

# Paired-recordings from synaptically coupled cortical and hippocampal neurons in acute and cultured brain slices

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Published online 11 September 2008; doi:10.1038/nprot.2008.147

**Analysis of synaptic transmission, synaptic plasticity, axonal processing, synaptic timing or electrical coupling requires the simultaneous recording of both the pre- and postsynaptic compartments. Paired-recording technique of monosynaptically connected neurons is also an appropriate technique to probe the function of small molecules (calcium buffers, peptides or small proteins) at presynaptic terminals that are too small to allow direct whole-cell patch-clamp recording. We describe here a protocol for obtaining, in acute and cultured slices, synaptically connected pairs of cortical and hippocampal neurons, with a reasonably high probability. The protocol includes four main stages (acute/cultured slice preparation, visualization, recording and analysis) and can be completed in ~4 h.**

## INTRODUCTION

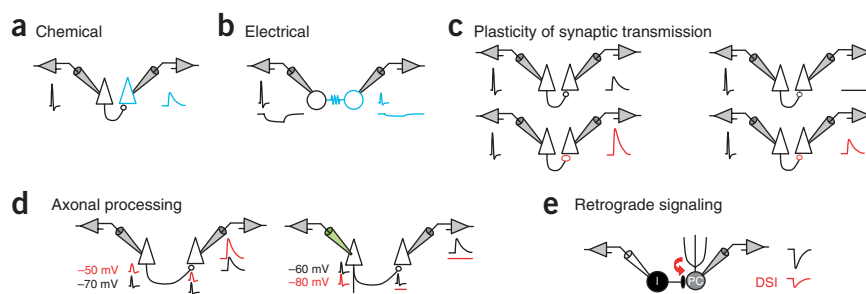
The use of paired-recording to study communication between individual neurons in small networks was first developed in the ganglion of *Aplysia in vitro*<sup>1</sup> and flourished 20 years later with the accession and spread of slice preparations from mammalian brain<sup>2</sup>. Paired-recording of connected neurons is a powerful and versatile tool allowing the study of many basic properties of neuronal communication in brain circuits with a high level of experimental rigor (Fig. 1). Synaptic interaction between neurons has been studied in many different brain areas including hippocampus<sup>3–5</sup>, cortex<sup>6–9</sup>, cerebellum<sup>10–12</sup>, striatum<sup>13,14</sup>, amygdala<sup>15,16</sup>, olfactory bulb<sup>17–19</sup> and spinal cord<sup>20</sup>.

Evaluation of the number of synaptic contacts involved in synaptic transmission constitutes a major motivation for recording pairs of connected neurons. The adjunction of biocytin in the recording pipettes allows a detailed reconstruction of the morphology of the presynaptic axon and the postsynaptic dendritic

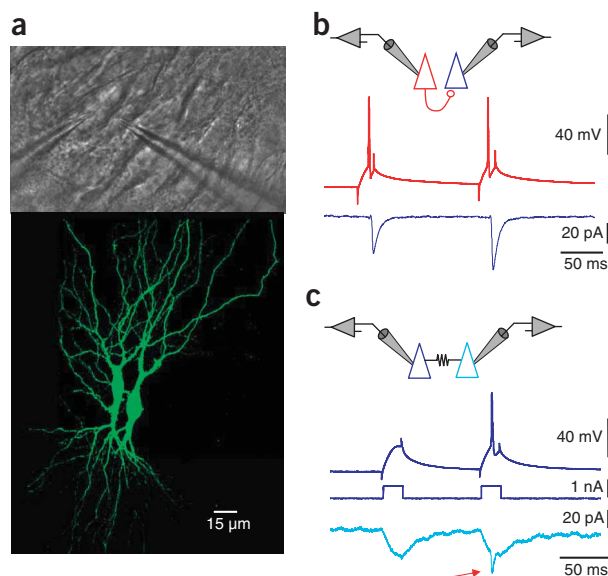
arborization (Fig. 2a) to identify the putative synaptic contacts<sup>7,8,21,22</sup>. Chemical synaptic transmission is probably the most studied function. Basic properties of synaptic transmission such as quantal analysis<sup>23–25</sup>, co-release of  $\gamma$ -aminobutyric acid (GABA) and Gly<sup>20</sup>, pharmacological characterization of postsynaptic receptors mediating unitary excitatory and inhibitory responses<sup>8,22,26,27</sup> and presynaptic mechanisms governing neurotransmitter release<sup>28–32</sup> or synaptic timing<sup>5,33</sup> can be studied with great accuracy.

