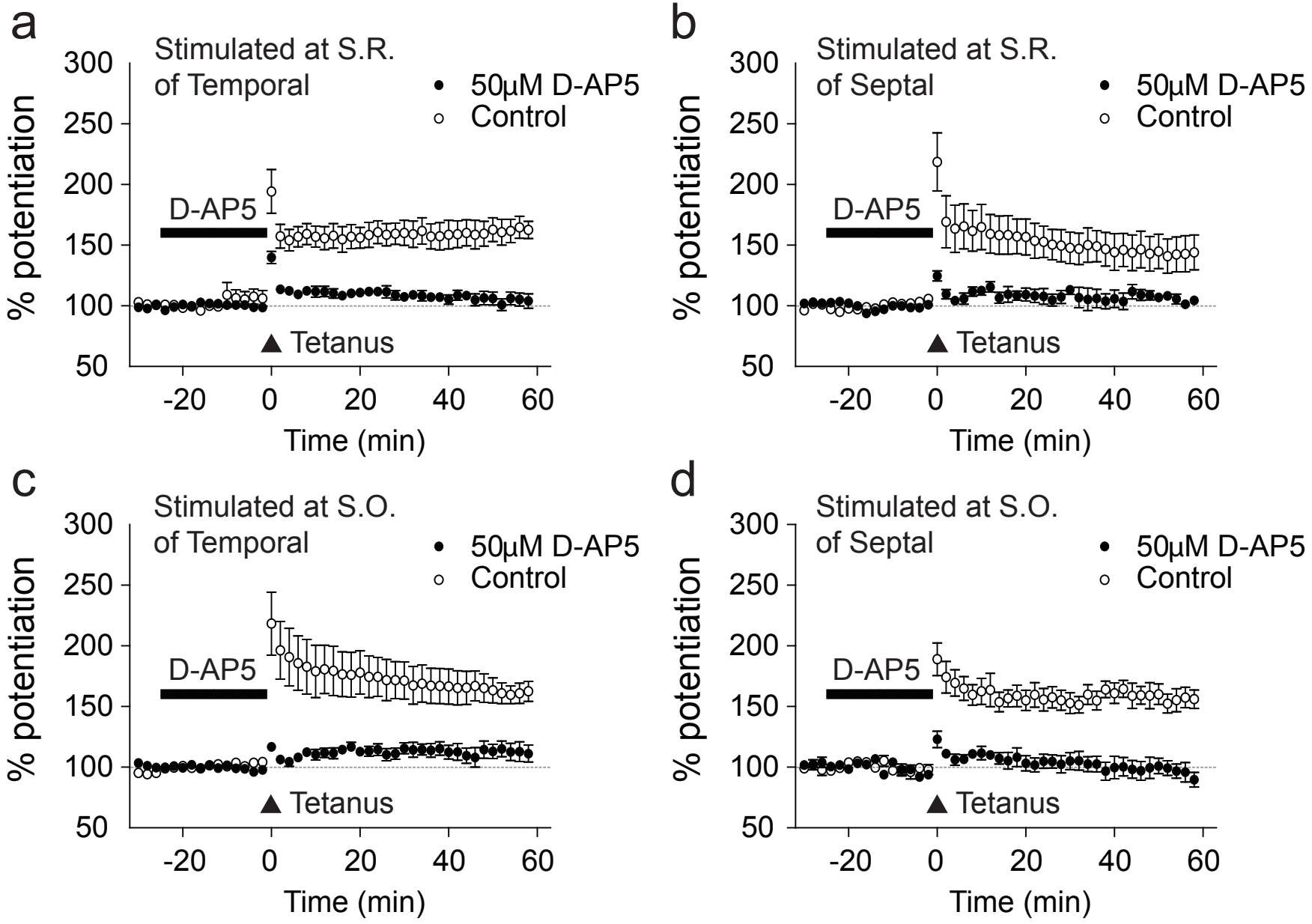

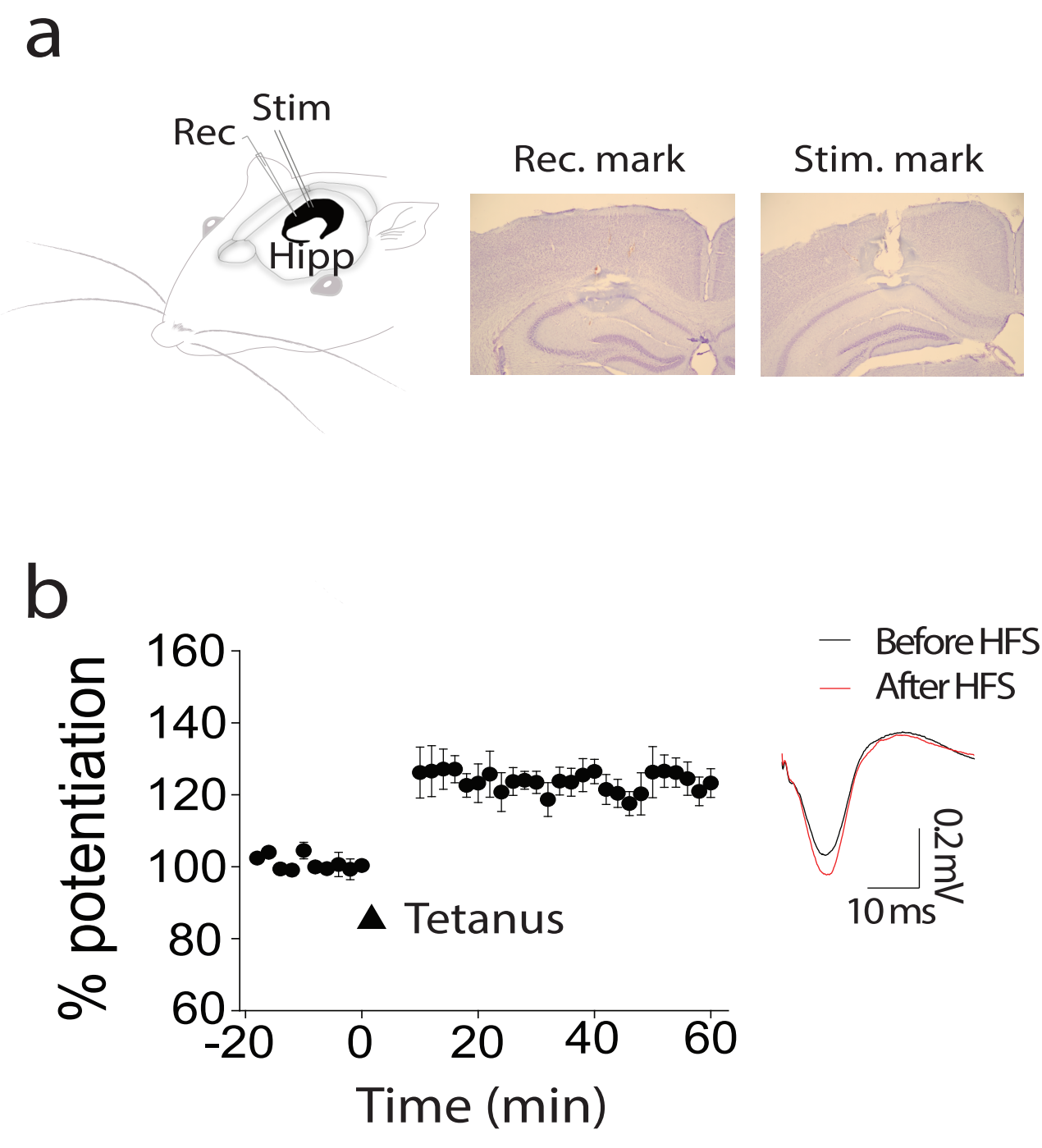

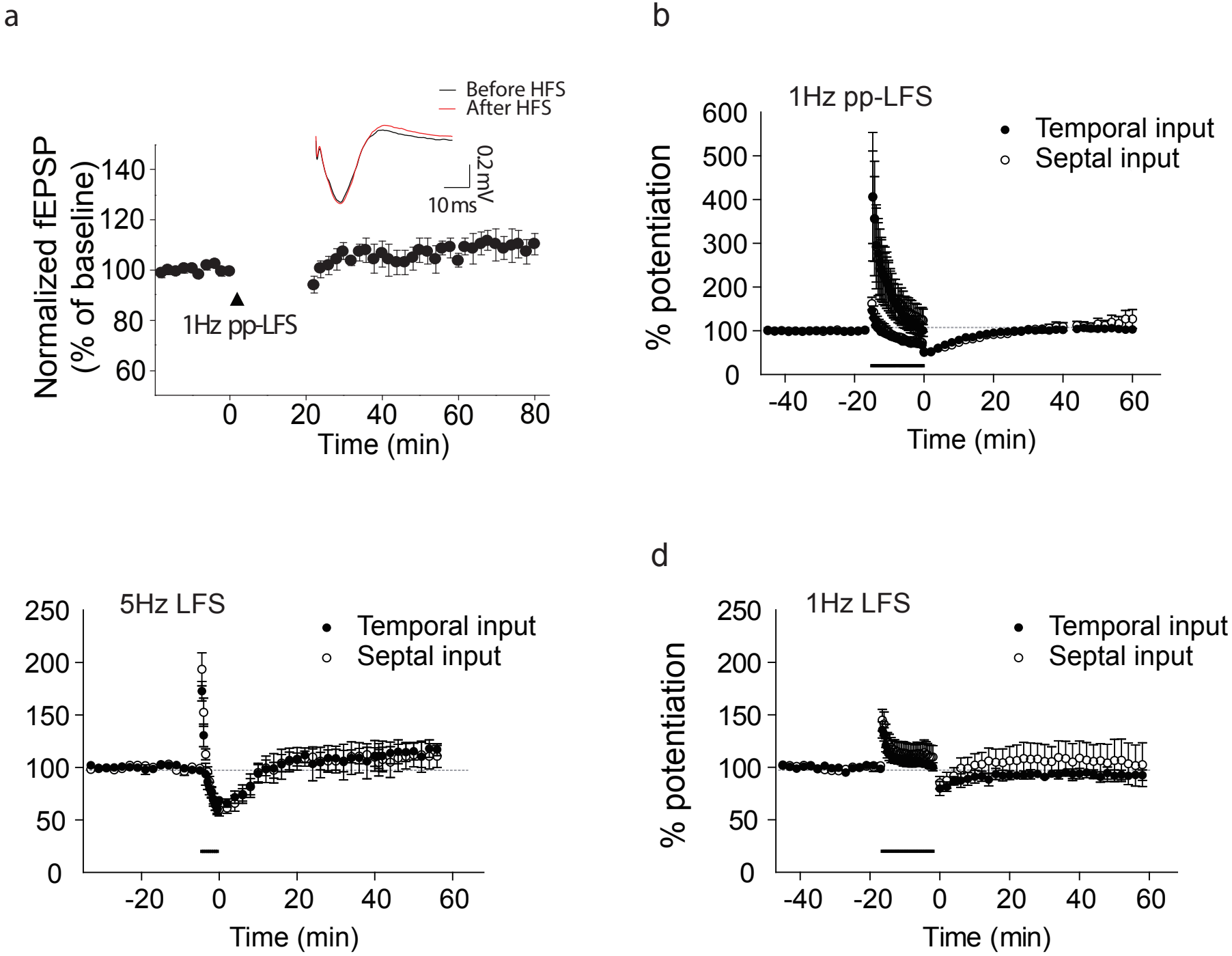

