Regenerating cortical connections in a dish: the entorhino-hippocampal organotypic slice co-culture as tool for pharmacological screening of molecules promoting axon regeneration.

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Abstract

We present a method for using organotypic slice co-cultures of the entorhino-hippocampal formation to analyze the axon-regenerative properties of a determined compound. The culture is based on the membrane interphase method, which is easy to perform and is generally reproducible. Possible changes in cell morphology after pharmacological treatment can be determined easily by *in vitro* electroporation. The degree of axonal regeneration after treatment can be seen directly by using transgenic mice or by axon tracing and histological methods. The well-preserved cytoarchitectonics in the co-culture facilitate the analysis of identified cells or axonal connections for up to 2 months.

Introduction

Brain slice cultures offer unique advantages over other *in vitro* methods, since they mimic numerous *in vivo* aspects [1, 2]. For most purposes, slices of developing brain, termed organotypic slice cultures (OSC), preserve a high degree of cellular differentiation and tissue organization. OSC have been prepared from a variety of brain regions, including hippocampus, neocortex, striatum, spinal cord, hypothalamus and cerebellum [3-10]. As OSC obviate the need for extensive animal surgery and equipment, their use in basic and applied research has increased over the years[1]. In addition, a number of neuropathological events (from genetic to infective) that affect specific brain regions have been reproduced in organotypic slice platforms[11-16]. Several methods and modifications have been developed for long-term OSC, from the *roller tube* technique of Gahwiler [17] to the interphase method of Stoppini and coworkers[1, 10]. OSC in *roller tubes* have proved tedious to prepare. In addition, cultured slices display high variability due to thinning of tissues to a near monolayer of cells. Furthermore, these plasma-clot embedded cultures show intense glial reactivity, which largely conditioned functional studies [18]. The membrane interface culture method (MICM) of Stoppini and coworkers facilitates access to the slice culture[1, 10]. The principle of the MICM is to maintain slices on a semiporous membrane (0.4 μm Ø pore) at the interface between the medium and the humidified atmosphere of the CO_2 incubator. In this method, the medium reaches cultures through the membrane *via* capillarity. This culture technique and its modifications [19, 20] yield nerve cells that are highly differentiated in terms of their morphological and physiological characteristics (see [4, 21, 22] for classic studies). Moreover, the presence of glial cells is believed to provide a microenvironment that facilitates differentiation of neurons. In this respect, dead cells and debris produced

during slice preparation disappear after 2 weeks *in vitro* [17], but glial (mainly astroglial) cells proliferate and a rim of laterally migrating astrocytes completely surrounds the cultures [18, 21]. While we can partly reduce their proliferation with antimitotics (see REAGENTS SETUP), glial scarring also occurs in MICM cultures [10]. However, scarred cells in MICM cultures are mainly located at the bottom and edge of the slice (Fig. 1). This is important since the culture medium is applied below the membrane and the dense glial network hinders the drug treatment.

In this protocol we focus on organotypic slice co-cultures containing entorhinal cortex and hippocampus as a way to explore axonal regeneration. Using pharmacological treatments, axonal regeneration can be monitored in MCIM cultures and some of the molecular and cellular mechanisms responsible for its absence after adult CNS lesions are revealed. In anatomical terms, the hippocampus and the dentate gyrus are discrete brain regions with a unique laminar organization of cell layers and afferent connections[23]. The entorhino-hippocampal connection (EHC) is the main afferent connection, albeit not the only one, to the hippocampus. The EHC has been analyzed in detail and various factors mediating its development have been determined. Moreover, as seen in studies by G. Raisman [24], P.L. Woodhams [25, 26] and M. Frotscher [27, 28], entorhino-hippocampal (EH) co-cultures have some specific features that make them particularly interesting in studies of axonal regeneration: i) the EHC is reproduced easily *in vitro* in MCIM cultures; ii) the EHC *in vitro* provides a degree of laminar specificity similar to that found *in vivo*; iii) in molecular terms, the connection is myelinated both *in vitro* in MCIM and *in vivo*; and iv) most of the cellular and molecular barriers to axon regeneration are present after the axotomy of the EHC *in*

vitro ([29]). Moreover, this system is used to demonstrate age-related decline in the ability of EH axons to regenerate, as happens *in vivo* [25, 26, 30].