Multiple configurations of neuronal connections have been explored: excitatory synapses between principal cells (i.e., pairs of cortical pyramidal neurons<sup>7,34</sup>), spiny stellate neurons<sup>9</sup>, excitatory synapses onto GABAergic interneurons<sup>3</sup>, inhibitory synapses onto principal cells<sup>22,35</sup> but also inhibition of GABAergic interneurons<sup>36</sup>. The main advantage of paired-recordings in the analysis of synaptic transmission resides in the fact that this is the only method that guarantees triggering of a presynaptic action potential (AP).

**Figure 1** | Configuration and possible applications of paired-recordings. (a) Chemical synaptic transmission between monosynaptically connected pyramidal neurons. The action potential (black trace) triggered in the presynaptic neuron (left) evokes an EPSP (blue trace) in the postsynaptic neuron (right). (b) Electrical coupling between two neurons. An action potential or current-induced hyperpolarization (black traces) triggered in the first neuron (left) produces attenuated voltage signals (blue traces) in the other neuron (right). (c) Plasticity of existing (left) or silent (i.e., synapses that contain NMDA receptors but no AMPA receptors and are therefore functionally silent during basal stimulation) (right) synapses. In control conditions, the presynaptic neuron (left) evokes an EPSP in the postsynaptic cell (left), or no response in the case of a silent synapse (right, symbolized by a dotted terminal). After potentiation (red symbols), the efficacy of synaptic transmission is enhanced (see traces). (d) Presynaptic membrane potential-dependent axonal integration (left) or conduction failures (right). Left, a presynaptic spike evoked from a depolarized potential (–50 mV, red traces) evokes a larger EPSP than at rest (–70 mV). Note the changes in the presynaptic action potential waveform at –50 mV (red traces). Right, the presynaptic spike fails to propagate in the axon (flat red traces) when it is evoked from a hyperpolarized (–80 mV) potential. (e) Retrograde signaling at a synapse established by an interneuron (I) and a Purkinje cell (PC). DSI, depolarization-induced suppression of inhibition. Red arrow indicates the release of endocannabinoids from the PC to the presynaptic terminal of I. Gray pipettes symbolize whole-cell patch-clamp recording; green pipette, sharp microelectrode; and white pipette, perforated-patch recording.



**Figure 2** | Synaptic interactions in pairs of CA3 pyramidal neurons from a cultured hippocampal slice. **(a)** DIC-infrared view of the CA3 region in a slice culture (top) and confocal reconstruction of two CA3 neurons filled with biocytin (bottom). **(b)** Short-term synaptic facilitation at a connection between two CA3 pyramidal neurons. Two action potentials triggered in the presynaptic neuron (red trace) evoked corresponding postsynaptic inward currents (trace in blue) in the other neuron. Note the facilitation on the second stimulation. **(c)** Example of electrical coupling between two neighbouring neurons in the CA3 region. The postsynaptic voltage-clamp signal (light blue trace) is a mirror of the presynaptic voltage deflection (dark blue trace). Note the electrical signature of the presynaptic spike (arrow).



Conventional extracellular stimulation techniques do not allow failures of axonal AP triggering (i.e., stimulation failures) to be distinguished from failures of axonal conduction or from failures of presynaptic release of neurotransmitter. Thus, experimental data obtained with extracellular stimulation must be interpreted with great care and any experimental manipulation that may potentially alter the recruitment of presynaptic axons should be avoided. However, limitations of paired recording in brain tissue maintained *in vitro* reside essentially in the exploration of functional connectivity. Because axons and dendrites might be severed during slicing, this protocol provides only a lower estimate of the real connectivity. Thus, low connection probability between two brain regions may simply result from slicing artifacts. To circumvent these problems, exploring connectivity *in vivo* should be envisaged<sup>37</sup> or in the whole structure maintained *in vitro*<sup>38</sup>.

