Journal of Visualized Experiments

A Model of Epileptogenesis in Rhinal Cortex-Hippocampus Organotypic Slice Cultures

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61330R1
Full Title:	A Model of Epileptogenesis in Rhinal Cortex-Hippocampus Organotypic Slice Cultures
Keywords:	Organotypic slices, epileptogenesis, propidium iodide, gliosis, CD68, C3d
Corresponding Author:	Cláudia Alexandra Valente Universidade de Lisboa Faculdade de Medicina Lisboa, Portugal PORTUGAL
Corresponding Author's Institution:	Universidade de Lisboa Faculdade de Medicina
Corresponding Author E-Mail:	cvalentecastro@medicina.ulisboa.pt
Order of Authors:	Cláudia Alexandra Valente
	Francisco José Meda
	Mafalda Carvalho
	Ana Maria Sebastião
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lisboa, Portugal

TITLE:

A Model of Epileptogenesis in Rhinal Cortex-Hippocampus Organotypic Slice Cultures

AUTHORS:

Cláudia A. Valente[1,2], Francisco J. Meda[1,2,*], Mafalda Carvalho[1,2,*], Ana M. Sebastião[1,2]

[1]Instituto de Farmacologia e Neurociências, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

[2]Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

* These authors contributed equally

cvalentecastro@medicina.ulisboa.pt
fmeda@medicina.ulisboa.pt
mafaldacarvalho@medicina.ulisboa.pt
anaseb@medicina.ulisboa.pt

CORRESPONDING AUTHOR

Cláudia A. Valente
cvalentecastro@medicina.ulisboa.pt

KEYWORDS:

Organotypic slices, epileptogenesis, propidium iodide, gliosis, CD68, C3d

SUMMARY:

Here, we describe the preparation of rhinal cortex-hippocampus organotypic slices. Under a gradual and controlled deprivation of serum, these slices depict evolving epileptic-like events and can be considered an ex vivo model of epileptogenesis. This system represents an excellent tool for monitoring the dynamics of spontaneous activity, as well as for assessing the progression of neuroinflammatory features throughout the course of epileptogenesis.

ABSTRACT:

Organotypic slice cultures have been widely used to model brain disorders and are considered excellent platforms for evaluating a drug's neuroprotective and therapeutic potential. Organotypic slices are prepared from explanted tissue and represent a complex multicellular ex vivo environment. They preserve the three-dimensional cytoarchitecture and local environment of brain cells, maintain the neuronal connectivity and the complex reciprocal interplay between all cells. Hippocampal organotypic slices are considered suitable to explore the basic mechanisms of epileptogenesis, but clinical research and animal models of epilepsy have suggested that the rhinal cortex, composed of perirhinal and entorhinal cortices, play a relevant role in seizure generation.

Here, we describe the preparation of rhinal cortex-hippocampus organotypic slices. Recordings of spontaneous activity from the CA3 area under perfusion with complete growth medium, at physiological temperature and in the absence of pharmacological manipulations, showed that these slices depict evolving epileptic-like events throughout time in culture.

Increased cell death, through propidium iodide uptake assay, and gliosis, assessed with fluorescent-coupled immunohistochemistry, was also observed. The experimental approach presented highlights the value of rhinal cortex-hippocampus organotypic slice cultures as a platform to study the dynamics and progression of epileptogenesis and to screen potential therapeutic targets for this brain pathology.

INTRODUCTION:

Epilepsy, one of the most prevalent neurologic disorders worldwide, is characterized by the periodic and unpredictable occurrence of synchronized and excessive neuronal activity in the brain. Despite the various antiepileptic drugs (AEDs) available, one-third of patients with epilepsy are refractory to therapy[1] and continue to experience seizures and cognitive decline. Furthermore, available AEDs hamper cognition due to their relatively generalized actions upon neuronal activity. Epileptogenesis is hard to study in humans, due to the multiple and heterogeneous epileptogenic injuries, long latent periods lasting months to decades, and the misleading effects of anticonvulsant treatment after the first spontaneous seizure.

The identification of potential therapeutic agents for the treatment of epilepsy has become possible due to animal models of epilepsy: 1) genetic models, which use genetically predisposed animals in which seizures occur spontaneously or in response to a sensory stimulus; 2) models of electrical stimulation-induced seizures; and 3) pharmacological models of seizure induction that use pilocarpine (a muscarinic receptor agonist), kainate (a kainate receptor agonist) or 4-aminopyridine (a potassium channel blocker), among others. These models were crucial in the understanding of the behavioral changes, as well as molecular and cellular mechanisms underlying epilepsy, and they have led to the discovery of many AEDs[2].

Ex vivo preparations are also a powerful tool to explore the mechanisms underlying epileptogenesis and ictogenesis. Acute hippocampal slices, which enable electrophysiological studies of living cells over a 6-12 h period, and organotypic hippocampal slices that can be preserved in an incubator over a period of days or weeks have been extensively used in studies of epileptiform activity[3]. Organotypic brain slices are prepared from explanted tissue and represent a physiological three-dimensional model of the brain. These slices preserve the cytoarchitecture of the region of interest and include all brain cells and their intercellular communication[4].

The most used region for long-term organotypic cultures is the hippocampus, as this region is affected by neuronal loss in multiple neurodegenerative conditions. They have been widely used to model brain disorders and are considered excellent tools for assessing a drug's neuroprotective and therapeutic potential[5-6]. Models of epileptogenesis, stroke and Aβ-induced toxicity were described in hippocampal organotypic slices[7-10]. Parkinson's disease was explored in ventral mesencephalon and striatum, as well as cortex-corpus callosum-striatum-substantia nigra, organotypic slices[11]. Organotypic cerebellar slice cultures mimic many aspects of axon myelination and cerebellar functions and are a widespread model for investigating novel therapeutic strategies in multiple sclerosis[12].

However, clinical research and animal models of epilepsy have suggested that the rhinal cortex, composed by perirhinal and entorhinal cortices, plays a role in seizure generation[13]. Thus, a model of epileptogenesis in rhinal cortex-hippocampus organotypic slices was

established[14]. Under a gradual and controlled deprivation of serum, rhinal cortex-hippocampus organotypic slices depict evolving epileptic-like events, unlike analogous slices always kept in a serum-containing medium.

In epilepsy, as in many acute and chronic diseases of the central nervous system, the neurocentric vision fails to elucidate the mechanisms underlying disease onset and progression. Clinical and experimental evidence point to brain inflammation, in which microglia and astrocytes play a relevant role, as one of the key players contributing to the epileptic process. Pharmacological experiments in animal models of epilepsy suggest that antiepileptogenic effects can be achieved by targeting pro-inflammatory pathways, and nowadays neuroinflammation is regarded as a novel option for the development of therapeutic approaches for epilepsy[15].

Here, we thoroughly describe the preparation of rhinal cortex-hippocampus organotypic slice cultures, as well as the details for recording spontaneous epileptiform activity from them. We highlight that this system mimics several neuroinflammatory aspects of epilepsy, being thus suitable to explore the role of glial cells and neuroinflammation in this pathology. Furthermore, it represents an easy-to-use platform for the screening of potential therapeutic approaches for epilepsy.

PROTOCOL:

The Portuguese law and European Union guidelines (2010/63/EU) were respected in all procedures regarding the protection of animals for scientific purposes. All methods described here were approved by the iMM's Institutional Animal Welfare Body (ORBEA-iMM) and the National competent authority (DGAV – Direção Geral de Alimentação e Veterinária).

1. Preparation of rhinal cortex-hippocampus slices

NOTE: The preparation of rhinal cortex-hippocampus slices uses P6-7 Sprague-Dawley rats.

1.1. Culture setup and medium preparation

1.1.1. On the day before the culture, prepare the required media and place them at 4 °C.

1.1.2. Prepare dissection medium: 25 mM glucose in Gey's Balanced Salt Solution (GBSS).

1.1.3. Prepare culture medium: 50% Opti-MEM, 25% HBSS, 25% Horse Serum (HS), 25 mM glucose, 30 µg/mL Gentamycin.

1.1.4. Prepare maintenance medium: Neurobasal-A (NBA), 2% B27, 1 mM L-glutamine, 30 µg/mL Gentamycin, HS (15%, 10%, 5% and 0%).

1.2. Brain harvesting

1.2.1. Just before starting the culture, add 1.1 mL of culture medium to each well of the 6-well plate with a pipette and place it at 37 °C.

1.2.2. Place all the equipment (dissection microscope, tissue chopper, dissecting lamp, dissecting tools, electrodes, plates, inserts and filter papers) inside the biological safety cabinet and sterilize under UV light for 15 minutes.

1.2.3. Adjust slice thickness to 350 µm.

1.2.4. Withdraw the GBSS from the fridge. Add 5 mL of GBSS to six Petri dishes. Six Petri dishes will be required per animal.

1.2.5. Euthanize the rat pup. Perform decapitation by using a sharp scissor at the base of the brain stem of the animal.

1.2.6. Wash the animal head three times in cold GBSS and take it inside the safety cabinet.

1.3. Tissue isolation and preparation of slices

1.3.1. Firmly insert sharp forceps into the eye sockets to hold the head.

1.3.2. Using a thin scissor cut the skin/scalp along the midline starting from the vertebral foramen towards the frontal lobes and put it aside.

1.3.3. Cut in the same way the skull and along the cerebral transverse fissure (space between brain and cerebellum). With curved long forceps, move it apart.

1.3.4. Discard the olfactory bulbs with a spatula. Remove the brain from the head and place it in ice-cold GBSS with the dorsal surface faced up (**Figure 1A**).

1.3.5. Insert the fine forceps into the cerebellum and go along the midline with the spatula opening each hemisphere very carefully (**Figure 1B**).

1.3.6. With short curve forceps, carefully remove the excess tissue that covers the hippocampi, without touching the hippocampal structure. Then with a spatula, cut below each hippocampus (**Figure 1C**).

1.3.7. Pick up one hemisphere and place it, with hippocampus facing up, onto a filter paper. Repeat the procedure with the other hemisphere and place it parallel to the first one, in the filter paper. Put the filter paper on the tissue chopper, with the hemispheres perpendicular to the blade, and cut the hemispheres in 350 µm slices (**Figure 1D**).