MATERIALS

Reagents

- Mice: newborn (P0-P1) mice. Regardless of the type of co-culture, we recommend not working with more than 5-6 pups per session. After brain dissection, two pups must be processed in parallel. We also recommend decreasing the number of pups for inexperienced researchers: two animals may be appropriate at the beginning. **CAUTION!** All animal experiments must comply with national and European regulations.
- Modified Minimum Essential Medium Eagle (MEM, Invitrogen cat. 11202-44).
- Hank's Balanced Salt solution (HBSS, Invitrogen cat. 24020083).
- Penicillin/Streptomycin solution 100 X (Invitrogen cat. 15140-22).
- HEPES 1M solution (Invitrogen cat. 15630-056).
- L-Glutamine 200 mM solution (Invitrogen cat. 25030-024).
- Sodium bicarbonate solution (Invitrogen cat. 25080-094).
- Heat-inactivated horse serum (Invitrogen cat. 26050-088).
- Ethanol (Merck cat. 108543).
- Xylene Substitute (Fluka cat. 78745).
- Sterile culture grade-H_2O (Sigma cat. W3500).
- Uridine (Sigma cat. U3750).
- Cytosine β-D-arabinofuranoside (Ara-C) (Sigma cat. C6645).
- 5-Fluoro-2´-deoxyuridine (5-Fluo) (Sigma cat. F0503).
- Biocytin (Sigma cat. B1578).

- 3,3′-Diaminobenzidine tetrahydrochloride (DAB) 10 mg tablets (Sigma cat. D5905).
- Hydrogen peroxide (32-33% in water, Sigma cat. 316989).
- Toluidine Blue (Fluka cat. 89640).
- Eukitt™ quick-hardening mounting medium (Fluka cat. 03989).

Equipment

- Dissection microscope with dark field optics (e.g., Olympus SZ51 with 10 x objectives or similar).
- McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, USA).
- Laminar flow hood. The laminar flow of the hood must be regulated.
- BTX ECM 830 Electro Square Electroporator (Harvard Apparatus, USA).
- Flaming/Brown micropipette puller (Model P-87, Sutter instruments CO., USA).
- Automatic pipette pumps (Gilson, Brand, Eppendorf or similar) and disposable 10 ml and 25 ml filter-containing sterile plastic pipettes.
- Culture plate inserts: 0.4 μm Millicell-CM membrane, 30 mm Ø (Millipore, cat. PICM01250).
- 6-well culture plates (Nunc cat. 140675).
- Large 100 mm Ø and small 35 mm Ø cell culture dishes (Nunc cat. 150679 and 150318, respectively).
- Razor blades for the tissue chopper.
- 1 large and 1 small curved scissors for initial skull dissection.
- Medium size forceps for holding head.
- 2 small straight dissecting scissors.
- One pair of fine straight forceps.

- One pair of curved fine forceps.
- Two pairs of fine spatulas for transferring brain pieces.
- Automatic pipettes and sterile filter tips (Gilson, Eppendorf or similar).
- Glass micropipettes (Word Precision Instruments, cat. 1B150-3).
- Disposable glass pipettes.
- Low-binding syringe filters (0.2 μm pore size).

REAGENT SETUP

MEMp (dissecting salt medium)

50% MEM, 25 mM HEPES and 2 mM glutamine without antibiotics. The pH is adjusted to 7.2-7.3 with 1 M NaOH. **CAUTION!** NaOH solution is toxic and must be handled carefully.▲ **CRITICAL STEP** Risk of contamination. Special care is recommended in maintenance of the temperature (4ºC) of the solution during the dissection. Prepare MEMp for single use and discard non-used or older MEMp.

MEMi (incubation culture medium)

50% MEM, 25 mM HEPES, 25% HBSS, 25% heat–inactivated horse serum, 2 mM glutamine, 1 ml of penicillin/streptomycin solution and 0.044% $NaHCO_3$. As above, the pH must be adjusted to 7.2-7.3 with 1 M NaOH. Prepared MEMi can be stored at 4ºC for at least one month.

Antimitotic stock solution

Stock solutions (10^{-3} M).

Uridine 1.21 mg in 5 ml sterile culture grade-H_2O
Ara-C 1.39 mg in 5 ml sterile culture grade-H_2O
5-Fluo 1.23 mg in 5 ml sterile culture grade-H_2O.

Make 1 ml aliquots of each. Store at – 20ºC.

Working solution (10^{-4} M).

Mix 1 ml of each compound with 7 ml of HBSS. Make aliquots of 500 μl and store at -20ºC.

PROCEDURE

Preparation of the 6-well culture plate and Millicell inserts ● TIMING 30-35 min

1| Remove the cover lid of the Millicell container and place 1 ml of warmed (36ºC) MEMi inside the Millicell and 1 ml between the insert and its plastic container. Place inserts in the dissecting hood under laminar flow.

2| Open the plastic box of the 6-well plate in sterile conditions and place 1 ml of warmed MEMi in each well. ■ **PAUSE POINT.** Before introducing the inserts into each well of the multi-well plate, wait a minimum of 15-20 min. During this time you can proceed with the protocol (points 5 to 9, see below).

3| Remove the excess of MEMi from the insert with the aid of a fire-polished glass micropipette and place the insert into the 6-well plate with a curved forceps.