The functional study of the mechanisms underlying presynaptic release of neurotransmitter has made huge progress with the development of paired recording from the presynaptic terminal and the postsynaptic compartment at giant mammalian synapses such as the Calyx of Held synapse<sup>39</sup> or the mossy fiber–CA3 cell synapse<sup>40</sup>. However, this method is inappropriate for studying the mechanisms of transmitter release at classical en passant boutons or at inhibitory synapses because their size is too small to allow whole-cell patch-clamp recording. For these synapses, the paired-recording technique represents a unique alternative. In fact, an attractive application of the dual-recording technique resides in the possibility of selectively injecting well-defined concentrations of calcium buffers<sup>41–43</sup>, ions<sup>44</sup>, inhibitory peptides<sup>45</sup> (O. El Far, S.B., D.D. & M. Seagar, unpublished data), small proteins<sup>46,47</sup> or neurotransmitter<sup>12</sup> in the presynaptic compartment to selectively target the release machinery. In some cases, synaptic transmission can be compared in control conditions and in the presence of the presynaptic compound in the same connection by repatching the presynaptic cell with different micropipettes<sup>41,43</sup>. However, alternative methods such as the perfusion of the presynaptic patch-pipette might also be used<sup>48</sup>. In this case, there is no risk of damaging the presynaptic neuron by repetitive whole-cell recordings. Autaptic hippocampal contacts that develop when neurons are cultured in isolated micro-islands have been used for addressing many functional questions about presynaptic mechanisms<sup>49</sup>. Although this technique appears convenient at first glance because a single pipette records both the pre- and postsynaptic sides, this approach is not fully appropriate because autaptic currents are unusually large and the interpretation of the data is often difficult because the selective targeting of the pre- or postsynaptic compartment with pharmacological agents is impossible.

A successful application of the paired-recording technique is the exploration of functional connectivity within or between brain regions. This approach has been developed with success for example to study the synaptic organization of the neocortex within or between different cortical layers<sup>50–53</sup> and to examine the reorganization that occurs in response to *in vivo* sensory experience<sup>54,55</sup>. However, functional exploration of long-distance synaptic connections such as hippocampal CA3–CA1 or granule cell–CA3 synapses is almost impossible in acute slices of brain tissue because the probability of severing axon collaterals increases with the distance between pre- and postsynaptic partners. A very low probability of connection has been reported at long-distance hippocampal synapses (~5% at CA3–CA1 cell synapses<sup>4,56</sup>; there is no published study reporting synaptic connection between pairs of granule cell–CA3 pyramidal neurons in acute slices). This problem can be circumscribed by using hippocampal slice cultures in which long-distance connections are re-established *in vitro* according to an *in vivo*-like connection pattern<sup>26,57,58</sup>. For instance, CA3–CA1 (refs. 26,59) and granule cell–CA3 neuron connections<sup>60–62</sup> are found in organotypic hippocampal slice cultures that have developed for at least 3 weeks *in vitro*. Thus, in comparison with acute slices, an increased neuronal connectivity may facilitate successful paired-recordings in cultured slices<sup>58</sup>. The search for connections can be improved by first recording the postsynaptic neuron and then systematically scanning the presence of evoked postsynaptic currents when neurons in the presynaptic territory are locally excited either with brief capacitance oscillations of the presynaptic sharp electrode recording or with puffs of extracellular K<sup>+</sup> with the presynaptic whole-cell patch-clamp pipette. Using these tricks, the probability of finding connections is increased to ~70 and 10%, respectively<sup>26,60</sup>.

Many different forms of short- and long-term plasticity of synaptic transmission can be investigated using paired-recording. The unambiguous identification of transmission failures constitutes a major advantage for the analysis of short- and long-term synaptic dynamics. Short-term synaptic plasticity at excitatory<sup>63,64</sup> and inhibitory<sup>22,29</sup> synaptic contacts can be easily tested with a pair

of presynaptic APs (Fig. 2b). Long-term synaptic plasticity has now been studied for >15 years by using dual recording. Important findings have been reported such as quantal analysis of long-term potentiation (LTP)<sup>23,56</sup>, induction of bidirectional long-term plasticity of synaptic transmission at connected pairs of neurons<sup>59</sup> and induction of LTP at the hippocampal granule cell–basket cell synapse<sup>65</sup>. The rules of spike-timing dependent plasticity (STDP) at excitatory<sup>59,66–71</sup> and inhibitory<sup>72</sup> synapses have been defined with dual recording because the activity patterns of pre- and postsynaptic activity can be precisely controlled. Finally, long-lasting, bidirectional plasticity of the excitability of the presynaptic neuron has also been characterized in connected pairs of cortical neurons when LTP or long-term depression is induced with STDP protocols<sup>73</sup>.