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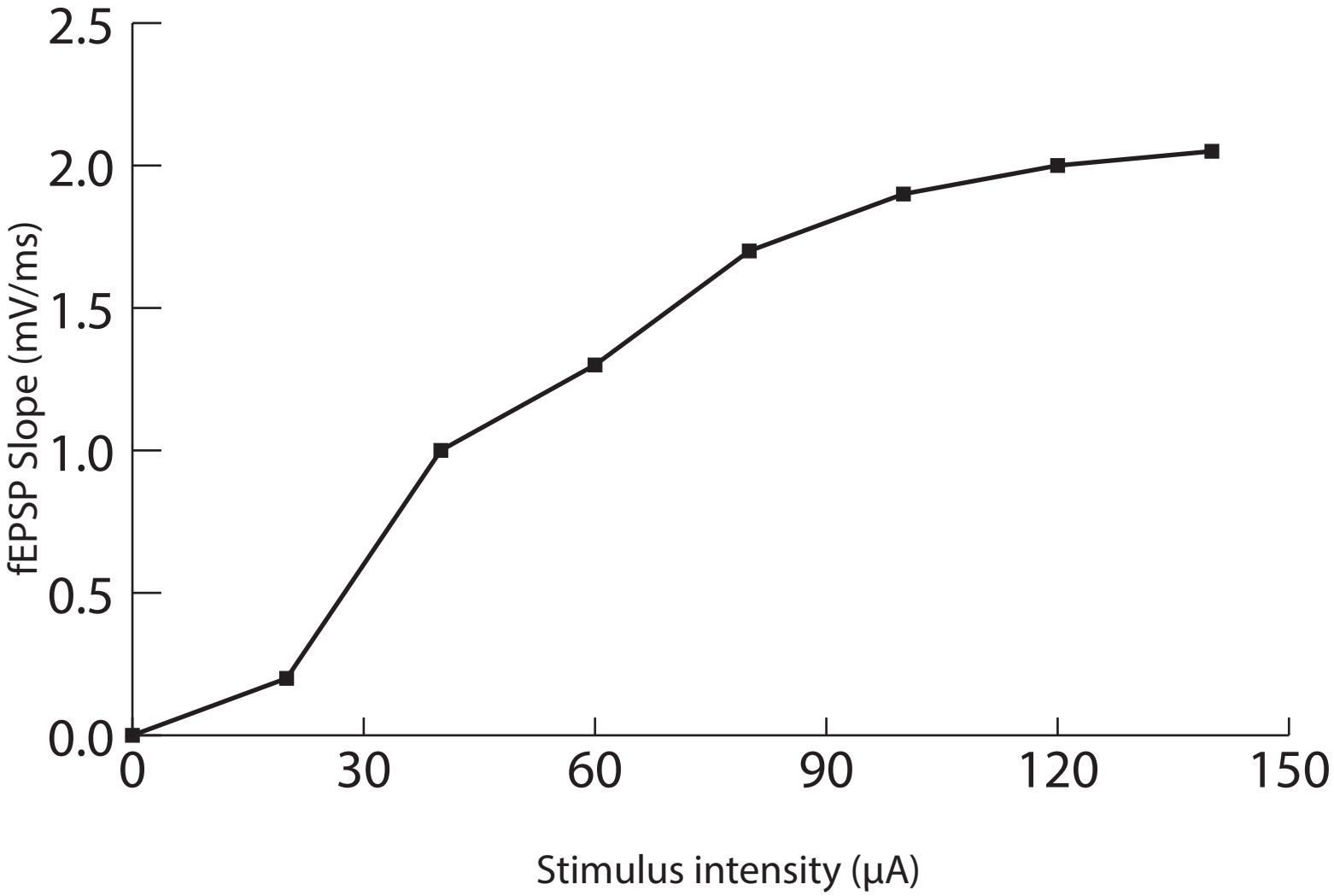