4| Place the multi-well plate containing the inserts into the CO_2 incubator at 36ºC. Plates containing MEMi can remain in the incubator for hours. We do not recommend keeping medium containing plates overnight.

Preparation of the material for dissection ● TIMING 15-25 min

5| Clean the surface area of the dissection hood with ethanol and turn on the laminar flow.

6| Carefully clean the razor blades of the tissue chopper with acetone and ethanol before mounting in the chopper. Fix the razor blade with the aid of the square key provided by the manufacturer and adjust the cutting thickness to 350-400 μm. After mounting, spray the blade with ethanol and leave to dry into the hood. ▲ **CRITICAL STEP.** As nervous tissue is very sensitive to oils, detergents and other substances that may remain as residue from previous experiments or mis-handling, take special care, check that all the ethanol has evaporated and change the razor blade in each session.

7| Carefully clean the teflon plate of the chopper with ethanol and leave to dry in the hood.

8| Open the box containing the surgical instruments inside the hood and wash all the instruments with saline, distilled water and 95% ethanol, then through an ethanol flame. This sterile technique must be used during surgery and dissection. **CAUTION!** Danger of burns. Do not flame fine forceps or scalpel knifes, as they will be damaged. If a surgical tool touches the dissecting hood surface or other non-sterile area, clean and flame it as above.

9| Keep the MEMp on ice in the dissection hood.

10| Prepare 4 or 5 35-mm Ø Petri dishes containing 3 ml of cold MEMp on ice in the dissection hood.

Entorhino-hippocampal dissection and slicing ● TIMING 10-15 min/pup

11| Decapitate one pup outside the sterile area by cutting with large scissors at the foramen magnum. Make this cut quickly and cleanly.

12| Insert a sharp forceps into the eyes to hold the head and swab the skin around the neck and the head with 70% ethanol to remove blood.

13| Make an incision into the skin along the midline of the head with small scissors and pull the skin to the lower side with the fine straight forceps.

14| Fell the point of the small scissors inside the skull and cut the bone bilaterally from the foramen magnum towards the front. ▲ **CRITICAL STEP**. Maintain sterility. Do not use the same scissors to cut the skin and to cut the skull or the brain. Maintain the scissors close to the skull to avoid damage to the brain.

15| Carefully peel off top of the skull. ▲ **CRITICAL STEP.** Be careful not to damage the cortex when removing the top of the skull.

16| Place the brain into a Petri dish containing cold MEMp and, under the dissecting microscope, remove the cerebellum and cut the brain in half along the midline with the scalpel. ▲ **CRITICAL STEP.** Cuts should be made quickly and cleanly. Avoid pulling on tissue.

17| Very carefully, remove meninges and major blood vessels from brain pieces with fine forceps. ▲ **CRITICAL STEP.** Any meninges not removed will make it difficult to cut the brain in the tissue chopper.

18| After dissection of the two hemispheres, separate the thalamus and the rostral portion of the striatum with a fine spatula by using the space between thalamus and cortex as anatomic reference. After discarding the dissected tissue, the lower portion of the hippocampus can be seen.

19| A second transverse cut is made following the hippocampal fimbria as reference and the remaining cortex is separated. After this second cut, we obtain the hippocampus and the adjacent cortex in a single tissue piece.

20| Transfer the block of tissue to the teflon plate of the chopper by using curved forceps.

21| Obtain sections in the horizontal plane though the caudoventral pole of the hemisphere. To perform these sections, place the hippocampus and adjacent cortex at a 45º angle to the razor blade. After sectioning, place the teflon plate in cold MEMp in a 100-mm Ø Petri dish. **? TROUBLESHOOTING.**

22| With two spatulas gently separate sections into the MEMp. Next, check under the microscope for quality of slices. ▲ **CRITICAL STEP.** Layering of the hippocampal formation and adjacent cortex can be clearly distinguished in the slices. Be sure to keep sections flat and unfolded and discard slices with cuts or crushes. **? TROUBLESHOOTING**

23| For *integral co-cultures*, select slices containing both the hippocampus and the entorhinal cortex; for *real co-cultures*, select slices as above and use a fine spatula to separate the hippocampus and the entorhinal cortex by cutting at the subicular level. Integral co-cultures maintain the entorhinal cortex in continuity with the hippocampus and retrohippocampal areas [24]. Selected slices must be stored in cold MEMp for 30-40 min.

Plating slices in the Millicell inserts ● TIMING 5-10 min / Transwell

23| Place the 6-well culture plate in the laminar flow hood. **▲ CRITICAL STEP** Control under the dark field microscope possible defects (e.g. different color or opacity) in the membrane that might affect nutrient transport across the surface. Discard membranes with poor quality, i.e. those that are not uniformly translucent.