Besides the study of chemical synaptic interaction (Fig. 1a), dual neuron recording is also a very powerful tool to study other forms of neuronal communication. Axonal processing (Fig. 1d) depending on the membrane potential of the presynaptic neuron has been revealed with the use of the paired-recording technique. The electrophysiological signatures of conduction failures or ping-pong propagation<sup>74–76</sup> and analog axon signaling at hippocampal<sup>77</sup> or neocortical synapses<sup>78,79</sup> are readily identified in the postsynaptic neuron upon variations in presynaptic membrane potential. In addition, direct evidence for electrical coupling (Fig. 1b) between GABAergic interneurons<sup>80–84</sup> has also been provided using simultaneous dual recordings (Fig. 2c); five distinct networks of electrically coupled interneurons have been identified in the

neocortex<sup>85</sup>. Functionally, electrical coupling enhances fast synchrony between connected neurons.

Today, most investigators use dual patch-clamp recordings at pre- and postsynaptic neurons. This technique presents many advantages (see above), but it also introduces the constraint that pipettes must obligatorily be changed after each recording. In the case of a low probability of connection, this approach may seem particularly tedious and discouraging. A possible alternative is the use of sharp microelectrodes for recording the presynaptic cells because several high-quality recordings can be obtained sequentially with the same pipette<sup>3,26</sup>. Alternatively, the loose-patch recording technique can be used to probe several presynaptic neurons to increase the sampling of possible connections<sup>10</sup>. More recently, transynaptic tracers have been developed to help identify the monosynaptic partners of a given neuron<sup>86,87</sup>.

Paired-recording of synaptically coupled neurons has the reputation of being frustrating and time consuming because earlier studies reported connection probabilities ranging between 1 and 5%<sup>4,34,56,88</sup>, meaning that 20–100 pairs must be tested to find a connection. Several factors may account for a low probability of connection (see TROUBLESHOOTING section). Here we report a protocol that allows us to obtain synaptically connected pairs of neurons in acute slices of the rat neocortex (L5–L5 pyramidal neurons) and hippocampal slice cultures (CA3–CA3, CA3–CA1 pyramidal neurons) with a probability >30%<sup>26,33</sup>. This protocol can be easily adapted with minor changes to record from pairs of synaptically connected neurons in many different brain areas.

## MATERIALS

### REAGENTS

- Experimental animals: rats (Wistar or Sprague-Dawley) or mice (C57/Bl6)
- **CAUTION** All animal experiments are to be performed in accordance with the guidelines on the use of animals by the relevant authorities.
- Artificial cerebrospinal fluid (ACSF; see REAGENT SETUP)
- Chloral hydrate (Sigma, cat. no. C8383; anesthetic, stock solution at 8% stored at 4 °C)
- Biocytin (Sigma, cat. no. B4261)
- CaCl<sub>2</sub> (Sigma, cat. no. C4901)
- Carbogen (95% oxygen, 5% carbon dioxide)
- D(+)-glucose (VWR, cat. no. 24370.294)
- EGTA (Sigma, cat. no. E4378)
- HEPES (Sigma, cat. no. H3375)
- KCl (VWR, cat. no. 26764.298)
- κ-Gluconate (Sigma, cat. no. G4500)
- KOH (Sigma, VWR, cat. no. 26668.296)
- Kynurenic acid (Sigma, cat. no. K3375; stock solution at 200 mM)
- MgATP (Sigma, cat. no. A9187)
- MgCl<sub>2</sub> (Sigma, cat. no. M0250)
- NaGTP (Sigma, cat. no. G8877)
- Na<sub>2</sub>ATP (Sigma, cat. no. A2383)
- NaH<sub>2</sub>PO<sub>4</sub> (VWR, cat. no. 28015.294)
- NaHCO<sub>3</sub> (VWR, cat. no. 27778.293)
- Phenol red (Sigma, cat. no. P3532; stock solution at 50 mM stored at room temperature; 19–23 °C)
- D(+) saccharose (VWR, cat. no. 27480.294)
- Cytosine β-D-arabinofuranoside (Ara-C; VWR, cat. no. 2510 10-1)
- Horse serum (BioWest, cat. no. S0900)
- Minimal essential medium (MEM; GIBCO-Invitrogen, cat. no. 21090-055)
- Hanks balanced salt solution (HBSS; GIBCO-Invitrogen, cat. no. 14185045)
- Penicillin–streptomycin (P: 10,000 U/ml, S: 10,000 µg/ml; GIBCO-Invitrogen, cat. no. 15140122)
- Ascorbic acid (Sigma, cat. no. A4544)
- B27 (GIBCO-Invitrogen, cat. no. 17504044)
- Natural spring water (Volvic)