1.3.8. Place the sliced tissue into a Petri dish with cold GBSS (**Figure 1E**).

1.3.9. Carefully separate the slices using the round tip electrodes (**Figure 1F**). Keep only the slices with a structurally intact rhinal cortex and hippocampus. DG and CA areas should be perfectly defined, as well as the entorhinal and perirhinal cortex (**Figure 1G**).

1.3.10. Place each slice onto the insert (**Figure 1H-I**), with a spatula and a round tip electrode. Remove excess dissection medium around each slice with a P20 pipette (**Figure 1J**). Four rhinal cortex-hippocampus slices can be cultured in a single insert (**Figure 1K**).

1.4. Culture maintenance

1.4.1. Change the medium every other day.

1.4.2. Warm up the medium at 37 °C.

1.4.3. Take the plates from the incubator. Pick up each insert by holding the plastic edge with forceps (**Figure 1L**).

1.4.4. Use a free hand to aspirate the medium from the well. Place the insert back into the well and add 1 mL, with a P1000 pipette, of fresh warmed medium (**Figure 1M**). Repeat for all the inserts. Make sure no air bubbles are trapped between the membrane and the medium.

NOTE: Epileptic-like slices undergo a gradual and controlled deprivation of serum in the medium. From 9 Days In Vitro (DIV) on, slices are maintained in NBA without HS[14].

[Place Figure 1 here]

2. Electrophysiological recordings

NOTE: Electrophysiological recordings were performed in rhinal cortex-hippocampus organotypic slices at 7, 14 and 21 DIV in an interface-type chamber. Recordings were obtained with an amplifier, digitized and analyzed with software. All recordings were band-pass filtered (eight-pole Bessel filter at 60 Hz and Gaussian filter at 600 Hz).

2.1. Setup preparation

2.1.1. Prepare 50 mL of NBA medium with 1 mL of B27 and 250 µL of L-Glutamine. Warm up at 37 °C.

2.1.2. Set the electrophysiology setup in close circuit. Verify if the flow rate is 2 mL/min.

2.1.3. Open the carbox (5% CO_2/95% O_2) valve and check the water level in the system.

2.1.4. Put the filter paper in the interface recording chamber to drain excess medium and the lens cleaning paper beneath the frame to supply medium to the slice.

2.1.5. Turn on the temperature controller, the amplifiers, and the micromanipulator.

2.1.6. Let the temperature in the interface chamber stabilize at 37 °C before starting the recordings.

2.1.7. Prepare artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 3mM KCl, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgSO_4$ with pH 7.4) and use it to fill the glass electrode with a syringe. Place it in the receiving electrode.

2.2. Recordings of spontaneous activity

2.2.1. Once the temperature is stable, remove the plate from the incubator and cut one slice from the insert with a highly sharp blade. Place it in a 60 mm plate with a drop of medium. Take it to the interface recording chamber.

2.2.2. Place the slice in the interface chamber with the hippocampus to the bottom right. Place the stimulating electrode in the mossy fibers and the receiving electrode in the CA3 pyramidal cell layer.

2.2.3. Proceed to the continuous acquisition protocol and record for 30 min.

3. PI uptake assay

NOTE: Cell death was assessed by monitoring the cellular uptake of the fluorescent dye propidium iodide (PI). PI is a polar compound, which enters cells with damaged cell membranes and interacts with DNA emitting red fluorescence (absorbance 493 nm, emission 630 nm). Since PI is not permeant to live cells, it is used to detect dead cells in a population.

3.1. PI incubation

3.1.1. Prepare, in culture medium, a fresh 1:10 dilution of PI stock.

3.1.2. For PI uptake assay remove the plate from the incubator and carefully raise the insert. Add 13 µL of PI to the medium, obtaining a final concentration of 2 µM, and agitate slowly the plate before putting the insert back in place. Make sure there are no bubbles beneath the slices.

3.1.3. Put the slices back in the 37 °C incubator for 4 h.

3.1.4. Proceed with the immunohistochemistry protocol, as described in the next section. Cover the plates with aluminium, since PI is light sensitive.

4. Immunohistochemistry

NOTE: In immunohistochemistry a neuron specific antibody, as well as antibodies able to discriminate resting and reactive phenotypes of microglia and astrocytes, were used to evaluate the extend of neuronal death and gliosis in rhinal cortex-hippocampus epileptic-like organotypic slices.

4.1. Tissue fixation

4.1.1. Remove the plate from the incubator and aspirate the medium. Fix the slices with 4% paraformaldehyde (PFA) for 1 h at RT, by adding 1 mL of PFA beneath and above the slices, with a pipette P1000.

4.1.2. Remove the PFA and add 1 mL of PBS. Also add PBS beneath and above the slices.

4.1.3. Keep the slices at 4 °C, in PBS, until further use. Always put parafilm around the plates to avoid drying.

4.2. Immunostaining steps

4.2.1. Wash twice, 10 min each time, with 1 mL of PBS.

4.2.2. Prepare permeabilization/blocking solution containing 1% Triton-X100, 10% HS and 10% BSA in PBS. Prepare 5% BSA solution.

4.2.3. Draw two rectangles with the hydrophobic pen (**Figure 2A**). Cut the slices from the insert (**Figure 2B**) with a highly sharp blade. Put two slices per slide (**Figure 2C**) and add 140 µL of permeabilization/blocking solution on the top of each slice, using a P200 pipette. Incubate for 3 h at RT.

4.2.4. Dilute the primary antibodies to the working dilution in 5% BSA in PBS. Incubate with the primary antibodies overnight at 4 °C.

4.2.5. Incubate with the secondary antibodies for 4 h at RT. From this step on, protect the plate from light since fluorophores are being worked with.

4.2.6. Place a 50 µL drop of Hoechst solution on the top of each slice and incubate for 20 min at RT.

4.2.7. Wash between incubations. Always wash three times, for 10 min each time, with PBS-T.

4.2.8. Remove Hoechst and wash as recommended.

4.2.9. Add 50 µL of mounting medium on the top of each slice. Cover with a glass coverslip and surround with nail polish (**Figure 2D**).

4.2.10. Let it dry at RT for 24 h.

4.2.11. Visualize the immunostaining under a confocal microscope. Keep the stained slices at -20 °C.

[Place Figure 2 here]

REPRESENTATIVE RESULTS:

Based on previous descriptions of epileptic signal analysis in organotypic hippocampal slices, interictal epileptiform discharges are here defined as paroxysmal discharges that are clearly distinguished from background activity, with an abrupt change in polarity and occurring at low frequency (<2 Hz). Paroxysmal discharges lasting more than 10 s and occurring at higher frequency (≥2 Hz) are characterized as ictal epileptiform activity. If an ictal event occurs within 10 s after the previous one, these two events are considered as only one ictal event.

Rhinal cortex-hippocampus organotypic slices at 7 DIV (**Figure 3A**) depict mixed interictal and ictal-like activity. At 14 DIV (**Figure 3B**), spontaneous activity is characterized by ictal discharges, which evolve to an overwhelming ictal activity at 21 DIV, with ictal events lasting >1 min (**Figure 3C**).

[Place Figure 3 here]

PI uptake assay followed by immunohistochemistry against the neuronal marker NeuN aimed at identifying neuronal death. PI uptake by granular and pyramidal neurons was observed in 7 DIV slices (arrows in **Figure 4A**), but the number of PI^+ neurons increased at 14 DIV (arrows in **Figure 4B**), corroborating an increased neuronal death with epileptogenesis progression.

[Place Figure 4 here]

A double staining of Iba1, together with CD68, was used to evaluate the M1 phenotype of microglia. Iba1 is a microglia/macrophages marker, while CD68 is a lysosomal protein expressed in high levels by activated M1 microglia and in low levels by resting microglia. At 7 DIV slices, ramified microglia with a low CD68 expression (arrows in **Figure 5A**) are more abundant than $Iba1^+/CD68^+$ reactive microglia (arrowheads in **Figure 5A**), whereas at 14 DIV, in all areas of the hippocampus, $Iba1^+/CD68^+$ bushy/amoeboid pro-inflammatory microglia (arrowheads in **Figure 5B**) exceed microglia with a low CD68 expression (arrows in **Figure 5B**). At 14 DIV some $Iba1^+/CD68^-$ cells with a hyper-ramification appearance can be pinpointed (open arrows in **Figure 5** - B4, B5, B6), which might suggest the occurrence of the M2 anti-inflammatory phenotype of microglia. However, this matter requires further study.

Recent studies demonstrated that different initiating CNS injuries can elicit at least two types of reactive astrocytes, A1 and A2, with A1 astrocytes being neurotoxic[16]. A1 subtype of astrocytes is characterized by an increased expression of Complement C3[16-18]. Complement C3, which plays a central role in the activation of the complement system, generates C3b, which is further degraded to iC3b, C3dg and C3d[19]. Thus, a double staining of GFAP and C3d was employed to assess astrogliosis. At 7 DIV the expression of C3d is barely detectable (**Figure 6A**), while in 14 DIV slices hypertrophic $GFAP^+/C3d^+$ astrocytes can be observed (arrowheads in **Figure 6B**), suggesting a progressive activation of A1 astrocytes.

Results demonstrate a progressive activation of microglia and astrocytes throughout the course of epileptogenesis, mimicking the events described in patients with epilepsy and in animal models of this pathology.

[Place Figure 5 here]

[Place Figure 6 here]

[Place Table 1 here]

Figure 1: Detailed procedure for the preparation of rhinal cortex-hippocampus organotypic slices. (**A**) Remove the brain from the head and place it in ice-cold GBSS with the dorsal surface faced up. (**B**) Insert the forceps into the cerebellum. Open the brain through the midline and remove the excess tissue over the hippocampus. (**C**) With a spatula cut below the hippocampus, as indicated by the arrows. (**D**) Place both hippocampi facing up and parallel to each other onto the filter paper and cut 350 µm slices on the tissue chopper. (**E**) Place the sliced hippocampus in ice-cold GBSS. (**F**) Separate the slices with the help of round tipped glass electrodes. (**G**) Choose only the slices that depict an intact rhinal cortex and hippocampus. (**H, I**) With the help of a round tipped glass electrode push each slice to the spatula and place it on the insert. (**J**) Remove the GBSS surrounding the slice. (**K**) Place four slices per insert. (**L**) To change the medium, lift the insert and aspirate the medium with a glass pipette. (**M**) Add fresh medium by placing the pipette between the insert and the walls of the 6-wells plate. Make sure there are no air bubbles beneath the slices.