24| Transfer the slices onto the humidified membrane of the insert with a fire-polished pipette and re-orient the slices with a fine spatula. Aspirate the excess of MEMi from the membrane surface. **▲ CRITICAL STEP.** Medium should not cover the membrane, because if it does the survival of the slices decreases steadily. **? TROUBLESHOOTING.**

25| Cultures are maintained at 36ºC in a 5% CO2, 95% air atmosphere at a relative humidity of 95%. The medium is changed after 24 h and thereafter every 48 h until cultures are ready to use. Although several slices can be cultured on the same transwell, we do not recommend more than three co-cultures per insert, as the long-term maintenance of the slices become tedious (changing medium every 24 hours, risk of contamination, etc). To reduce glial proliferation, 500 µl of the anti-mitotic stock is dissolved in 40 ml of MEMi for a final concentration of 10^{-6} M. Co-cultures can be treated the first 3-4 days *in vitro*.

Axotomy of the EHC in MCIN organotypic co-cultures● TIMING 3 h

26| After 15-21 days *in vitro* (DIV), reduce the laminar flow of the hood. Place the 6-well plate under the stereomicroscope. Axotomize the EHC by cutting the co-culture from the rhinal fissure to the ventricular surface along the entire EH interface with a sterilized tungsten knife or a fine spatula (Fig. 3). ▲ **CRITICAL STEP.** During this procedure the Millicell membrane must not be damaged with the knife and the co-cultures must not dry.

27| Place the 6-well plate in the CO_2 incubator at 36ºC for 15-20 minutes and warm the MEMi media.

28| After 15-20 min, change the MEMi (1 ml) and place the plate in the incubator and wait for 1 hour.

29| After 1 hour, change the medium again with warm MEMi. Then return the plate to the incubator. Cultures must be analyzed for integrity and defects next day. **? TROUBLESHOOTING**

Pharmacological treatment of the axotomized co-cultures. ● TIMING 1 h/6-well plate

Depending on the drug (Fig. 3), final concentration should be calculated and evaluated in each experimental case. Due to the glial scar (Fig. 1), drug must be applied on top of the culture (Fig. 3).

30| In a clean hood with reduced laminar flow, place the 6-well plate containing the axotomized cultures and prepare the drug solution in MEMi without antibiotics. When the drug interacts with serum proteins, MEMp can be used. Drug solution should be sterile by filtration using low-binding syringe filters.

31| Open the plate and change the media with 1 ml of warm MEMi.

31| Under binocular observation, place a drop (not more than 1-2 μL final volume) of the drug dissolved in MEMi in each culture and put the plate into the incubator. Use a fine tip (p2 Gilson micropipette or similar). Repeat steps 31-32 for at least 10-15 days.

Fast morphological analysis of cultured neurons by electroporation after drug treatment. ● TIMING 24 h

The intracellular labeling in organotypic slices with horseradish peroxidase HRP or other intracellular stains gives us data about the health status of labeled neurons and possible changes in morphology [31]. However, the intracellular labeling is usually tedious. From this method, additional procedures were developed to label individual cells in slices, including the use of viral vectors (lentivirus, herpes virus, etc) or gene transfer methods by using biolistics, lipotransfection or electroporation [32]. For example, single cell electroporation of neurons has been developed with good results [33]. The following short protocol is a modification developed in our labs of the device used for focal electroporation of embryonic slices [34]. In these studies, a Petri dish and a cover square platinum plate electrodes are currently used. These electrodes can be obtained from SONIDEL (refs. CUY701P5E/L) or other companies. In addition, a square electroporator is needed: BTX ECM 830, SONIDEL CUY21 or similar. Fig. 2 shows our device. A platinum electrode (connected to the - pole) that is 0.5 mm Ø and 30 mm long ends in a flat area of 9 square millimeters (3 x 3 mm) (A). At the flattened end the platinum wire describes waves that allows us to retain for capillarity 1-3 μL of an eGFP-expressing plasmid (pCDNA3-eGFP; 500 ng/μL final concentration) or similar. In the Petri dish, an aluminum plate, 25 mm long x 15 mm wide, is located and connected to the + pole by a cable.

32| Clean the surface area of the hood with ethanol and turn on the laminar flow. After the ethanol has evaporated, decrease the flow of the hood. Warm a HBSS solution at 36ºC.

33| Place the Petri dish of the electroporator system under the diseecting binocular and clean the electrode with ethanol. Connect the Petri dish electrode connector to the + pole of the electroporator. **CAUTION**! Danger of electric shock**.** The electroporator must be switched off at this moment. Verify that the foot switch of the electroporator is close to you.

34| Clean the wire electrode with ethanol and allow it to dry inside the hood. Connect the electrode to the – pole. **CAUTION!** The electroporator must be switched off at this moment.

35| Put the 6-well tray into the hood, remove the media from one of the Millicell and change for 1 ml of HBSS for 5 min. Repeat this operation twice. Adjust the electroporation conditions in the electroporator unipolar current of 100 V, 50 ms pulse, half second interval, 5 times.