### EQUIPMENT

- Dissection tools for removing the brain (scissors, forceps, spatula and Pasteur pipettes)<sup>89–93</sup>
- High-quality vibrating slicer (VT1000S or VT1200; Leica Microsystems)
- Incubation chamber: a submerged chamber optimized to ensure sufficient oxygenation of the tissue during the recovery period. Usually, it consists of a small plastic cylinder ending in a nylon net
- Cyanoacrylate glue (Super Cyano, Jelt)
- Microfilters (World Precision Instruments, cat. no. MF3465)
- Millicell culture plate inserts (Millipore)
- Filter paper (VWR, cat. no. 516-0015)
- Plastic Petri dishes (diameter 35, 60 and 145 mm)
- Upright, fixed-stage microscope configured for differential interference contrast (DIC)-infrared video microscopy (Olympus BX51 WI; Olympus)
- Temperature-controlled slice chamber (Luigs & Neumann) to maintain the temperature at 34 °C
- Grid of nylon threads glued to a U-shaped platinum frame to maintain the slice in the bottom of the recording chamber. Details on its manufacture are provided elsewhere<sup>92</sup> **▲ CRITICAL** The space between adjacent nylon threads must allow the positioning of two recording electrodes in the region of interest. The platinum U-frame should be centered to avoid any mechanical interaction between its walls and the recording pipettes.
- Vibration isolation table (with a pneumatic system; TMC or Newport)
- XY stage for microscope to allow the independent movement of the microscope from the slice chamber and the recording pipettes (Luigs & Neumann)
- Water-immersion objective: ×40 or ×60, high numerical aperture (≥0.8) with long working-distance (≥2 mm) and specific design (cone shaped) to allow the insertion of two recording electrodes between the objective and the recording tissue
- Infrared-sensitive camera (IR-CCD camera; TILL Photonics or CoolsNAP HQ; Roper Scientific)
- Video monitor (black and white)
- Thermal videoprinter (UP-890CE; Sony) or video acquisition board to store images

## PROTOCOL

- Two peristaltic pumps (Minipuls 3; Gilson) *Note:* Independent inflow and outflow facilitates the adjustment of the liquid height in the recording chamber.
- Two patch-clamp amplifiers (Axopatch 200B (one channel), Axoclamp 2B (two channels) or MultiClamp 700B (two channels); Molecular Devices)
- Gain amplifiers (at least two channels) to amplify small postsynaptic signals (Sigmann)
- Two stable micromanipulators (usually left and right, LN mini 25; Luigs & Neumann) **▲ CRITICAL** They should be arranged such that the pipette on one side can be removed and changed without disturbing the pipette on the other side.
- Acquisition equipment (AD/DA converter and computer with data acquisition software)
- Thick-walled borosilicate glass tubing (Harvard Apparatus, cat. no. GC150F-10; outer diameter 1.5 mm, inner diameter 0.86 mm). Patch-pipettes are pulled freshly every day and stored in a pipette container (WPI)
- Two three-way pressure valves for maintaining pressure in the lines behind the patch pipettes
- Pipette puller: it should reproducibly pull patch-pipettes. Vertical pullers (PB-7; Narishige or L/M-3P-A; List-Medical) are generally user-friendly and reliable. Excellent results may also be obtained with horizontal pullers (Brown-Flaming P-97; Sutter Instruments)
- Patch-pipette fillers (MicroFil; WPI) with solution filter (0.2- $\mu$ m pore)

### REAGENT SETUP

**Standard ACSF** 125 mM NaCl, 2.5 mM KCl, 0.8 mM  $\text{NaH}_2\text{PO}_4$ , 26 mM  $\text{NaHCO}_3$ , 3 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 50  $\mu$ M phenol red and 11 mM D-glucose.