Figure 2: Specific procedure for the immunohistochemistry assay. (**A**) With the hydrophobic pen draw two squares in the slide. (**B**) Cut the piece of insert that contains the slice. (**C**) Place each slice in the squares drawn with the hydrophobic pen and start the permeabilization/blocking step. (**D**) After concluding the protocol, finish by mounting the slices in mounting medium, covering with a glass coverslip and surrounding it with nail polish.

Figure 3: Spontaneous epileptiform activity of rhinal cortex-hippocampus organotypic slices. Representative electrographic seizure-like events, recorded from CA3 area in an interface-type chamber, after (**A**) 7 DIV, (**B**) 14 DIV and (**C**) 21 DIV. Seizure details are shown in lower traces.

Figure 4: Representative images of NeuN and PI stained rhinal cortex-hippocampus organotypic slices. Images of NeuN stained mature neurons and PI-positive cells were acquired at (**A**) 7 DIV and (**B**) 14 DIV, on a confocal laser microscope with a 20x objective. Magnified images of the dashed areas are shown. Arrows point to death neurons (in orange). Scale-bar, 50 µm.

Figure 5: Representative images of Iba1 and CD68 stained rhinal cortex-hippocampus organotypic slices. Images of Iba1 and CD68 stained microglia, and Hoechst stained nuclei, were acquired at (**A**) 7 DIV and (**B**) 14 DIV, on a confocal laser microscope with a 20x objective. Magnified images of the dashed areas are shown. Arrows point to ramified $Iba1^{+}/CD68^{-}$ microglia, arrowheads indicate $Iba1^{+}/CD68^{+}$ M1 microglia (in yellow) and open arrows reveal hyper-ramified microglia. Scale-bar, 50 µm.

Figure 6: Representative images of GFAP and C3d stained rhinal cortex-hippocampus organotypic slices. Images of GFAP and C3d stained astrocytes, and Hoechst stained nuclei, were acquired at (**A**) 7 DIV and (**B**) 14 DIV, on a confocal laser microscope with a 20x objective. Magnified images of the dashed areas are shown. Arrowheads point to $GFAP^{+}/C3d^{+}$ reactive A1 astrocytes (in yellow). Scale-bar, 50 µm.

DISCUSSION

Animal models of epilepsy have been crucial for the discovery of many AEDs, however they require many animals and most of them are time-consuming due to the latent period for seizure onset. The low-magnesium induction of epileptiform activity in hippocampal acute slices has also been thoroughly revised in the literature[3], but acute slices have a 6-12 h viability making it impossible to assess long-term changes. Organotypic slices can be maintain in culture from days to weeks, allowing to overcome the short viability time of acute slices, and models of epileptogenesis in organotypic hippocampal slices have been proposed[3,7,8].

Here we describe the preparation of organotypic slices, comprising the rhinal cortex and the hippocampus. These slices take 15-20 min to prepare per animal, starting from animal sacrifice until placement of slices onto the inserts, and 6-8 slices per hemisphere can be obtained. Extra care must be taken when opening the hemisphere to expose the hippocampus and when removing the tissue from the filter paper after slicing. Excess tissue above the hippocampus can also compromise the slice integrity during slicing.

Rhinal cortex-hippocampus organotypic slices depict an evolving epileptic-like activity resembling in vivo epilepsy. After one week in culture, most slices depict mixed interictal and ictal-like activity, which progresses to solely ictal-like events with time in culture. So far, we have recorded few interictal discharges in slices with 2-3 weeks. In this system, epileptic-like activity appears to develop faster than in organotypic hippocampal slices. This might be attributed to the presence of the rhinal cortex, which preserves most of the functional input to the hippocampus. To fully address this issue, a complete characterization of the epileptic signals displayed by these slices throughout time in culture, such as number and duration of ictal events, together with their amplitude and frequency, is currently being performed.

This system can be maintained in culture for more than three weeks, and mimics many molecular correlates of epilepsy, such as neuronal death, activation of microglia and astrocytes and increased production of pro-inflammatory cytokines[14], allowing a long-term characterization of these aspects. It also represents an easy-to-use screening platform, where pharmacological interventions targeting specific cellular pathways can be implemented and potential therapeutic targets can be tested. Undoubtedly, the system herein presented can help to further enlighten the mechanisms of epileptogenesis.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Bioimaging Unit of Instituto de Medicina Molecular João Lobo Antunes, for all the suggestions concerning image acquisition.

The work was funded by Fundação para a Ciência e Tecnologia (FCT) through Project PTDC/MED-FAR/30933/2017, Faculdade de Medicina da Universidade de Lisboa, and Project 952455-EpiEpiNet (Epileptogenesis and Epilepsy Network: from genes, synapses and circuits to pave the way for novel drugs and strategies)/H2020-WIDESPREAD-2020-5.

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Fisher, R. S. et al. Electrical stimulation of the anterior nucleus of thalamus for treatment of refractory epilepsy. *Epilepsia*. **51**, 899-908 (2010).

2. Loscher W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure*. **20**, 359-368 (2011).

3. Heinemann, U., Kann, O., Schuma, S. An overview of in vitro seizure models in acute and organotypic slices. In: Pitkänen A, Schwartzkroin PA, Moshé SL, editors. Models of seizures and epilepsy. Cambridge: Elsevier Academic Press. 35-44 (2006).

4. Sundstrom, L., Morrison, BIII, Bradley, M., Pringle. A. Organotypic cultures as tools for functional screening in the CNS. *Drug Discovery Today: Targets*. **10**, 993-1000 (2005).

5. Holopainen, I. E. Organotypic hippocampal slice cultures: a model system to study basic cellular and molecular mechanisms of neuronal cell death, neuroprotection, and synaptic plasticity. *Neurochemical Research*. **30**, 1521-8 (2005).

6. Noraberg, J. et al. Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *CNS Neurological Disorders Drug Targets*. **4**, 435-52 (2005).

7. Dyhrfjeld-Johnsen, J., Berdichevsky, Y., Swiercz, W., Sabolek, H., Staley, K. J. Interictal spikes precede ictal discharges in an organotypic hippocampal slice culture model of epileptogenesis. *Journal of Clinical Neurophysiology*. **27**, 418-24 (2010).

8. Chong, S. A. et al. Intrinsic Inflammation Is a Potential Anti-Epileptogenic Target in the Organotypic Hippocampal Slice Model. *Neurotherapeutics*. **15**, 470-488 (2018).

9. Li, Q., Han, X., Wang, J. Organotypic hippocampal slices as models for stroke and traumatic brain injury. *Molecular Neurobiology*. **53** (6), 4226-37 (2016).

10. Cho, S., Wood, A., Bowlby, M. R. Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics. *Current Neuropharmacology*. **5**, 19-33 (2007).

11. Humpel, C. Organotypic brain slice cultures: a review. *Neuroscience*. **305**, 86-98 (2015).

12. Doussau, F., Dupont, J. L., Neel, D., Schneider, A., Poulain, B., Bossu, J. L. Organotypic cultures of cerebellar slices as a model to investigate demyelinating disorders. *Expert Opinion on Drug Discovery*. **12** (10), 1011-22 (2017).

13. Vismer, M. S., Forcelli, P. A., Skopin, M. D., Gale, K., Koubeissi, M. Z. The piriform, perirhinal, and entorhinal cortex in seizure generation. *Frontiers in Neural Circuits*. **9**, 27 (2015).

14. Magalhães, D. M., Pereira, N., Rombo, D. M., Beltrão-Cavacas, C., Sebastião, A. M., Valente, C. A. Ex vivo model of epilepsy in organotypic slices - a new tool for drug screening. *Journal of Neuroinflammation*. **15**, 203 (2018).

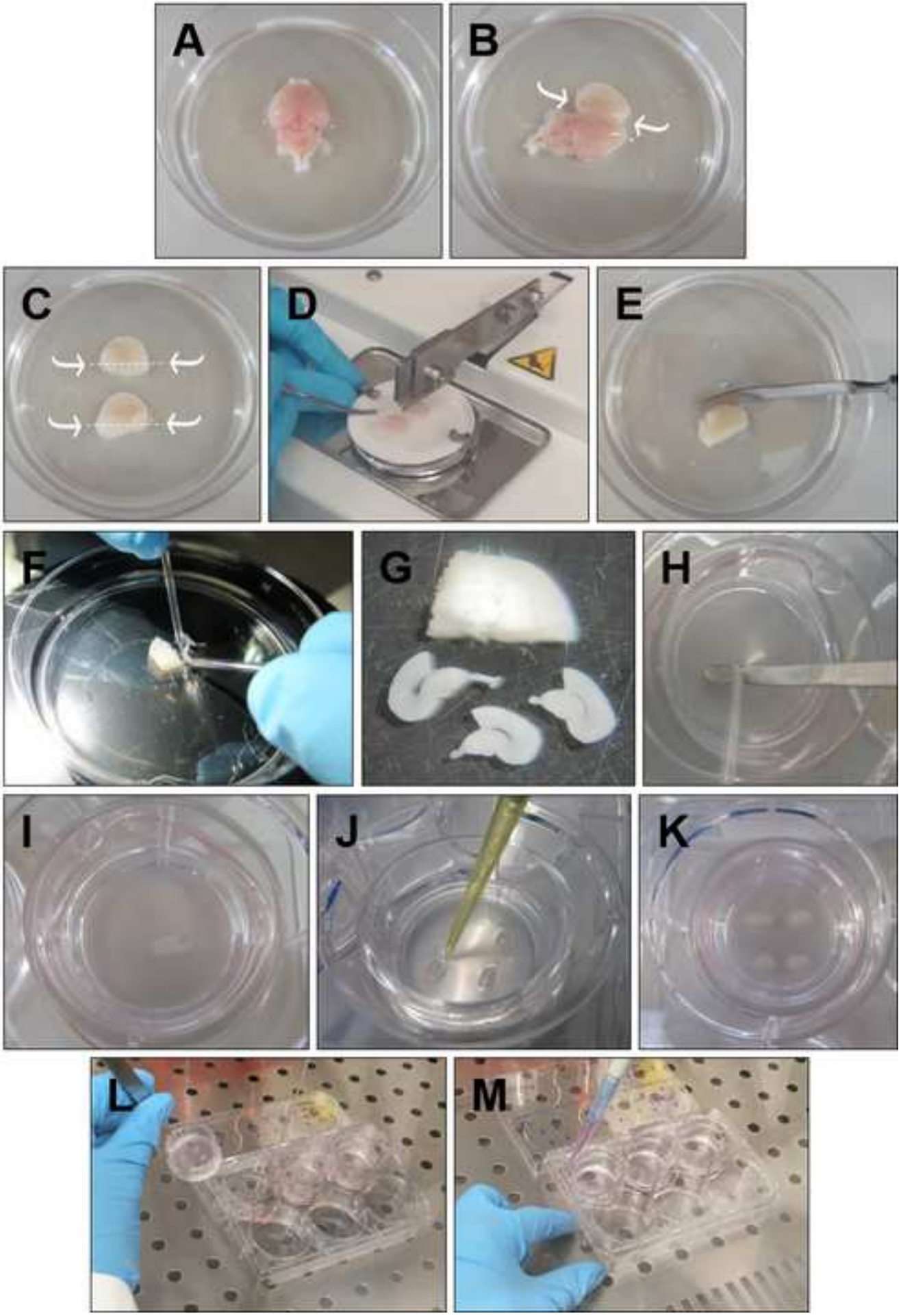
15. Ravizza, T., Balosso, S., Vezzani, A. Inflammation and prevention of epileptogenesis. *Neuroscience Letters.* **497** (3), 223-30 (2011).