36| Aspirate the HBSS with a fire-polished pipette and place the insert in the Petri dish in close contact with the aluminum electrode. ▲ **CRITICAL STEP.** The culture must be located in the area of the electrode and a thin layer of HBSS has to wet the membrane area in contact with the electrode.

37| Put 1-2 μL of the DNA plasmid at the plated end of the electrode with a p2 pipette and draw the flat end of the electrode close to the culture (usually 1-2 mm distance) to maintain a drop over the culture by capillarity. Push the foot switch to start the electroporation. **CAUTION!** Risk of electric shock.

38| Switch off the electroporator, remove the insert from the Petri dish and directly place the insert in the 6-well culture plate containing 1 ml of warm MEMi.

39| Put the 6-well plate into the CO_2 incubator at 36ºC. After 24-36 hours, electroporated cells can be seen in an inverted fluorescence microscope without fixation (Fig. 3). Alternatively, cultures can be fixed with 0.1 M phosphate-buffered 4% paraformaldehyde for 10 min, rinsed in 0.1 M pH 7,3 PBS, mounted in fluorescence mounting media and processed for further eGFP observation. **? TROUBLESHOOTING.**

Histological corroboration of EHC regeneration after drug treatment. ● TIMING 48 h

Once it is established whether the drug alters the neuronal or glial morphology, we can analyze the degree of axonal regeneration obtained after our treatment/s in two ways, depending on how co-cultures were prepared: i) by using eGFP transgenic animals in which the entorhinal slice is eGFP-positive and the hippocampus is eGFP-negative (Suppl. Fig. 1) or ii) by using normal non-transgenic animals (Suppl. Fig. 1). In the first case, the evaluation of axon regeneration is easy by direct observation with fluorescence microscopy. In the second case, the EHC must be labeled with an anterograde axonal tracer. In our experience use of Biocytin is the best, fastest and easiest method for labeling the EHC in MCINN. Biocytin (biotinyl-lisine) is a powerful anterograde tracer in the mammalian central nervous system [35]. As indicated above, we and others have used Biocytin to trace the EHC in MCINN [24, 36, 37]. In the following paragraphs we briefly indicate the most important steps of the protocol.

40| Place a small amount of Biocytin in a 35 mm Ø Petri dish.

41| Remove the 6-well plate from the CO_2 incubator and place it in the clean hood under low laminar flow.

42| Under the binocular and using a glass micropipette, pick up a small crystal of Biocytin with the tip of the pulled micropipette.

43| Place the 6-well plate under the microscope and open the plate.

44| Place the crystal of Biocytin in the entorhinal slice. ▲ **CRITICAL STEP**: slightly press the Biocytin crystal into the slice. This process is delicate and requires experience. We recommend that inexperienced researchers do not perform this step at the beginning.

45| Proceed as in points 27 to 29.

46| After 24 hours, remove the media with a fire-polished pipette, rinse the culture with 0.1 M phosphate buffer pH. 7.3 and fix the cultures for 6-7 hours with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3, adding 2 ml of the fixative inside and below the Millicell.

47| Rinse the cultures rapidly 3-4 times in 0.1 M phosphate buffer, pH 7.3 and detach the culture from the membrane with the aid of a fine spatula. Transfer the culture to a glass vial.

48| Incubate the cultures for two days with an Avidin-Biotin Peroxidase complex (ABC-elite^{TM}, Vector Labs., USA). Alternatively, HRP-tagged Streptavidin can be used. Reagent concentration and dilutions should follow manufacturer's instructions. We recommend a dilution buffer containing 10% Normal Horse serum and 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.3.

49| After incubation, rinse the cultures 3-4 times in 0.1 M phosphate buffer, pH 7.3, for 1 hour, taking care not to touch the culture with the glass pipette. Rinse culture with 0.1 M Tris-HCl buffer twice (5 min each).

50| Incubate the cultures for 10 min with a solution of 0.07% DAB and 0.1% Nickel ammonium sulphate in 0.1 M Tris-HCl, pH 7.6. **CAUTION!** As DAB is toxic, gloves

and mask should be used. Use the minimum amount of DAB solution to prevent risks. Follow local rules for treatment of waste material and chemicals.

51| After DAB incubation, add 10 μL of a 1% aqueous solution of H_2O_2 and wait 10 min.

52| Stop the reaction with 0.1 M Tris-HCl buffer, pH 7.6. **? TROUBLESHOOTING**

53| (**Optional**) Cultures can be stained in 0.1% toluidine blue for 1 or 2 min. Staining is followed by quick rinses in distilled water and differentiation of the stain in 30-50% ethanol for 1-10 min (Suppl. Fig 1). This differentiation process must be monitored under a microscope.