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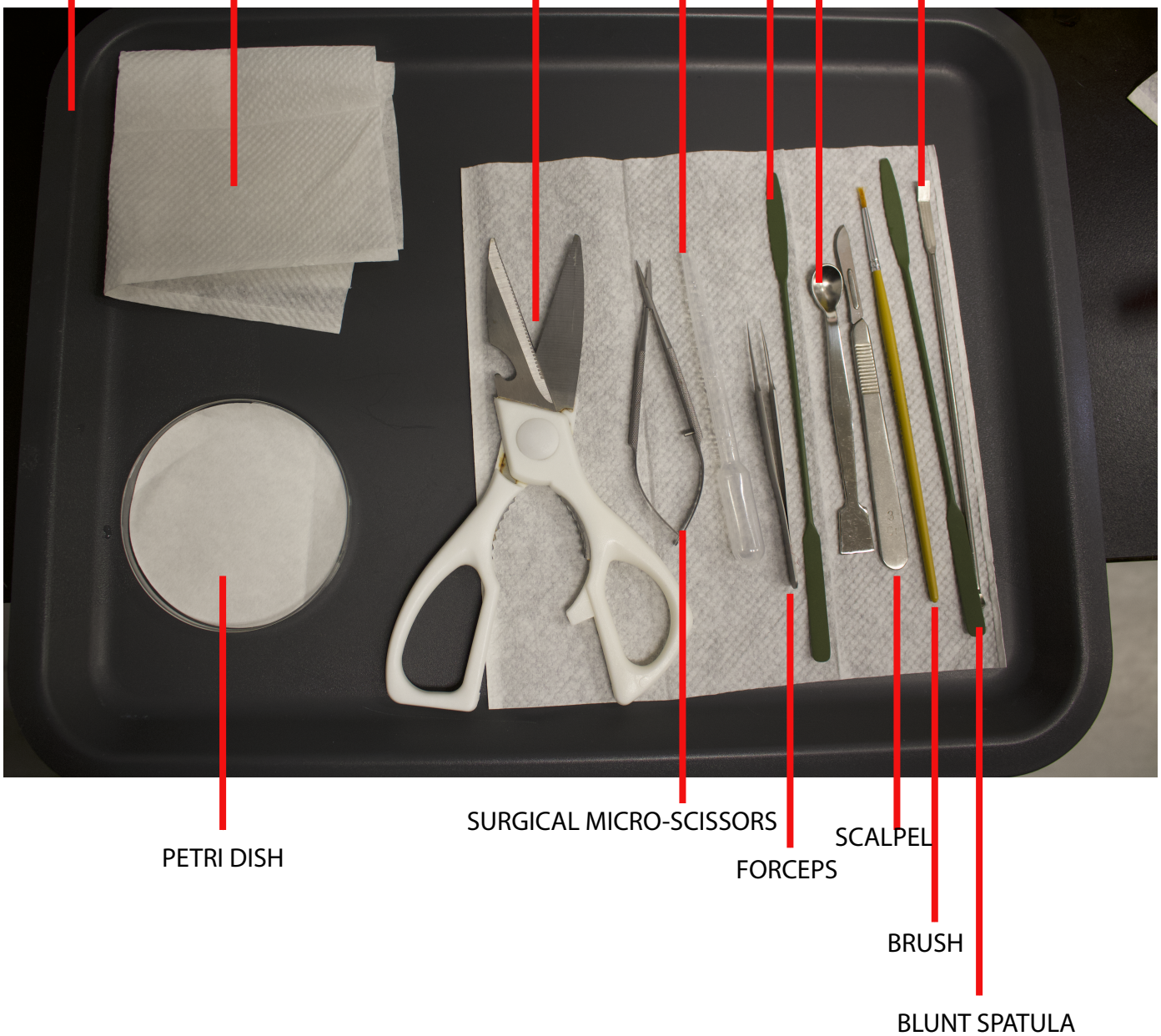