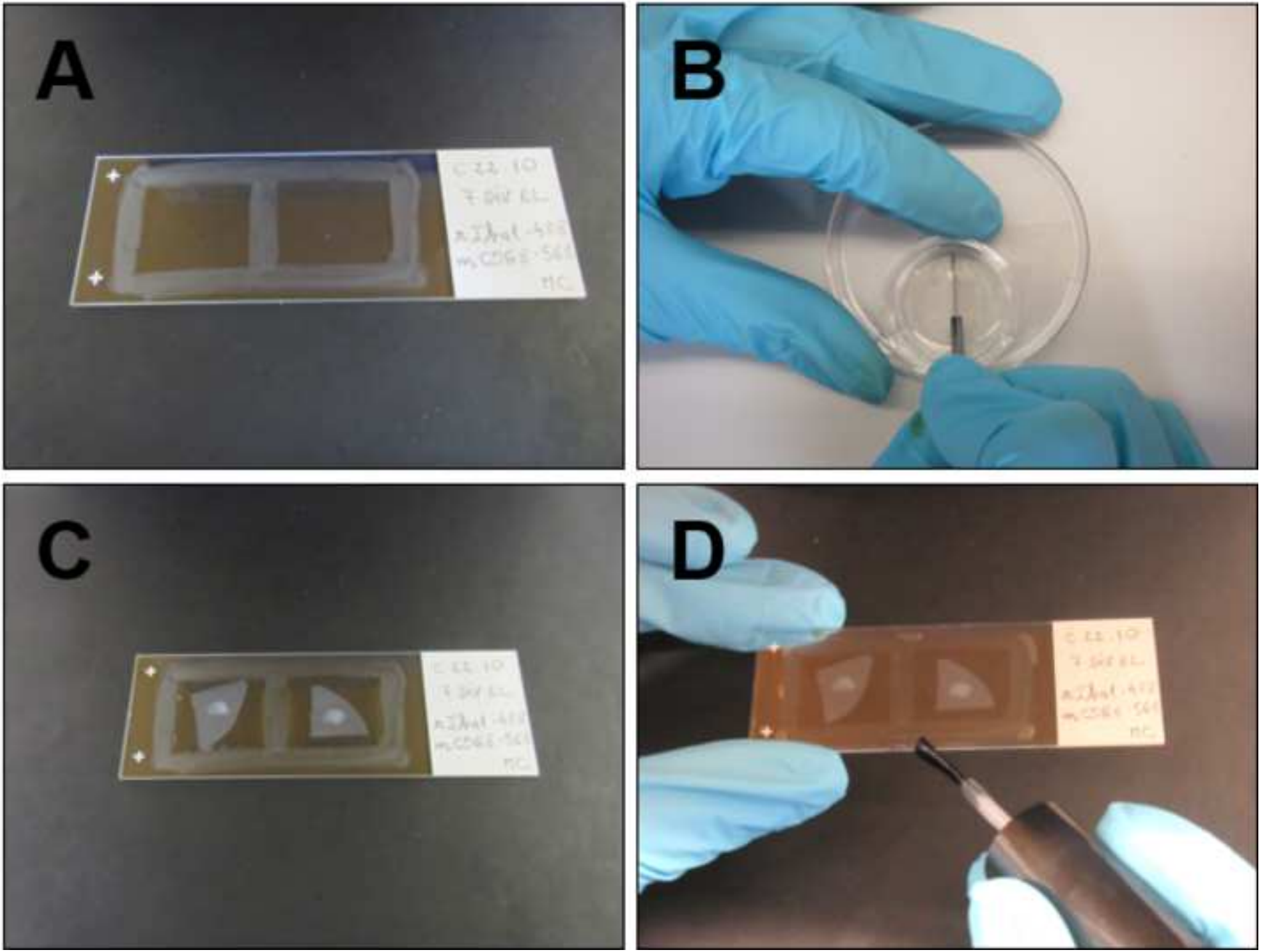
16. Liddelow, S. A. et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*. **541**, 481-487 (2017).

17. Wu, T. Complement C3 is activated in human AD brain and is required for neurodegeneration in mouse models of amyloidosis and tauopathy. *Cell Reports*. **28** (8), 2111-2123 (2019).

18. Hartmann, K. et al. Complement 3^{+}-astrocytes are highly abundant in prion diseases, but their abolishment led to an accelerated disease course and early dysregulation of microglia. *Acta Neuropathologica Communications*. **7**, 83 (2019).

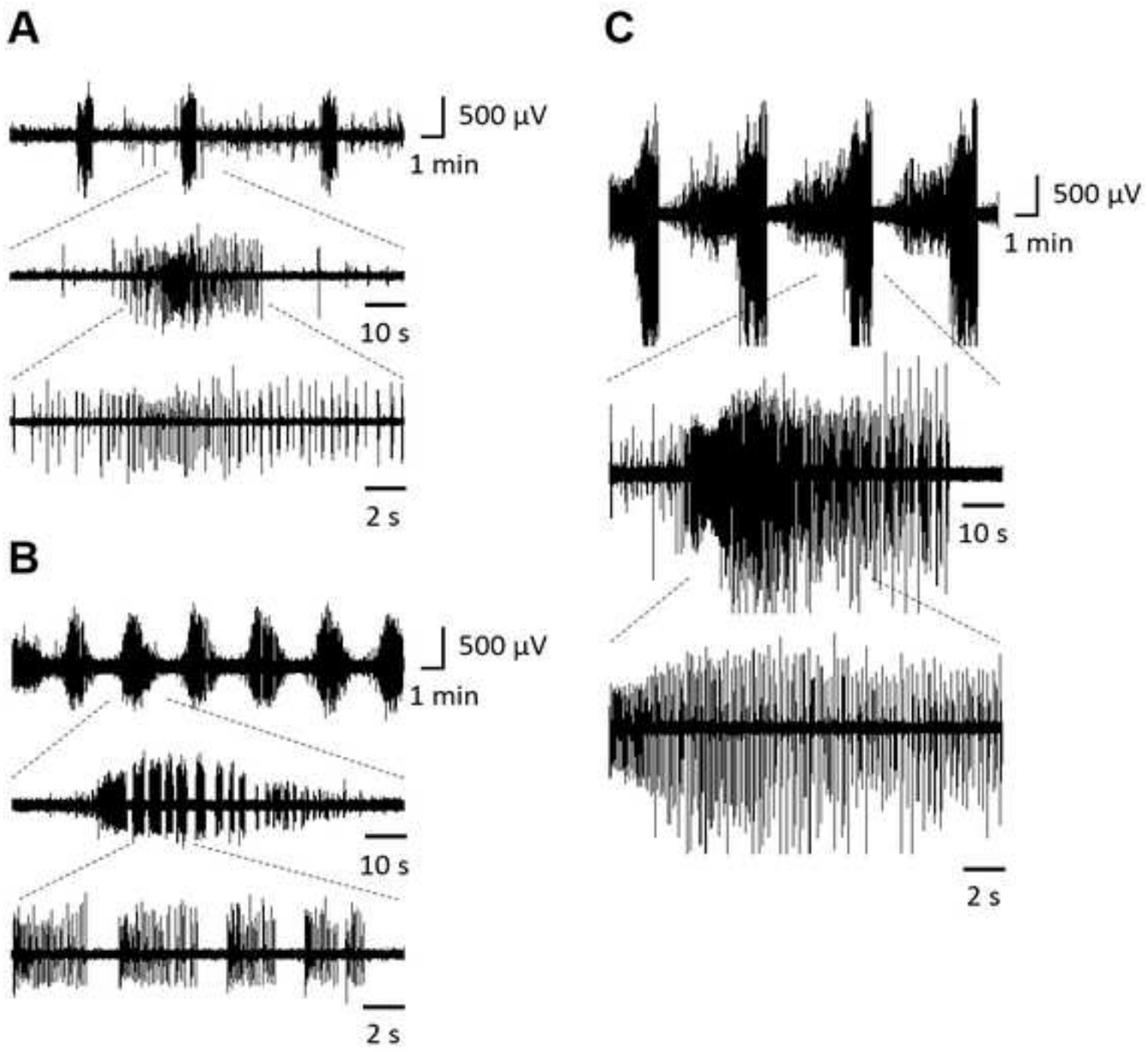
19. Nilsson, U. R., Funke, L., Nilsson, B., Ekdahl, K. N. Two conformational forms of target-bound iC3b that distinctively bind complement receptors 1 and 2 and two specific monoclonal antibodies. *Upsala Journal of Medical Sciences*. **116**, 26-33 (2011).