54| Mount the cultures in glass slides and dehydrate stepwise in ethanol (70%, 95%, 100%), clear in xylene substitute and embed in mounting media (Eukitt™ or similar).

? TROUBLESHOOTING advice can be found in Table 1

TABLE 1| Troubleshooting table

Problem	Possible reason	Solution
Slices are not translucent after sectioning in the tissue chopper. The slice color is white homogeneously.	Teflon plate or the razor blade contains traces of acetone or alcohol, they have not been well cleaned or they are covered with remnants of the protective oil.	Remove the razor blade and replace with a new clean one. Make sure that it is clean and dry. Increase the drying time in the incubation hood.
After sectioning, the pyramidal layers of the hippocampus cannot be seen in the slices.	There is a problem with the composition of the medium.	Discard slices and prepare new fresh and sterile MEMp. Maintain sterile conditions and temperature of the MEMp, the dissection hood and the surgical material.
After a few days in culture, the center of the co-culture appears dark.	The thickness of the cultured slices is greater than 400 μm and anoxia is affecting cell survival.	Discard slices and prepare new ones at correct thickness. Check the tissue chopper.
After a few days in culture, the co-culture becomes white.	The co-culture is contaminated.	Discard slices and prepare new ones. Sterilize all surgical material and equipment including reagents before preparation.
	The amount of media is not appropriate and the oxygenation is not right.	Discard slices and prepare new ones. Reduce the amount of media to 1 ml.
24 hours after axotomy, the entorhinal cortex has separated from the hippocampus.	The knife removed tissue during axotomy because it was too thick.	Discard slices. Check whether the membrane is affected or not and improve the technique in younger thicker slices.
After axonal tracing and DAB development, few axons are labeled in the hippocampus and the injection site is difficult to determine.	Too small an amount of Biocytin was applied in the entorhinal slice.	Increase the number of crystals in the entorhinal cortex. We recommend 3 per slice.
	ABC does not penetrate the slice due to weak permeation of the slice.	Increase permeabilization by incubating co-cultures with 10% DMSO in 0.1 M phosphate buffer for 20 min. Increase the percentage of Triton X-100 in ABC solution.

ANTICIPATED RESULTS

Although monolayer cultures of dissociated cells permit electrophysiological and biochemical characterization of neurons, some specific cell-cell interactions with crucial roles during development are lost. We and others have demonstrated the proper formation of the EHC *in vitro* [8, 24, 25, 36-38]. Anterograde labeling with Biocytin or Tau-eGFP cultures (Suppl. Fig. 1) showed that, after 5-7 DIV, the EH axons formed the typical parallel bundles in the hippocampus proper and the dentate gyrus. Studies by Woodhams and Atkinson described how EHC development may be slightly faster and more extensive in the MICM culture than in roller tube OC [25]. Here we described a method in which a lesion can be easily performed in cultured EH co-cultures after two weeks *in vitro* allowing for the screening of molecules designed to promote axon regeneration. Current methods of screening mainly use monolayer cultures of cerebellar neurons or dorsal root ganglia cells growing over a coated surface containing a particular inhibitory molecule or compound [39]. Though relevant, these methods do not bring the researcher close to the *in vivo* situation, since most factors in these preparations are lacking. The loss of regeneration of the EHC *in vitro* closely followed those seen *in vivo* [26, 30, 40] and most of the factors involved in the absence of regeneration are present in these culture platforms [29]. In technical terms, in our experience, *Tau-eGFP* cultures are more appropriate than Biocytin-labeled cultures for a fast evaluation of a molecular screening, since all possible regenerating axons will be fluorescent. However, we recommend use of *Tau-eGFP* +/- rather than *Tau-eGFP* +/+ mice, since increased expression of eGFP may affect neuron viability. In addition, the modified procedure of electroporation is fast and reproducible, giving rise to a relevant number of electroporated cells to check morphology. In our hands, approximately 30-35 cells are

labeled in each slice after electroporation. These cells are viable and their morphology can be analyzed for several days. Compared with other non-viral methods such as gen-gun transfection [41] or single-cell electroporation [33], our method is easy and fast and could help neuroscientists to analyze specific modifications of several neurons at the same time 24 hours after a treatment without compromising the survival of the culture, which in addition offers us the opportunity to determine whether a molecule promotes axon regeneration.

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Figure 1

GFAP-positive glial scar in long-term MICM EHC co-cultures.

A-C) Distribution of the GFAP-positive elements in standard EH co-cultures growing over an interphase membrane for 15 days. Each panel corresponded to the respective region labelled in the central diagram. At a medial culture level (A), the distribution of GFAP-positive cells is very similar to those reported *in vivo*. However, in transversal observation of the lateral portion of the culture (B), intense labelling of GFAP can be seen in the lower portion of the slice in close contact with the transwell membrane. This dense network of glial cells can be seen in a horizontal section parallel to the membrane (C). To perform the GFAP-staining in A and C, we used the protocol described in [29]. The protocol used in B is published in [18]. Abbreviations, EC: entorhinal cortex; S: Subiculum; DG: Dentate gyrus; CA1-3: hippocampal regions 1 and 3. Scale bar: A = 250 μm, B and C = 100 μm.