### PROCEDURE

#### Brain dissection and acute slice/slice culture preparation ● TIMING ~ 90 min

**1** | To prepare the slicing setup, the day before the experiment prepare an iced platform with two Petri dishes (145 mm and 60 mm) filled with ice. Store it at  $-20^\circ\text{C}$  overnight (**Fig. 3a,b**). Also store the slicing stage and inner slicing chamber at  $-20^\circ\text{C}$ , and prepare cubes of iced sucrose-based slicing solution using ice-cube bags and store at  $-20^\circ\text{C}$ .

**▲ CRITICAL STEP** For preparation of slice cultures, the dissection tools, slicer bath and filter paper should be sterilized.

**2** | On the day of the experiment (**Fig. 3a**), fill a 120-ml beaker with oxygenated ACSF with FCS (4%) at room temperature. Place a filter paper on the iced platform. Prepare an inverted suction pipette to water the brain with a Pasteur pipette broken at the narrow tip and mounted on a rubber bulb. Drop 1 ml of cutting solution on the filter paper. Fill the outer chamber of the slicer with ice and water and fill the inner slicing chamber with the sucrose-based solution. Add two ice cubes for acute slices (not for preparation of slice cultures).

**3** | Anesthetize the animal with chloral hydrate (intraperitoneal 200 mg  $\text{kg}^{-1}$ ). We usually use 15–20-d-old rats for acute cortical slices and 5–10-d-old rats for hippocampal slice cultures. Brain slices from juvenile animals usually contain a high proportion of healthy neurons. In addition, electrophysiological characteristics (input resistance, noise level) are generally better in neurons recorded from young animals. For slice cultures, clean the neck and head of the animal with ethanol and sodium hypochlorite (bleach).

**4** | Kill the animal by decapitation with either scissors or a guillotine at the level of the medulla. Put the head on the iced platform and water the isolated tissue every 10 s with the oxygenated ice-cold slicing solution during this step. With a scalpel blade, cut the scalp bilaterally in a caudal direction

**Figure 3** | Preparation of neocortical or hippocampal slices. **(a)** General view of the slicing setup: from left to right, top: the ice cold and bubbled cutting/slicing solution containing the phenol red pH indicator (1); the container for freshly cut slices containing normal solution complemented with FCS (2); the slicing stage where the neocortex or dissected hippocampi will be glued (3); the vibratome (4). Bottom: the iced platform with a filter paper on the top (5); set of dissection tools (6). **(b)** Zoomed view of a rat brain before dissection on the iced platform. During dissection the brain is periodically watered with cooled dissecting solution. **(c)** Zoomed view of two pieces of hippocampus glued on the slicing stage before being transferred to the vibratome slicing chamber. **(d)** View of a rat brain hemisphere during slicing. Waves and bubbles can be seen at the surface of slicing solution due to the oscillations of the razor blade and the carbogen bubbling, respectively.

This solution is prepared every day and saturated with carbogen at least 10 min before use.

**Sucrose-based slicing solution** 280 mM sucrose, 26 mM  $\text{NaHCO}_3$ , 1.3 mM KCl, 1 mM  $\text{CaCl}_2$ , 10 mM  $\text{MgCl}_2$ , 11 mM D-glucose, 50  $\mu$ M phenol red and 2 mM kynurenate. This solution is usually prepared each week and stored at  $4^\circ\text{C}$  for 1 week. It is also bubbled with carbogen and used for the cutting procedure. Antibiotics (penicillin–streptomycin) are added to the slicing solution for hippocampus slice cultures.