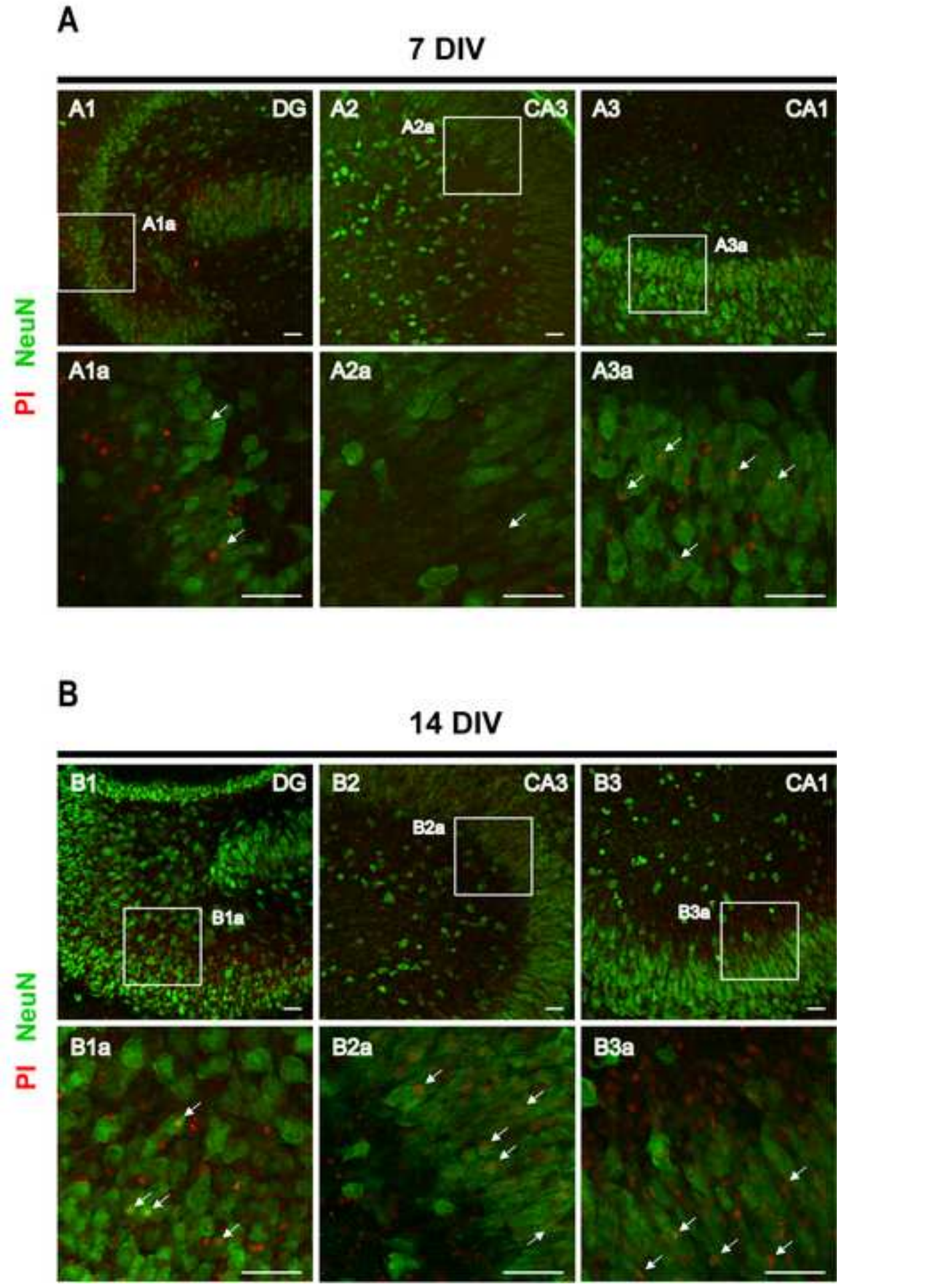


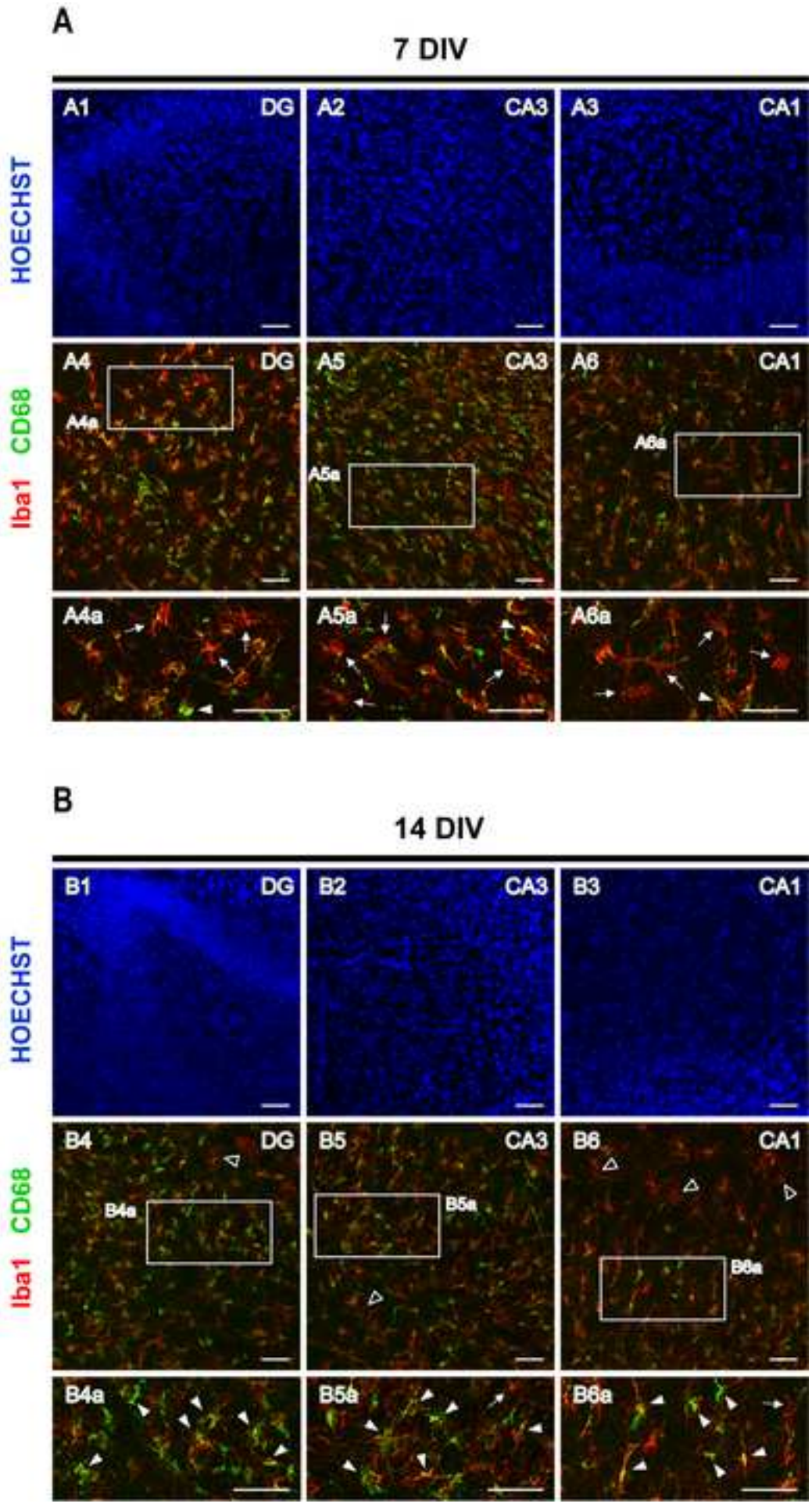


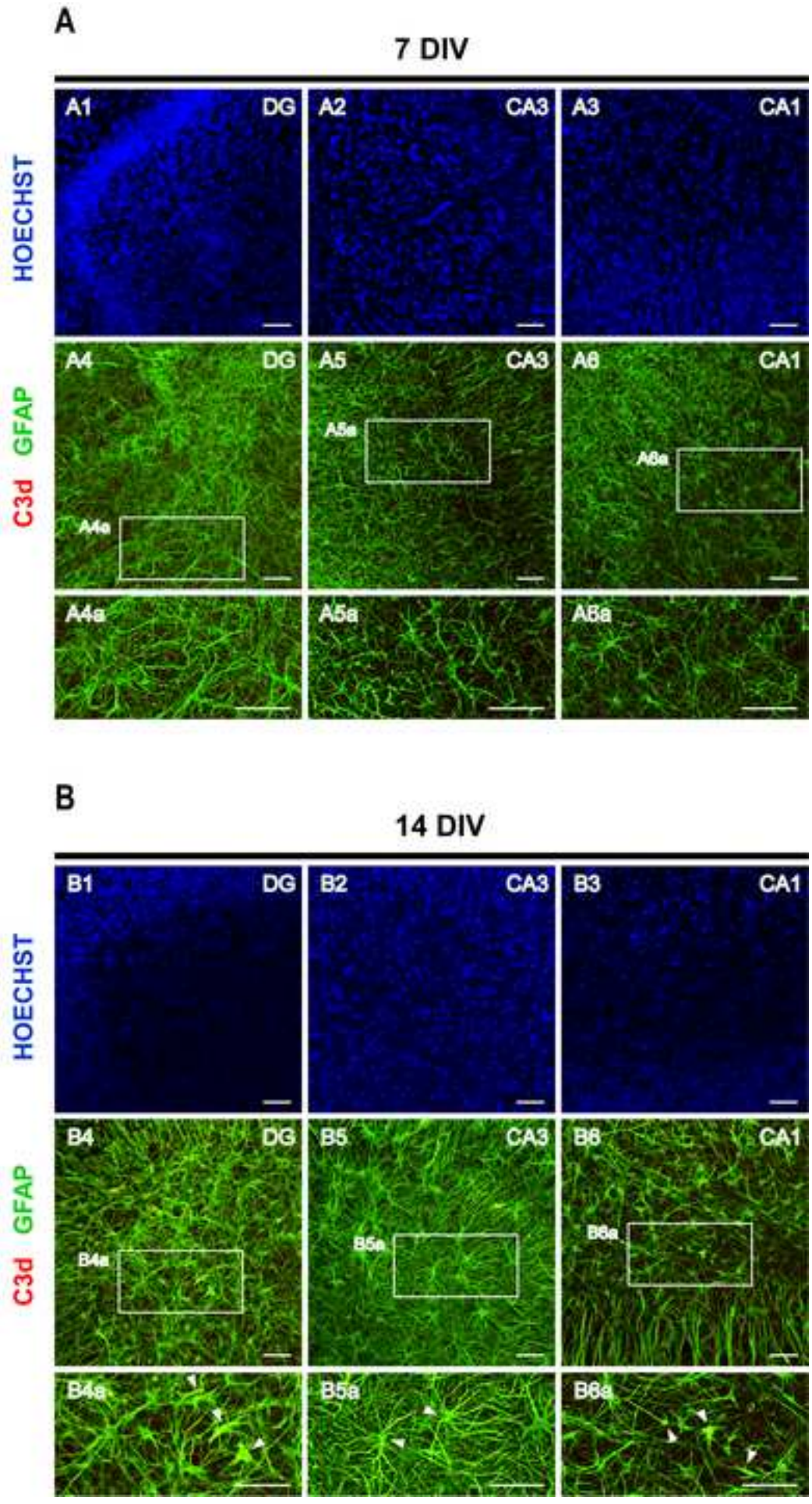
Recordings of spontaneous activity

Click here to access/download;Figure;Figure 3.tif









List of materials and equipments used

Click here to access/download;Table of Materials;Material and Equipment Table.xlsx

Name of Material/ Equipment	Company
50 mL Centrifuge Tube, Conical Bottom	Corning
70% Ethanol	Manuel Vieira, Lda
Amplifier	Axon Instruments
Amplifier	Axon Instruments
Anti-C3d (goat)	R&D Systems
Anti-CD68 (mouse)	Abcam
Anti-GFAP (mouse)	Millipore SAS
Anti-Iba1 (rabbit)	Abcam
Anti-NeuN (rabbit)	Werfen
Artificial cerebrospinal fluid (aCSF)	
B-27™ Supplement (50X), serum free	Thermo Fisher Scientific
Blades for scalpel handle	Fine Science Tools
Bovine Serum Albumin (BSA)	NZYTech
Brain/Tissue Slice Chamber System	Warner Instruments
Calcium chloride dihydrate	Merck Millipore
Cell culture inserts, 30 mm, hydrophilic PTFE	Millipore SAS
Cold light source	SCHOTT
Confocal laser microscope	Zeiss
Conventional incubator	Thermo Scientific Heraeus
D(+)-Glucose monohydrate	Merck Millipore
D-(+)-Glucose solution, 45% in water	Sigma
di-Sodium hydrogen phosphate dihydrate	Merck Milipore
Dissecting microscope/magnifier	MEIJI TECHNO CO. LTD
Donkey anti-goat IgG (H+L) coupled to Alexa Fluor 568	Invitrogen
Donkey anti-mouse IgG (H+L) coupled to Alexa Fluor 488	Invitrogen
Donkey anti-mouse IgG (H+L) coupled to Alexa Fluor 568	Invitrogen
Donkey anti-rabbit IgG (H+L) coupled to Alexa Fluor 488	Invitrogen
Donkey anti-rabbit IgG (H+L) coupled to Alexa Fluor 568	Invitrogen
Dumont #5 Fine Forceps Biologie Inox	Fine Science Tools
Dumont #5 Forceps Standard Inox	Fine Science Tools
Dumont #7 Forceps Standard Dumoxel	Fine Science Tools

Dumont Medical #7S Forceps Short Curve Inox	Fine Science Tools
Gentamycin stock solution, 50 mg/mL	Thermo Fisher Scientific
Gey's Balanced Salt Solution (GBSS)	Biological Industries
Glass Electrodes	Science Products
Glass Pasteur pipettes, 230 mm	VWR International
Hank's Balanced Salt Solution (HBSS)	Thermo Fisher Scientific
Hoechst 33342	Invitrogen
Horse Serum, Heat Inactivated (HS)	Thermo Fisher Scientific
Hydrochloric acid	Merck Milipore
Hydrophobic Pen	Dako
INCU-Line IL10	VWR
Interface chamber	Warner Instruments
Iris Spatula Curved	Fine Science Tools
Labculture Class II Biological Safety Cabinet	HERASafe
Lens Cleaning Paper	TIFFEN
L-Glutamine solution 200 mM (Q)	Thermo Fisher Scientific
Magnesium sulfate heptahydrate	Merck Millipore
Micro tube 0.5 mL, PP	SARSTEDT
Micro tube 1.5 mL, PP	SARSTEDT
Micro tube 2.0 mL, PP	SARSTEDT
Micromanipulators	Sutter Instrument
Miroscope Cover Glasses, 24 mm x 60 mm	Marienfeld
Nail polish	Cliché
Neurobasal-A Medium (NBA)	Thermo Fisher Scientific
Opti-MEM® I Reduced-Serum Medium	Thermo Fisher Scientific
Paraformaldehyde, powder	VWR Chemicals
Peristaltic pump	Gilson
Phosphate saline buffer (PBS)	
Phosphate standard solutions, PO_4^{3-} in water	BDH ARISTAR
Pipette set	Gilson
Platinum 5 blades	Gillette
Potassium chloride	Sigma-Aldrich

Propidium iodide (PI)	Sigma-Aldrich
Qualitative Filter Paper, Cellulose, Grade 1, 55 mm	Whatman
Qualitative Filter Paper, Cellulose, Grade 1, 90 mm	Whatman
Scalpel handle	Fine Science Tools
Slip Tip Insulin Syringe without Needle 1 mL	SOL-M
Sodium chloride	VWR Chemicals
Sodium dihydrogen phosphate monohydrate	Merck Millipore
Sodium hydrogen carbonate	Merck Millipore
Sodium Hydroxide	Merck Milipore
Stimulator	Astro Med Inc GRASS Product Group