Figure 2

MICM electroporation for morphological studies.

A) Low-power photography showing the electroporation device containing a Millicell insert in the Petri dish over the aluminium electrode and the wire electrode (see protocol for details). **B)** Diagram illustrating the procedure of MCIM electroporation indicated in the protocol. **C)** Example of eGFP-positive electroporated cells (arrows) 24 h after electroporation in the entorhinal slice of an EH co-culture after 15 DIV. Abbreviations as in Figure 1. Scale bar C = 100 μm.

Figure 3

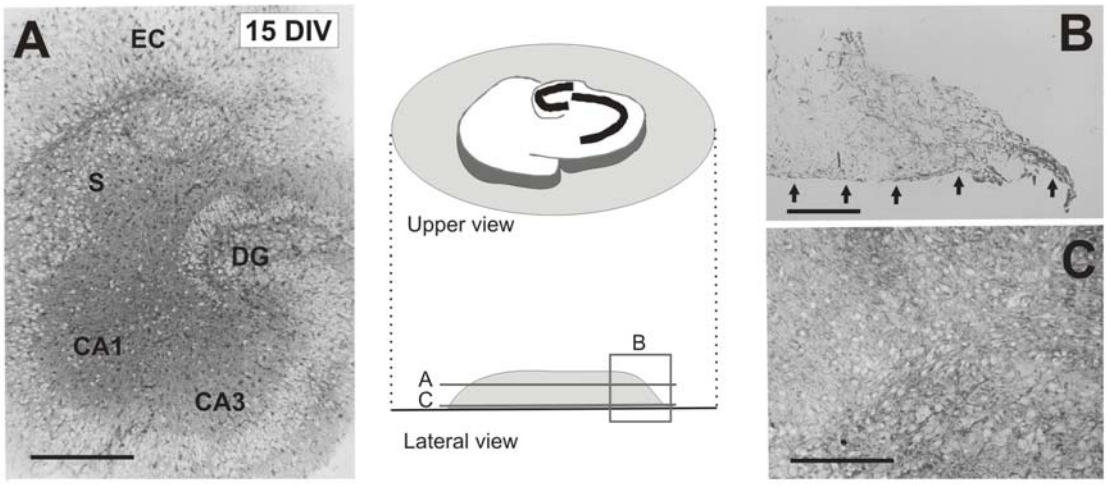
Axotomy of the EHC in MCIM platforms, drug treatment and corroboration of EHC regeneration

A) Diagram illustrating the procedure of axotomy, drug delivery and Biocytin tracing in EHC. Asterisks indicate the location of the Biocytin crystals. **B)** Example of a control EH co-culture treated only with MEMp. After 10 days of treatment with MEMp the EHC do not regenerate. **C-D)** Example of a parallel experiment showing the regeneration of the EHC after treatment with the NEP1-40 peptide (see [42] for details of the peptide). Numerous axons can be seen entering the hippocampus after Biocytin tracing. D is a high magnification of the boxed area in C. Arrowhead point shows the growth cones of regenerating axons. **E)** Example of the quantification of the experiment. To perform this quantification, we counted the mean number of Biocytin-labeled fibers that crossed a 400-μm segment in the hippocampus (located at a distance of 75–80 μm from the axotomy) for each section, using a 40× oil-immersion objective. The asterisk indicates statistical significance ($p< 0.05$ Student *t*-test) Abbreviations as in Figure 1. Scale bar C = 250 μm pertains to B and D = 75 μm. The dashed lines in B and C label the axotomy trajectory.

Supplementary Figure 1

EH co-cultures using the MCIM method and EHC labeling, intact *vs* complex co-cultures.

A) Example of EHC at 3 DIV (A-B) and after 15 DIV (C-D). A is a phase contrast image of the culture and B is the corresponding fluorescence photomicrograph. Complex co-cultures in A-C were prepared using a Tau-eGFP expressing mice kindly provided by Prof. Manson (Edinburgh, UK) [43]. In these co-cultures the entorhinal cortex is taken from an *eGFP* +/- mouse and the hippocampus from an *eGFP* -/- mouse. Growing axons (arrows in C) in the hippocampal fields can be seen directly under the fluorescence microscope. **D)** Example of a Biocytin-labeled EHC in an intact co-culture after the above-mentioned protocol. The location of the Biocytin crystal in the entorhinal cortex is labeled by an asterisk. Arrows point to anterogradely labeled axons in the hippocampus. Abbreviations as in Figure 1. Scale bar: A = 250 μm, pertains to B-D.