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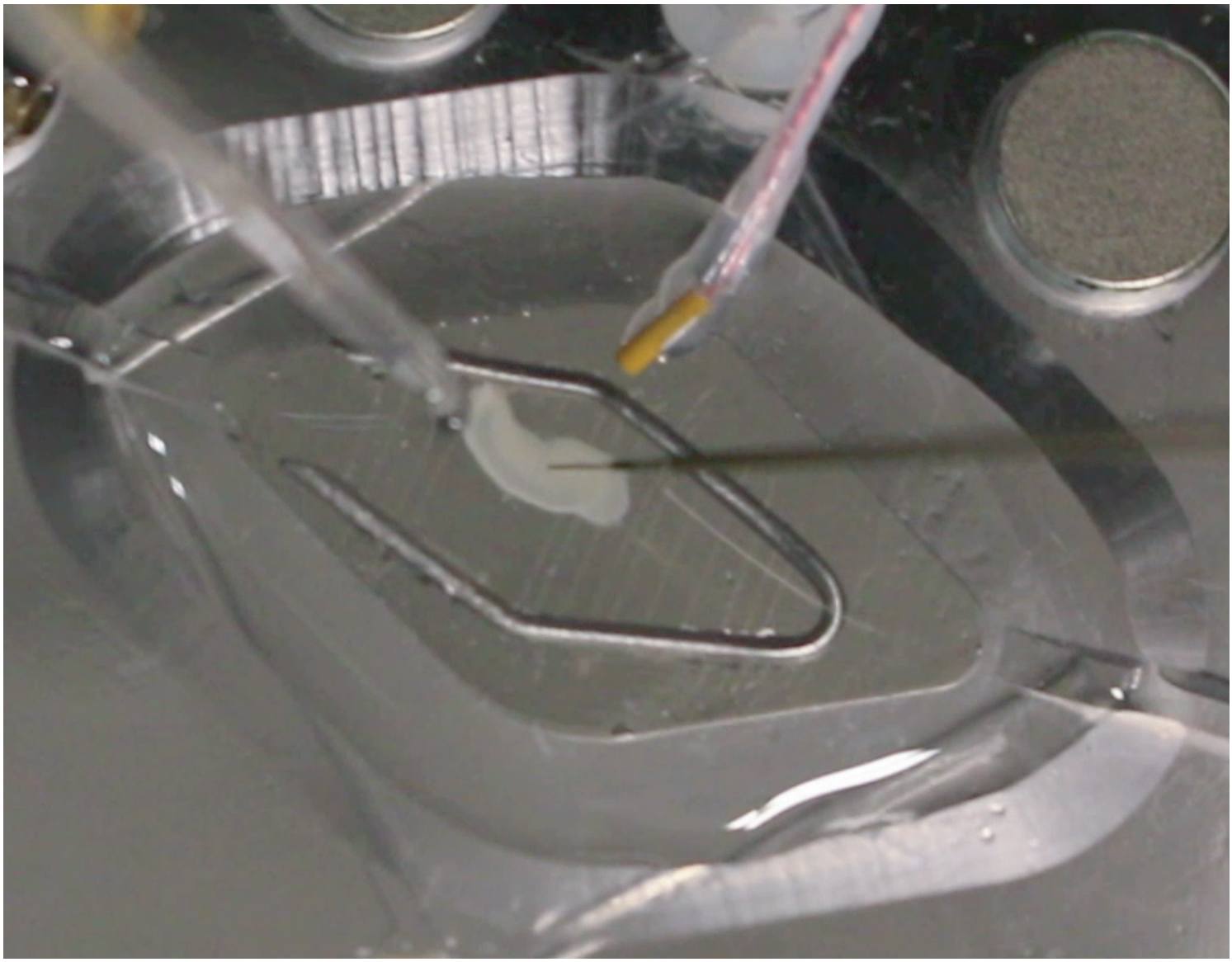

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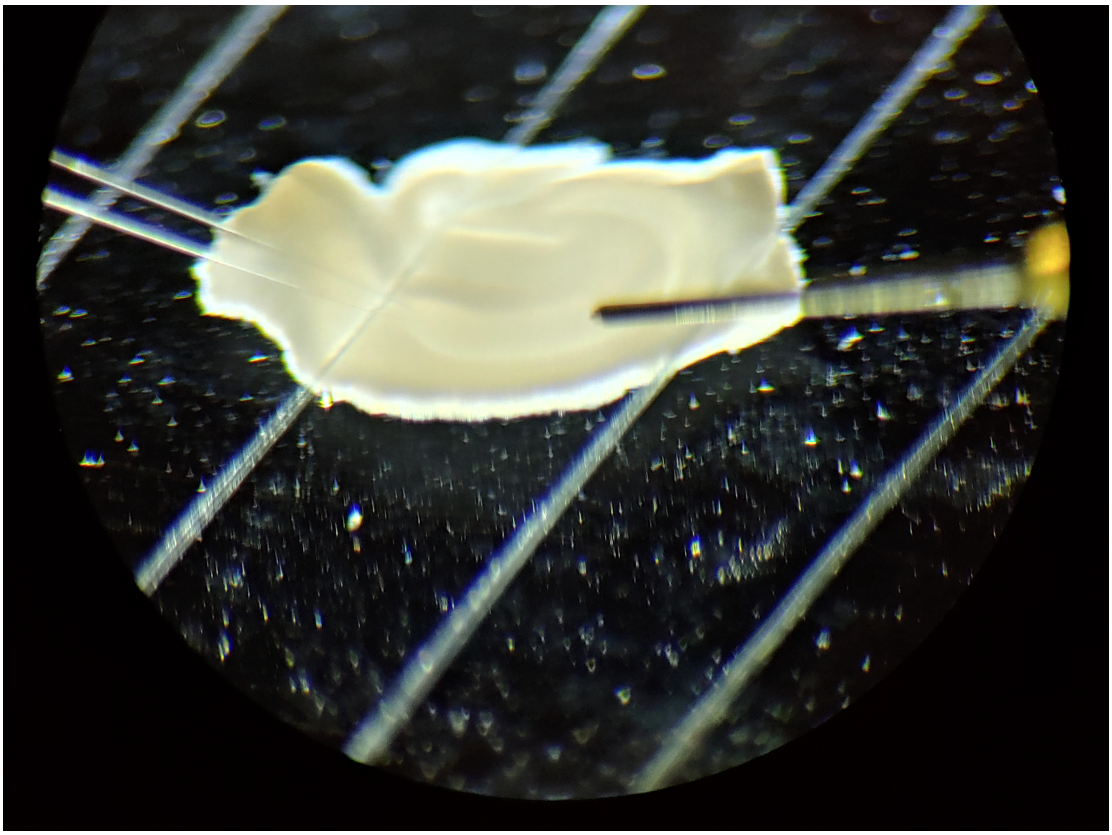