Student Scissors Straight SharpSharp 12cm	Fine Science Tools
SuperFrost Plus™ Adhesion slides	Thermo Fisher Scientific
TC-Treated Sterile 60 x 15mm Tissue Culture Dish	Corning
TC-Treated Sterile 6-Wells Plates	Corning
Temperatue controller	MEDICAL SYSTEMS CORP.
Tissue Chopper	The Mickle Laboratory Engineering CO. LTD.
Triton X-100	BDH
Tween-20	Sigma

Catalog Number

430829

UN1170

Axoclamp 900A

Digidata 1440A

AF2655

ab31630-125ug

MAB360

ab108539

16712943S

17504-044

10011-00

MB04602

1.02382.0500

PICM03050

KL 300 LED

LSM 710

BB15, Function Line

1.08342.1000

G8769

1.06580.1000

122285

A11057

A21202

A10037

A21206

A10042

11254-20

11251-20

11271-30

11273-22
15750-037
01-919-1A
GB150F-10
612-1702
24020-091
H1399
26050-088
1.09057.1000
S200230-2
390-0384
BSC-HT Haas Top
10092-12
HS 12

25030-024
1.05886.0500
72,699
72.690.001
72.691
MP-285
102242

10888-022
31985-047
28,794,295
M312

452232C
P2, P10, P20, P100, P200, P1000

P5405-250g

P4170-25MG
1001-055
1001-090
91003-12
161000
27800.360
1.06346.1000
1.06329.1000
535C549998
S48 Stimulator
91402-12
J1800AMNZ
CORN430166
CORN3516
TC-102
MTC/2
14630
P2287

Comments/Description
Dilute at a ratio 1:1000
Dilute at a ratio 1:250
Dilute at a ratio 1:500
Dilute at a ratio 1:600
Dilute at a ratio 1:500
Homemade
5% BSA is used to dilute the primary antibodies. Add 0.5g BSA in 10 mL PBS.
Set to 37 °C and 5% CO_2
Dilute at a ratio 1:200
Dilute at a ratio 1:200
Dilute at a ratio 1:200
Dilute at a ratio 1:500
Dilute at a ratio 1:500

Round tips homemade

Stock solution at 2 mg/mL in PBS

Homemade. PBS with 0.5% Tween-20 (PBS-T) is used to wash slices during the immunohistochemistry assay.

Stock solution at 1 mg/mL in water.

Medium retention 11µm

Medium retention 11µm

Set to 350 µm

April 15th, 2020

Dear Doctor Nam Nguyen,

Attached please find our revised version of the manuscript JoVE61330 entitled "A Model of Epileptogenesis in Rhinal Cortex-Hippocampus Organotypic Slice Cultures" by Cláudia A. Valente, Francisco J. Meda, Mafalda Carvalho and Ana M. Sebastião, for reconsideration by The Journal of Visualized Experiments.

All points raised by the Editorial Office and Reviewers #1, #3 and #4 were addressed. Reviewer #2 asked to confirm that the recordings were epileptic-like events by applying standard antiepileptic drugs to the system. Unfortunately, due to the worldwide epidemic situation, Instituto de Medicina Molecular João Lobo Antunes is closed, with no prediction of reopening. To better show the occurrence of an evolving epileptic-like activity, Fig. 3 now shows the spontaneous activity of rhinal cortex-hippocampus organotypic slices after 7, 14 and 21 days in culture and the definition of interictal and ictal-like epileptiform activity considered by the authors was also included in the manuscript. If the experiment asked by Reviewer #2 is still considered of utmost relevance to the paper, I am forced to ask for much more time to do it.

Furthermore, Reviewers complained about the poor quality of the representative images for the immunohistochemical assays, which I believe is attributed to the PDF that was sent for review. If this is not the case, please let me know, so that I contact Bioimaging Unit to find out what might have occurred. Following their advice, z-stacks were acquired in Zeiss 710 Confocal microscope and the maximum intensity projection was obtained in the ZEN software (from Zeiss). Panels were prepared in Adobe Illustrator and exported having into consideration the 300 dpi quality.