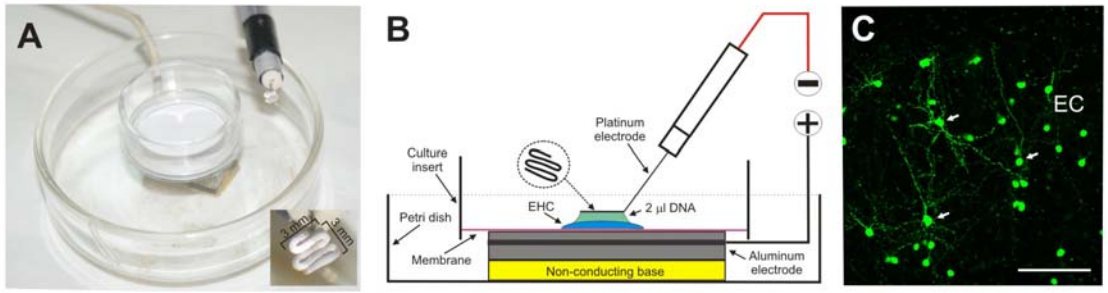


Figure 2

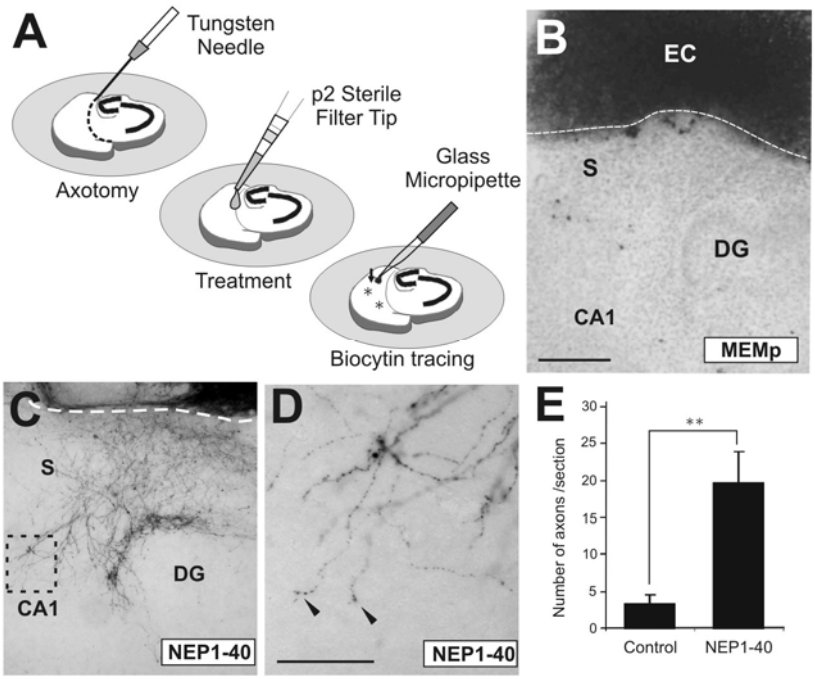
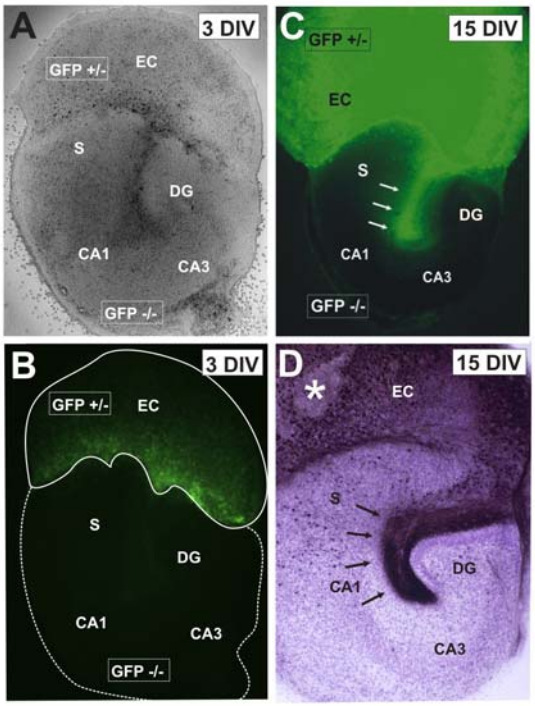


Figure 3


A
3 DIV
GFP +/-
EC
S
DG
CA1
CA3
GFP -/-
C
GFP +/-
15 DIV
EC
S
DG
CA1
CA3
GFP -/-
B
3 DIV
GFP +/-
EC
S
DG
CA1
CA3
GFP -/-
D
*
EC
15 DIV
S
DG
CA1
CA3

Suppl Fig 1.