Chemical concentrations of slicing and ACSF solution

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Compound	Slicing Solı	ACSF (mM)
$CaCl_2.2H_2($	0.5	2
Glucose	25	25
KCl	2.5	2.5
$MgCl_2.6H_2$	7	1
NaCl	87	125
NaH_2PO_4	1.3	1.3
$NaHCO_3$	25	25
Sucrose	75	

Name of Reagent/Material	Company	Catalog Number	Comments
Atropine Sulphate salt mono	Sigma-Aldr	5908-99-6	Stored in Dessicator
Axon Digidata 1550B			
Calcium chloride	Sigma-Aldr	10035-04-8	
Clampex 10.7			
D-(+)-Glucose ≥ 99.5% (GC)	Sigma-Aldr	50-99-7	
Eyegel	Dechra		
Isoflurane	RWD Life S	R510-22	
Magnesium chloride hexahy	Sigma-Aldr	7791-18-6	
Matrix electrodes, Tungsten	FHC	18305	
Multiclamp 700B Amplifier			
Potassium chloride, BioXtra,	Sigma-Aldr	7447-40-7	
Potassium phosphate monob	Sigma-Aldr	7778-77-0	Stored in Dessicator
Pump	Longer pre	T-S113&JY10-14	
Silicone oil	Sigma-Aldr	63148-62-9	
Sodium Bicarbonate, BioXtra, 99.5-100.5%	Sigma-Aldr	144-55-8	
Sodium Chloride, BioXtra, ≥99.5% (AT)	Sigma-Aldr	7647-14-5	
Sodium phosphate monobasic, powder	Sigma-Aldr	7558-80-7	
Sucrose, ≥ 99.5% (GC)	Sigma-Aldr	57-50-1	
Temperature controller	Warner Ins	TC-324C	
Tungsten microelectrodes	FHC	20843	
Urethane, ≥99%	Sigma-Aldr	51-79-6	
Vibratome	Leica	VT-1200S	
Water bath	Grant Instr	SAP12	

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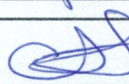
Signature:	
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Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures?

In Section 1.1.1., Lines 127 -132, the authors use urethane as the primary form of anesthesia. Although they provide a cautionary statement about its carcinogenic properties, I would recommend that they also add a statement as to why urethane is used for these procedures, since it is an antiquated anesthetic agent.
Comment: Urethane is used currently in our lab due to structural challenges which cannot permit the experimenter's safe usage of isoflurane vaporizer as anesthesia for experiments with long duration. Isoflurane is used only for short period of time under the fume hood with an improvised way to reach approximately 5%. We currently use isoflurane only during *in vitro* experiment to anesthetize the mouse just before decapitating the head for brain isolation. This issue is currently being addressed by moving to another lab which can facilitate the safe use of isoflurane vaporizer for lab members. It is however, permissible to use urethane in City University of Hong Kong.

Line 128, no dosage and route of administration is provided for Atropine.
Comment: The route of atropine administration was previously stated as intramuscular. The dosage (0.05mg/kg) has now been added in the manuscript.

General question: How is the depth of anesthesia monitored to ensure adequate plane is reached?
Comment: The depth of anesthesia is monitored by intermittently checking for mouse reflex using toe pinch, tail pinch, ear pinch and corneal touch. When the mouse is fully anesthetized, there will not be any reflex or voluntary movement when performing the above tests. The mouse is ensured to reach a surgical plane of anesthesia before experiment begins.

Line 136, 1.1.3. Is this heating pad a warm water circulating heating pad or electric? Electric heating pads are no longer recommended for use in animals due to the "hot spots" and risk of thermal injuries.
Comment: We use an electric heating pad. A layer of cloth or tissue paper is placed between the heating pad and the mouse to prevent direct contact with the skin of the mouse. The

applied layer is just enough to enable sufficient heat conductance to the anesthetized mouse. This reduces the risk of thermal injuries to the mouse.

Line 249, 1.3.19, what is PFA?
Comment: Paraformaldehyde (PFA). This has now been addressed in the manuscript.

Line 267, 2.1.4, what is ACSF (artificial cerebrospinal fluid)? It should be spelled out for the first use of acronym.
Comment: Artificial cerebrospinal fluid (ACSF). This is already spelt out in section 2.1.

Line 302, 2.2.8, states that mouse is anesthetized with Isoflurane. What is the % Minimal Alveolar Concentration (MAC) and what is the setting for carrier gas oxygen?
Comment: As mentioned above, isoflurane is used only for short period of time under the fume hood with an approved improvised method to reach approximately 5%. We currently use isoflurane only during *in vitro* experiment to anesthetize the mouse just before decapitating the head for brain isolation.

Line 348, 2.2.10 please remove the "s" on While
Comment: This has been addressed in the manuscript.

Line 480, the word "getting" should be "obtaining", just a suggestion.
Comment: This has been addressed in the manuscript.

Line 499, finding "a" cure or finding "cures" for brain disease is still "ongoing".
Comment: This has been addressed in the manuscript.

Under Name of Material/Equipment, Atropine is available in pharmaceutical grade, what about the ACSF manufacturer? Table needs cleaning up.
Comment: ACSF is prepared locally in the laboratory, hence there is no manufacturer for the finished solution. I however provided the pharmaceutical grades and manufacturers for each compound or chemical used in the preparation of ACSF.

Please provide additional comment, if necessary.
Comment: The video has been re-edited. Hopefully it meets the standards of the journal.