I hope that now our MS proves suitable for publication in The Journal of Visualized Experiments.

Yours sincerely,

Cláudia Valente de Castro

Cláudia Alexandra dos Santos Valente de Castro, PhD
Researcher / Invited Assistant Professor
Instituto de Farmacologia e Neurociências
Faculdade de Medicina da Universidade de Lisboa

Email: cvalentecastro@medicina.ulisboa.pt
Tel: +351 217985183

Response to Reviewers

Editorial comments

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.

Answer. The authors have revised the whole manuscript carefully.

2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 33-40, 59-66, 70-80, 82-85.

Answer. The referred sentences were restructured.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Answer. This issue was addressed.

4. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Answer. The authors have changed the highlighted protocol steps.

5. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Answer. The highlighted steps were revised. However, the authors have always excluded the references to forceps, blades and pipettes which appear in the middle of some sentences.

6. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Answer. The discussion is now more extensive.

Reviewer #1

This manuscript introduces experimental methods of rat rhinal cortex-hippocampal organotypic slices as a model of epileptogenesis. The described culture and relevant experimental methods are useful for experimenters to set up such a model. I believe the methods and the results are reliable and can be reproducible. However, the manuscript needs to be revised to improve the quality.

Major Concerns:
1. Although this is a method article, the information given in the introduction and discussion sections are limited. The authors claimed that 'in-vivo like' epileptic slices can be obtained by a gradual deprivation of serum in the medium that is a key and unique part of this method I believe. I am wondering whether the authors have used control groups (using one of the standard OHSC medium- either MEM with serum- or Neurobasal with B27-based) to compare with their method and to show their method can produce reliable epileptogenesis closer to the in vivo situation. So far most published articles for the in vitro epileptogenesis slice models use the serum free medium (Neurobasal based), therefore this comparison will be necessary to justify the method. If there is previously published data from the authors for example, it will be useful to mention in Introduction or Discussion.
Answer. Rhinal cortex-hippocampus organotypic slices were not viable upon an abrupt change to serum-free medium at 3 DIV, as described in pre-existing models. This was extensively tested by the authors and hence the gradual serum deprivation was implemented. Furthermore, the authors have shown in Magalhães et al., 2018 (reference #14), that slices maintained in serum-containing medium depicted a physiological activity. This remark is now included in the Introduction.

2. The results shown here is from slices cultured up to 2 weeks. The author claim that this model can be used for new target and drug discovery in epileptogenesis and neuroinflammation. 2 weeks can be little short to design an experiment to test drugs to see efficacy in the epileptogenesis. It would be useful to share more background information (or at least to address in the discussion section) about, 1) how long can these slices be maintained in the culture? 2) What proportion of slices is expected to show interictal and ictal in different time points? 3) Are there any long-term characterization data regarding neuroinflammtion and epileptiform activity? 4) If these cultures cannot be maintained for more than 2 weeks, this has to be mentioned as a limitation of the method in the discussion section.

Answer. These cultures are always maintained until 3 weeks. Fig. 3 now has a representative trace of the spontaneous epileptiform activity depicted by a 21 DIV slice.

Minor Concerns:

1. In general, the manuscript is not well reviewed by the authors. The sentences need to be polished.

Answer. The manuscript was revised by the authors and some sentences were refined.

2. Fig. 1A does not give any information compared to 1B. The respective sentences (line 141) is not relevant to Fig.1A

Answer. Fig. 1A was eliminated from the panel. The former Fig. 1B is now Fig. 1A and all figures were renamed accordingly in the panel and in the manuscript.

3. For beginners, it will be hard to understand the steps of Fig. 1C and 1D. However, I assume that a video material will help.

Answer. Indeed, the video will help to better understand these steps.

4. Reference need to be checked in line 171 (normally it should be numbered)

Answer. Reference was replaced by the adequate number.

5. In line 186, it is not clear what ' close circuit' means. Is it an electronic circuit or perfusion flow circuit? Because you mentioned flow rate 2mL/min...

Answer. “Close circuit” means that the medium is in a container and after passing through the system, tubing and interface chamber, returns to the same container. Flow rate is set at 2mL/min.

6. I do not understand line 191. It need to be rephrased.

Answer. The electrophysiology set-up was first perfused with water, to ensure that there were no leaks throughout the tubing. After 15 min, the perfusion fluid was changed to pre-warmed Neurobasal A. To avoid confusions the sentence "Switch to the previously prepared NBA medium" was removed.

7. I found that Fig. 3B does not give any value. It can be removed

Answer. Fig. 3 aims to show the representative electrographic seizure-like events throughout time in culture. Fig. 3A shows the representative discharges recorded from CA3 region of the hippocampus after 7 DIV, while Fig. 3B depicts the discharges at 14 DIV. Fig. 3 now has a Fig. 3C that illustrates the recordings obtained at 21 DIV.

8. For interical and ictal activity the authors can mention what analysis criteria they used based on what background information

Answer. Based on previous descriptions of epileptic signal analysis in organotypic hippocampal slices, the authors have defined interictal epileptiform activity as paroxysmal discharges that are clearly distinguished from background activity, with an abrupt change in polarity and occurring at low frequency (<2 Hz). Paroxysmal discharges lasting more than 10 s and occurring at higher frequency (≥2 Hz) are characterized as ictal epileptiform activity. If an ictal event occurs within 10s after the previous one, the authors have considered the two events as only one ictal event. These definitions are now included in the manuscript (line 267).

9. In Fig. 5A and B, it is not clear to see the difference between 7 DIV and 14 DIV. The morphology indicated by arrows and arrowheads is not very visible even in the magnified images.

Answer. Arrows point to ramified microglia with low CD68 expression. According to the fluorophores used in the immunohistochemistry assay these $Iba1^{+}/CD68^{-}$ microglia are bright red and are much more abundant at 7 DIV. Arrowheads indicate less ramified or bushy/amoeboid microglia with higher CD68 expression. These $Iba1^{+}/CD68^{+}$ microglia have greenish or yellow regions and can be observed at 14 DIV.

The authors believe that the difficulty in seeing these morphologies is attributed to the PDF that was sent for review. Z-stacks were acquired in Zeiss 710 Confocal microscope and the maximum intensity projection was obtained in the ZEN software (from Zeiss). Panels were prepared in Adobe Illustrator and exported having into consideration the 300 dpi quality.

10. The first sentence in the discussion section (line 360) is completely irrelevant.

Answer. This sentence was removed.

Reviewer #2:

Manuscript Summary:
In the present study, authors introduced a method for preparing the rhinal cortex-hippocampus organotypic slice cultures in which they induced epileptiform activity and claimed that it is a good model for investigating "the dynamics and progression of epileptogenesis" and wrote that this model "can used for a deeper understanding of the mechanisms of epileptogenesis onset and progression". Although they did not pay to these aspects of epileptogenesis and just re-confirmed "a progressive 303 activation of microglia and astrocytes throughout the course of epileptogenesis", their method is also very useful for finding the mechanisms of epileptogenesis. On the whole the study seems to perform carefully and many aspects of methods have been considered carefully.

Major Concerns:
1- In the title and whole text it is better to write "organotypic brain slice culture" instead of "organotypic brain slices".
Answer. This alteration was addressed in the title and in some other sentences throughout the manuscript.

2- The present study is very similar to a previous study that authors referred to it (Magalhães, D.M., Pereira, N., Rombo, D.M. et al. Ex vivo model of epilepsy in organotypic slices—a new tool for drug screening. J Neuroinflammation 15, 203; 2018). The similarities between these two studies is so high that it is necessary to write exactly and precisely what is the innovation of the present study. In addition, they have to explain the methodologically differences between the present study and Magalhães et al. Meanwhile, in the "Culture maintenance", section 4.5., it is necessary to write briefly the method of epileptiform activity induction (controlled deprivation of serum in the medium).
Answer. The first author of the present study, Cláudia A. Valente, is the corresponding author of the paper Magalhães et al., 2018. There was an invitation from JoVE to better illustrate the model of epileptogenesis presented in *J Neuroinflammation*. Thus, there are no methodological differences between the present study and the one described in Magalhães et al.
Regarding the serum deprivation protocol, Opti-MEM medium supplemented with 25% of horse serum (HS) is used to maintain the slices up to 3 DIV. In the first medium exchange (3 DIV), Opti-MEM based medium is replaced by Neurobasal A (NBA) supplemented with 2% B27, 1 mM L-glutamine (Q) and 15% HS, in the second medium exchange (5 DIV) NBA/10% HS is added to slices, at 7 DIV NBA/5% HS is used and from 9

DIV on, slices are kept in NBA with no HS. Thus, HS decreases from 25% (at 3 DIV) to 0% at 9 DIV.

3- Introduction, Line 3: The authors wrote that "30-40% of patients are refractory to therapy and continue to experience seizures". However, according to most of references this percentage is 20-30%. Please correct it and cite to a valid and suitable reference.
Answer. The authors changed the sentence to *"one-third of patients with epilepsy are refractory to therapy and continue to experience seizures".* Reference #1, Fisher et al., 2010, was added to the manuscript. All the other references were changed accordingly.

4- Introduction, Paragraph 2, Lines 3-4: Authors claimed that "animal models of epilepsy are expensive and time-consuming" and concluded that organotypic brain slices are better to be used. I think it is not a good reason for encouraging the researcher to use organotypic slices. Having a mouse or rat and using its brain slices is not more expensive than preparing an organotypic slice. In addition, it is possible to induce epileptiform activity in brain slices easily by using high-K+ or zero-Mg2+ artificial cerebrospinal fluid, it is not time-consuming. Please write logic reasons here.
Answer. Introduction now explains better the various models of epilepsy.

5-In "Immunostaining steps", section 2.3., figure citations are wrong! This section relates to figure 2; but not figure 3.
Answer. Figure citations were corrected.