1. Please be specific in your comments. If possible, divide your comments into 2 categories:
   a) Absolutely not acceptable - for serious errors and deviations from the animal research standards.
   b) Improvement requires - for minor deviations, missing parts, etc.... I am not certain if it were my computers or not, but the accompanying video was "jumpy", "choppy"or "pixelated" throughout the video. I reviewed it on two different computers.

   For each comment, please specify if the changes in video are required, or if only changes in the complementary text are necessary. **Obviously, changes in the video are more difficult so it is important to note if changes in the text are sufficient.** Please use the chart below to provide details on each issue (replace examples listed):

#	**Time in the video**	**comment**	**Change in video required Yes/No**	**Change in text is sufficient Yes/No**	**Suggested Changes**
Example	2:20 – 2:34	Name of drug used for anesthesia is not mentioned	No	Yes	
1		Entire video was jumpy. It may have been my computers. No other significant issues, but it was difficult to assess.			
2					

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Comment: Okay

2. Please sort the Materials Table alphabetically by the name of the material.

Comment: Okay. This has been addressed.

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Comment: Okay. The steps for most protocols have been expanded to add more details previously overlooked in the manuscript.

4. 1.1.1: What is the age/gender/strain of the mouse? How much atropine is used?

Comment: C57BL6/J male mice were used for this experiment. The ages used ranged between 5–9 weeks old for *in vitro* and 6–12 weeks old for *in vivo* experiments. 0.05mg/kg of atropine was administered intramuscularly. This has now been addressed in the manuscript.

5. Please mention how proper anesthetization is confirmed.

Comment: To ensure whether a surgical level of anesthesia has been obtained, the response of the mouse to physical stimuli is done. The reflex of the mouse to toe pinch, tail pinch, ear pinch and corneal touch is done. This has now been addressed in the manuscript.

6. Please specify all surgical coordinates and tools used.

Comment: Okay. This has been addressed.

7. 1.2.1: How large is the cut?

Comment: The size of the cut area is 2mm * 3mm.The stereotaxic coordinates used for this are (AP: 1,3 and ML: 3,3).

8. Please avoid commercial language: Tucker Davies Technology system, Multiclamp, Clampex, etc.

Comment: Okay. This has been addressed.

9. What happens to the mouse at the end of step 1?

Comment: The mouse is euthanized by cervical dislocation at the end of experiment. This has been addressed.

10. 2.2.8: How much isoflurane is used?

Comment: Isoflurane is used only for short period of time under the fume hood with an improvised way to reach approximately 5%. We currently use isoflurane only during *in vitro* experiment to anesthetize the mouse just before decapitating the head for brain isolation.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Comment: Okay. This will be sent as an attachment.

Changes to be made by the Author(s) regarding the video:

Please note that the video is severely below the publication standard for JoVE videos. Hong Kong is within our videographer's network so we are able to come to your university to film. This would be a JoVE produced video where we handle the scripting, filming, and production of the video for you. Please consider this as there are severe videographer issues (constant change of focus, jump cuts, etc.).

1. Please homogenize the written protocol with the video narration. Ideally, the video narration is a word for word reading of the written protocol.

Comment: Well noted. This has been addressed.

2. 0:14-0:22 - Rather than announcing the demonstrators' names and then showing their faces over silence, this would do a better job of presenting the demonstrators if we see their faces while Hannah Tetteh is saying their names. Why does the camera move in these parts?
Comment: Okay. This has been addressed

3. 0:41, 2:08, 2:12, 2:13, 2:27, 2:40, 2:59, 3:01, 4:59, 7:17, 7:37, 8:03, 8:05, 8:28, 8:50, 8:59, 9:20, 10:41 - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.

Comment: Okay

4. The voiceover narration audio does not meet our quality standards. It sounds heavily compressed, and was likely recorded on a small microphone (like a webcam or cell phone microphone). This will need to be rerecorded at a higher quality.

Comment: Okay. I used a different microphone for this recording.

5. 1:45-1:51 - It is not clear what is being shown in this shot. Either narration should be added to explain this shot, or the shot should be cut out.

Comment: This has been addressed.

6. 3:19-4:00, 9:33-9:51, 11:03-11:33, 12:07-13:23 There is the image of a mouse cursor toward the bottom of the frame here. This should be erased.

Comment: Okay. This has been addressed.

7. 5:08-6:45, 11:34-12:03 - This screen recording portion needs to be redone. Some of the edits are confusing, the camera often moves, and the framing of the screen changes from shot to shot. Ideally, the recording would be done with screen capture software. If that is not possible and a camera must be used, the screen should be framed with the entire screen visible in the frame. This angle should be maintained throughout the steps involving a computer.

Comment: Well noted. This is being worked on

8. 9:52 - The drastic camera move that is at the beginning of the clip should be removed.

Comment: Okay.

9. Please repeat the title frame at the end. Please remove the frame with the word title at the beginning of the video as well.

Comment: Okay

If you choose to revise the video instead of switching to a JoVE produced video, please upload the revise high-resolution video here:
https://www.dropbox.com/request/FCq3VxNLmLR0hk0Id9X6?oref=e

Comment: In as much as it will be great for us to have a JoVE produced video, unfortunately, we do not have sufficient funding allocated to enable us do a JoVE produced video at the moment.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:
Manuscript Summary:
Electrophysiological field recording of synaptic plasticity in the interlamellar hippocampal CA1 in vivo and in vitro

Major Concerns:
The protocol lacks specific information about materials/reagents used.
1.1.1) Inject urethane (0.06 g per 25 g weight) intraperitoneally to anesthetize mouse - Why urethane used as a anesthetic? its a known carcinogen and personnel protection gear is a must while handling it. A note about use of alternate like isoflurane could be added.

Comment: Urethane is used currently in our lab due to structural challenges which cannot permit the experimenter's safe usage of isoflurane vaporizer as anesthesia for experiments with long duration. Isoflurane is used only for short period of time under the fume hood with an improvised way to reach approximately 5%. We currently use isoflurane only during *in vitro* experiment to anesthetize the mouse just before decapitating the head for brain isolation. This issue is currently being addressed by moving to another lab which can facilitate the safe use of isoflurane vaporizer. We however have permission for the use of urethane in City University of Hong Kong.

2.2.12 Separate the two brain hemispheres along the midline with a scalpel blade. Isolate the
317 hippocampus - size of hippocampus should be incorporated

Comment: Thank you for the suggestion. We believe the size of the isolated hippocampus will not have significant influence on the protocol or the outcome of the protocol hence our failure to incorporate it in the manuscript.

Authors have to mention that the procedure is terminal somewhere in the protocol. Aseptic procedure is also not discussed.

Comment: This has now been addressed in the manuscript.

Minor Concerns:
140 1.1.4) Apply eye gel to moisten the eyes of the mouse. - Eye gel brand needs to be specified

Comment: This has now been specified in the table.

150 1.1.7) Gently puncture the cisterna magna to drain the cerebrospinal fluid (CSF) - What are they using to puncture cisterna magna needs to be specified

Comment: A sharp pointed scalpel blade is used. This has been now been addressed in the manuscript.

Reviewer #2:
Manuscript Summary:
The authors overview synaptic plasticity along the lamellar and longitudinal axis of hippocampus highlighting technique for longitudinal recordings in vitro and in vivo.

Major Concerns:
There is not much new here and the details of longitudinal slicing, most critical aspect, and recording techniques are not adequately presented as compared to available literature(

*Are the title and abstract appropriate for this methods article?
Quite appropriate
*Are there any other potential applications for the method/protocol the authors could discuss?
Procedure is pretty specific for hippocampal anatomy and circuitry and electrophysiological investigations of such, applications beyond such are minimal.

Comment: The protocol can be applied in the investigations of neurological conditions in which the hippocampus is involved such as epilepsy and schizophrenia. The unique synaptic properties will provide new insights into approaching such neurological conditions.

*Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)
While most of hardware and software equipment is pretty standard, the table of materials

includes mostly chemicals which are even more standard, but if such are listed, amplifier and recording system and software should be tabled, I think.

Comment: Okay. This has been addressed.

*Do you think the steps listed in the procedure would lead to the described outcome?
This is unclear. Most of the steps and procedures have been in common use for well 30+ years, the only relatively novel aspect is the longitudinal slices and unfortunately the details, images of both slicing longitudinally and care with respect to electrode placement in different proximal(CA3) to distal (subiculum) longitudinal cuts is not presented. Variation in slicing and recording placement here will make considerable difference and it is the reason very few have conducted experiments in this regard.

Comment: Okay. This has been addressed.

*Are the steps listed in the procedure clearly explained?
Yes but the key items that are different from well-established procedure are not presented.

Comment: Well noted.

*Are any important steps missing from the procedure?
Greater visualization and description of the longitudinal slicing and recordings needs to be presented. Importantly, what variation is there in the few longitudinal cuts obtained? Do researchers use all longitudinal cuts? How many are obtained? Clearly researchers don't use each longitudinal cut, how many are obtained/ What is the variation across longitudinal cuts? This is reason most new researchers are focused on well characterized lamella cuts?

Comment: 2 hippocampal brain slices with intact parameters can be obtained. We have not observed any significant difference between the brain slices obtained when used for recordings.. Usually one brain slice is used during experiment however the second brain slice can serve as backup.

*Are appropriate controls suggested?
Well describing variation in different longitudinal cuts would go a long way here!

Comment: No significant observable difference is seen between the longitudinal brain slices.

*Are all the critical steps highlighted?
As noted the key ones are not.

Comment: This has hopefully been addressed.
*Is there any additional information that would be useful to include? As described.

The authors may want to update there basic references to include much more recent reviews (still fairly old) on hippocampal organization compared to Andersen et al., 1971; 1973). Dr. Amaral review quite well in Amaral & Witter, 1989 and more recently it was well reviewed by Sloverter & Lomo (2012). Others have also published exploring plasticity in longitudinal slices and some references to such should be included (see Papaleonidopoulous and coclleagues (2017) BMC Neuroscience for references).

Comment: Well noted.

*Are the anticipated results reasonable, and if so, are they useful to readers?
This is unclear, most are standard, the finding of LTP, but no LTD in longitudinal CA1 projections seems highly unlikely as I know of no known glutamatergic pathway that potentiates but does not depotentiate, so I am quite skeptical. Are the authors aware of any other paper indicating such?

Comments: It has been demonstrated in vitro that some synapses presenting with one subunit of NMDA receptors may present only LTD or LTP but not both. (Ref: Liu *et a*l., 2004). Lack of LTD could be speculated to be because, these synapses lack inducing mechanisms necessary for LTD induction. Also, it is possible there is LTD in under natural conditions but could not be induced using the induction protocols which are artificial. The reason as to why this synapse did not present with LTD needs further investigation.

*Are any important references missing and are the included references useful?
As noted above.

Comment: Well noted.
Minor Concerns:
as noted above

Reviewer #3:
Manuscript Summary:
The manuscript provides a concise description of methods to achieve in vivo and in vitro electrophysiological recordings from the hippocampal CA1 area in order to test properties of longitudinal (intralamellar) excitatory connectivity.
The manuscript is based on two previous publication involving the authors in which the longitudinal connectivity was described in detail (Yang et al., 20014 PNAS) and later synaptic plasticity further characterized (Sun et al., 2018 Sci Rep).
In fact, the representative results and figures panels are from these two papers.

Major Concerns:
1) Although the primary aim of in vivo and in vitro recordings is the same, I'm not convinced

that combining these two technically divergent approaches in a single methodological paper is beneficial to the readers/potential users.
The in vivo recording is actually a very generic approach, the only specific aspect being the choice of the appropriate stereotactic coordinates for the stimulating and recording electrodes for optimal placement for investigating the longitudinal connectivity. Moreover, under these conditions I'm not convinced that a selective stimulation ad recording of longitudinal connection is possible. CA3 fibers (Schaffer collaterals) fan out along the septotemporal axis, therefore the stimulation may very well activate CA3 axons that run across the laminae. In particular in the str. radiatum, where very little of the CA1 axons are observed it is questionable if these are not in fact such CA3 axons.

Comments: It is true. Possibility of activating the CA3 fibers is a shortfall for the *in vivo* approach. This method is better demonstrated *in vitro*. However, using the exact stereotaxic coordinates and depth, the probability is reduced. There is no way of knowing exactly except by utilizing some experimental means such as CRISPR-Cas9 system with optogenetics which is target specific and can be able to label target neurons within the interlamellar ca1 axis. This is one question that highly on our minds and have a possibility of being investigated should the need arise for a research involving the *in vivo* system.