6-To confirm that recorded LFPs are epileptiform activity, the authors can apply one or two standard antiepileptic drugs and show their effectiveness.
Answer. At the moment, the application of antiepileptic drugs to the system cannot be performed. Unfortunately, due to the worldwide epidemic situation, Instituto de Medicina Molecular João Lobo Antunes is closed, with no prediction of reopening.
To better show the occurrence of an evolving epileptic-like activity, Fig. 3 now shows the spontaneous activity of rhinal cortex-hippocampus organotypic slices after 7, 14 and 21 days in culture. The definition of interictal and ictal-like epileptiform activity was also included in the manuscript (line 267).

7- In "Electrophysiological recordings", section 2.3., why did the authors stimulated the slice with 1-4 V? If the "controlled deprivation of serum in the medium" leads to epileptiform activity, they have to record only spontaneous LFPs. Please explain it.
Answer. The authors did not stimulate these slices, only recorded the spontaneous activity.

Minor Concerns:

-in "2. Brain harvesting" section, change numbering 2.1.1. to 2.1.

-All abbreviations (e.g. DIV etc) needs tone written in complete form when appeared in the text for the first time.

Answer. These issues were addressed.

Reviewer #3:

Manuscript Summary:

This protocol describes the preparation of rhinal cortex + hippocampal slice cultures as well as procedures for recording from them electrophysiologically and techniques for quantifying cell death and neuroinflammation.

Major Concerns:

1. The manuscript focuses on the aspect of the protocol wherein the concentration of horse serum in the culture medium is gradually decreased over the first nine days in vitro. It's not clear from this manuscript or the referenced Magalhães et al that HS depletion produces different levels of epileptiform activity vs control slices (only representative traces are shown from single slices). Published literature on hippocampal slice cultures demonstrates epileptogenesis without modifications to the culture medium (https://doi.org/10.1016/0165-0270(89)90051-4) and in a wide variety of serum-free media formulations (https://doi.org/10.1371/journal.pone.0172677). It is possible that slices including the rhinal cortices are different, necessitating the additional perturbation of serum-deprivation, but I could find no such evidence. Presumably other groups would find the same, limiting the utility of this protocol in it's current form.

Answer. Indeed, the authors based their model in pre-described models of epileptogenesis in organotypic slices, but the model presented here is different from those. It is different because it uses rhinal cortex-hippocampus organotypic slices, instead of hippocampal organotypic slices, and because it involves a gradual deprivation of serum, instead of an abrupt change to serum-free medium. Rhinal cortex-hippocampus organotypic slices were not viable upon an abrupt change to serum-free medium at 3 DIV, as described in pre-existing models. This was extensively tested by the authors and hence the gradual serum deprivation protocol was implemented.

A complete characterization of the epileptic signals displayed by these slices throughout time in culture, such as number and duration of ictal events, together with their amplitude and frequency, is currently being performed. As there are several animal models of epilepsy (thoroughly revised in https://doi.org/10.1124/pr.110.003046 and

https://doi.org/10.1007/s11064-017-2222-z), this model of epileptogenesis in rhinal cortex-hippocampus organotypic slices can become an alternative to other models already published in hippocampal slices.

2. The abstract and introduction suggest that organotypic slice cultures exhibit no difference in synaptic connectivity. However the literature shows robust dendritic sprouting (https://dx.doi.org/10.1113%2Fjphysiol.2003.039099) and a >20-fold increase in the fraction of interconnected CA3 pyramidal cells (https://doi.org/10.1152/jn.1995.73.3.1282). This reflects the authors' apparent bias towards neuro-inflammation (vs. synaptic re-organization) as the mechanism of epileptogenesis.

Answer. The authors have altered the introduction.

3. It is likely that inflammation is related to epileptogenesis. And, it does seem that serum deprivation increases cell death and inflammatory markers. But it is unclear that this enhanced inflammation is necessary or contributes to epileptogenesis. Epileptogenesis in unchanged culture medium is viewed as a model of deafferenting injury, wherein the deafferentation comes from slicing. It is unclear what pathophysiological process serum deprivation is modelling.

Answer. In this model, the authors believe that epileptogenesis is not only driven by deafferentation injury. If it were, all slices would demonstrate epileptic-like activity. As the authors explained in Magalhães et al. 2018, most rhinal cortex-hippocampus organotypic slices, maintained in a serum-based medium do not display paroxysmal discharges, precluding the possibility that slice preparation per se could be the only cause for spontaneous activity. The pathophysiological process that serum deprivation is modelling is currently under study.

Overall, the protocols for slice preparation, recording, and staining are clearly presented. But the utility of the serum-deprivation protocol as a model of epileptogenesis is low, unless the authors can provide statistical evidence of this. Perhaps if this were presented as a model of enhanced neuroinflammation, it would find broader acceptance?

Answer. As already said, the authors verified that rhinal cortex-hippocampus organotypic slices were not viable upon an abrupt change to serum-free medium at 3 DIV, as described in hippocampal slices, and decided to remove the serum gradually. However, the biological process that serum deprivation, either abrupt (as in other models) or gradual (as in this model), is modelling is still unknown.

Neuroinflammation is a molecular correlate of epilepsy, but nowadays is also considered a potential cause for this pathology. Indeed, this model mimics several

neuroinflammatory features of epilepsy and thus might open new horizons for investigating the contribution of inflammatory pathways to epileptogenesis.

Minor Concerns:

1. Is the horse serum replaced with something in the serum deprivation protocols? If not, is there substantial osmolarity shift during the deprivation?

Answer. Yes, Opti-MEM medium supplemented with 25% of horse serum (HS) was used to maintain the slices up to 3 DIV. From the first medium exchange, at 3 DIV, Opti-MEM based medium is replaced by Neurobasal A supplemented with 2% B27, 1mM Q and decreasing concentrations of HS until it reaches 0% HS at 9 DIV.

Regarding osmolarity, the authors have not measure it.

2. The overall quality of the figures is low (pixellation of subpanel labels, etc.) - this may just be an artifact in the pdf?

Answer. The authors believe that this is attributed to the PDF that was sent for review. Z-stacks were acquired in Zeiss 710 Confocal microscope and the maximum intensity projection was obtained in the ZEN software (from Zeiss). Panels were prepared in Adobe Illustrator and exported having into consideration the 300 dpi quality.

Reviewer #4:

Manuscript Summary:

The manuscript of Valente and colleges describes the preparation and maintenance of combined rhinal cortex-hippocampus slice cultures with a serum free medium formulation following the Stoppini method (1991). In addition they describe ongoing changes associated with spontaneous epileptogenesis.

The description of the electrophysiology, the culturing as well as the (immuno)fluorescent labeling procedures of the diverse cell types are clear and concise and there is enough information to successfully repeat the procedure in any other lab. The major findings i.e. ongoing cell loss, microglial activation and transformation of astrocytes open up new horizons for investigating the inflammatory pathways of epileptogenesis.

Major Concerns:

The abstract of the manuscript implicates (page 3 lines 43-44) that the serum free procedure (over other slice culture protocols) would be critical for the spontaneous epileptogenesis (see also Magalhaes et al., 2018). Indeed, serum deprivation was shown

to induce ROS formation and subsequent cell loss and might therefore account for many of the observed changes in microglia activation and network reorganisation underlying the appearance of the spontaneous activity. However, increased seizure-susceptibility and the occurrence of spontaneous epileptiform activity (especially in slices aged >DIV14) have been described in several laboratories using serum containing media formulations.

Is the present model simply accelerating the degradation of the cultures or does it represent a specific mechanism with regard to epileptogenesis?
It is to note that the presence of a certain type of network activity in the recording chamber (with a completely different aCSF) does not indicate presence or absence of the same activity in the incubator (Albus et al., 2013).

Answer. Several researchers have shown the occurrence of spontaneous activity in hippocampal organotypic slices, but most of them change slices at 2-3 DIV to a serum-free medium. Rhinal cortex-hippocampus organotypic slices were not viable upon an abrupt change to serum-free medium at 3 DIV, as described in pre-existing models. This was extensively tested by the authors and hence the gradual serum deprivation was implemented. The pathophysiological process that serum deprivation is modelling is currently under study.

In this system, epileptic-like activity seems to evolve faster than in published models of epileptogenesis in organotypic hippocampal slices. This might be attributed to the presence of the rhinal cortex, which preserves most of the functional input to the hippocampus and contributes to epileptogenesis. To fully address this topic, a complete characterization of the epileptic signals displayed by these slices throughout time in culture, such as mean number and duration of ictal events, together with their amplitude and frequency, is currently being performed. The authors have added this paragraph to the discussion.

No quantification is given for the changes in PI (page 8, "..but the number of PI+ neurons increased..") and in immunolabeling for CD68, C3d between DIV7 and DIV 14. Although a quantitative comparison might not be the main aim of the study, it would be helpful for other laboratories using different culturing protocols.

Answer. Representative images of PI uptake/NeuN, Iba1/CD68 and GFAP/C3d were just qualitative. The authors decided to show some representatives images of progressive neuronal death and gliosis in this system, but this was not the aim of the invitation from JoVE.

Minor Concerns:

The role of rhinal cortex in epileptogenesis has been described in the introduction but no recording from the cortex is presented. Usually, keeping the adjacent cortex improves quality of the slice, but in our hands it had no effect on the presence or absence of epileptiform activity.

Answer. By keeping the rhinal cortex, the authors aimed at preserving most of the functional input to the hippocampus. Furthermore, the presence of the rhinal cortex was described by Vismer and co-workers (Reference #13) to be relevant for epileptogenesis. In this system, epileptic-like activity seems to evolve faster than in published models of epileptogenesis in organotypic hippocampal slices. To fully address this topic, a complete characterization of the epileptic signals displayed by these slices throughout time in culture, such as mean number and duration of ictal events, together with their amplitude and frequency, is currently being performed. The authors have added this paragraph to the discussion.

The authors did not record from cortex. That is something that can be performed in the future.

The quality of the histo images in the pdf for review were too poor to judge the statements. It might be just a problem of this version, but for the publication one will need higher resolution images. You might also think about changing LUT for the red channel, to improve its visibility.

Answer. The authors believe that this is attributed to the PDF that was sent for review. Z-stacks were acquired in Zeiss 710 Confocal microscope and the maximum intensity projection was obtained in the ZEN software (from Zeiss). Panels were prepared in Adobe Illustrator and exported having into consideration the 300 dpi quality.