The in vitro approach is novel in in its concept and offers a new type of longitudinal slice preparation. Also as the CA3 is removed and the stimulation is applied to a longitudinal slab of the CA1, it more convincingly demonstrates the proposed connectivity.
Therefore i would recommend the authors to focus on this approach and extended it with (a) intracellular recordings, if possible in the paired configuration to directly assess unitary synaptic connections, and/or (b) combined with photostimulation.

Comment: Well noted

2) All figures focus on the representative results. Please consider making additional figures to illustrate some methodological aspects after all in this paper the methods should take centrestage!

Comment: Well noted.
[
Minor Concerns:
1) The title is very much results centric, please reformulate to emphasize the methods. Also while the representative results focus on plasticity, this methods can and should be used to assess other properties of the synapse.

Comment: Noted.

2) Introduction suggests that the connectivity is widely considered lamellar, missing the fact that it is long known that axons of mossy cells of the hilus and CA3 pyramids show extensive interlamellar spread.

Comment: Well noted. This has now been addressed briefly. The focus of this protocol is on the interlamellar connections of the CA1 region which has received relatively little attention unlike the CA3 region.

3) The motivation and potential use of the approach is not spelled out very well in the introduction, only the investigation of plasticity is reiterated.

Comment: Well noted. This has been addressed.

Protocol

4) Point 1.1.6: Is the microscope meant to be a binocular microscope?

Comment: A stereo microscope is much applicable here. I believe experimenters working on electrophysiology will be aware of the type of microscope to use hence we did not specify.

5) Point 1.3: This is actually the in vivo recording - data acquisition is of course part of it. Please reword this heading.

Comment: Okay. This has now been addressed

6) Point 1.3.3: Can you provide exemplary coordinate combinations?

Comment: CA1-CA1 coordinates. R (1.5, 1.0) and S (2.0, 1.7) or R (1.5,1.0) and S (1.7, 1.5).

7) Point 1.3.6: Can any other recording system be used?

Comment: Yes. It depends on the laboratory and their choice of recording system. This has now been addressed.

8) Point 1.3.7: Description here sounds very specific to the recording system - not informative to anybody not using that brand of system.

Comment: This has now been addressed.

9) Point 1.3.8: Use "depth" instead of "distance"?!

Comment: Okay. This has been addressed.

10) Point 1.3.11: "Perform an Input-ouput curve" is technical slang. Could you please reword and also explain less experienced readers what and how is it done? A figure panel would be also helpful.

Comment: This has been addressed in the manuscript.

11) Point 1.3.15-16: This sequence seems to suggest that high frequency stimulation is followed by low frequency stimulation - please indicate that only one of the two should be applied.

Comment: This has been addressed.

13) Point 1.3.16: What is the physiological relevance of the three different LTD induction protocols?

Comment: In the hippocampus, in addition to NMDA receptors, metabotropic glutamate receptors (mGluRs) and cannabinoid-1 receptors are believed to participate in LTD induction. Utilising the three different LTD induction protocols was to ensure that both NMDA- dependent and NMDA-independent forms of LTD were investigated.

14) Point 2.2.8: Animal procedures should follow local institutional and national guide lines.

Comment: This has been addressed.

15) Point 2.2.14-15: Indicate that the two points are alternatives?!

Comment: This has been addressed.

16) Point 2.2.16: Please give units for speed and amplitude. A question here is how specific these values are for a specific slicer type/brand. Also, please check the units of the thickness (definitely not micromolar)!

Comment: This has been addressed. The unit for the thickness was an oversight.

17) Point 2.2.19: Please provide temperature for recovery.

Comment: This has been addressed.

18) Point 2.3: Again this is actually the in vitro recording - data acquisition is part of it. Please reword this heading.

Comment: This has been addressed.

19) Point 2.3.2: Use e.g. "transfer" instead of 'pick'. Also, "macrochamber" is the slice holding chamber.

Comment: This has been addressed.

20) Point 2.3.2: Provide concentration of NaCl solution!

Comment: As an update on the protocol, we now use only the ACSF as internal solution. This has been addressed in the manuscript.

21) Point 2.3.4: Can any other software data acquisition system be used?

Comment: Yes. This has now been addressed.

22) Point 2.3.7-8: Indicate that the two points are alternatives!?

Comment: This has now been addressed.

23) Point 2.3.9: Is the "isolater" an isolated stimulus generator?

Comment: Yes. This has now been addressed.

24) Point 2.3.10: Again: "Perform an Input-output curve" is technical slang. Could you please reword and also explain less experienced readers what and how is it done? A figure panel would be also helpful.

Comment: Yes. This has been addressed.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please provide reprint permissions for Figures 1-5.

Comment: This was published under creative common licenses (CC BY) which gives the right to authors to reuse figures provided it has been duly cited or acknowledged. Below is the link given by the permission team of SpringerNature.

https://creativecommons.org/licenses/by/4.0/

Also below is the link to such claims by Scientific Reports journal for our previous publication in which we adapted Figures 1-5.

https://s100.copyright.com/AppDispatchServlet?imprint=Nature&oa=CC%20BY&title=Long%20term%20potentiation%2C%20but%20not%20depression%2C%20in%20interlamellar%20hippocampus%20CA1&author=Duk-gyu%20Sun%20et%20al&contentID=10.1038%2Fs41598-018-23369-4&publication=Scientific%20Reports&publicationDate=2018-03-26&publisherName=SpringerNature&orderBeanReset=true

Changes to be made by the Author(s) regarding the video:

This video revision is a marked improvement from the initial submission.

1. Please reduce the video length to 15 min or less.

Comment: Okay. This has been done.

2. Why is Gona Choi not listed in the Author list? The introductions of Gona Choi and Jihwan Lee are not needed in the video.

Comment: According to enquiries made from JoVE, the recorded video can have people not on the author list, but they must be acknowledged. In our case, Gona Choi assisted with a little part of this video and she was accordingly acknowledged in the manuscript even though she is not part of the authors.

3. 0:04-0:12, 0:18-0:48, 1:41-2:00, 15:23-15:49 - The audio for the on-screen interviews of Hannah Tetteh is not publication grade. This should be re-recorded.

Comment: Okay. The on-screen interviews have been re-recorded.

4. 12:00 - Please fix the font and formatting of the o in the vitro

Comment: This has now been corrected.

5. Please normalize the volumes of the audio throughout the video. The audio should be peaking between -6 and -12 dB.

Comment: Okay. This has been done.