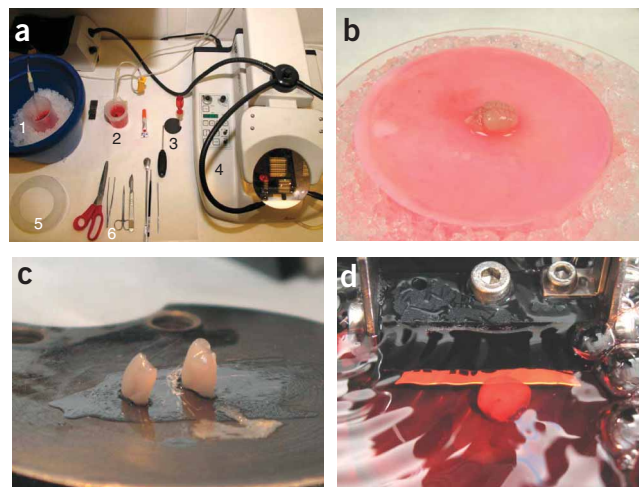
**Culture medium** 25 ml MEM, 12.5 ml HBSS, 12.5 ml horse serum, 0.5 ml penicillin/streptomycin, 0.8 ml glucose solution (1 M), 0.1 ml ascorbic acid solution (1 mg/ml), 0.4 ml HEPES (1 M), 0.5 ml B27 and 8.95 ml Volvic water. Solutions must be filter-sterilized (Steritop) before use<sup>91,93</sup>. Fresh culture medium is prepared every week.

**Patch-pipette solution for whole cell recording** 120 mM  $\kappa$ -gluconate, 20 mM KCl, 10 mM HEPES, 0.5 mM EGTA, 2 mM  $\text{MgCl}_2$ , 2 mM  $\text{Na}_2\text{ATP}$  and 0.5 mM NaGTP, titrated with KOH to pH 7.4. EGTA can be reduced to 0. This solution is frozen in 1 ml aliquots and can be stored for 6–8 months at  $-20^\circ\text{C}$ .

Biocytin (0.2–0.4%) can be added to visualize the morphology of the recorded pairs of connected neurons (**Fig. 2c**).

### EQUIPMENT SETUP

**Patch-clamp setup** Useful indications for the assembly of a functional patch-clamp setup and perfusion system can be found elsewhere<sup>89</sup>. The detection of small postsynaptic currents or potentials will be possible only if the recording noise is minimized. The peak-to-peak noise level should be  $\leq 200$ –500  $\mu\text{V}$  in current clamp and  $\leq 5$ –10 pA in voltage clamp. Details on the procedure for earthing the electrophysiological rig are given elsewhere<sup>94</sup>.



from above the eyes toward the posterior of the skull. Push the scalp aside and using small, thin scissors, cut the skull bilaterally in a rostral direction from the vertebral foramen toward the frontal lobes. Make four lateral cuts from the midline, two anterior and two posterior. Be sure these final cuts are sufficiently anterior and posterior so as not to damage the brain. Using blunt forceps, open the skull from the midline in a lateral direction. Extract the brain from the skull with a spatula and put it on the ice platform (**Fig. 3b**).

**▲ CRITICAL STEP** While cutting the skull, keep the tip of the scissors as close to the skull as possible with upward motion. The extraction of the brain should be accomplished within ~1–2 min after decapitation.

**5| Slicing.** At this step, two options are available: (A) for neocortical acute slices and (B) for cultured hippocampal slices (**Fig. 3c,d**).

**(A) Preparation of neocortical acute slices**

- (i) Isolate the hemispheres and place one in the cooled slicing chamber for anterior slicing. With a scalpel, create a base by making a cut in the frontal plane ~2 mm from the posterior part of the cortex. The anterior part of the hemisphere is removed to keep a block of 6–7 mm width.
- (ii) Slide the base of the tissue block from the spatula onto a thin film of cyanoacrylate glue on the cooled slicing stage. Submerge the stage with the block of tissue in the bath of the slicing chamber.
- (iii) Cut slices at 300–350 μm. The vibration rate of the blade is usually fast (~60–70 Hz or graduation 6–7 on the VT1000S) and the speed is nearly minimal (0.1–0.2 mm s<sup>-1</sup> or graduation 1–2 on the VT1000S). The amplitude of the horizontal blade movement is 0.8 mm.
- (iv) Transfer the slices into the incubation chamber containing FCS for 60 min at room temperature. Then, transfer in the definitive incubation chamber filled with standard ACSF at room temperature.

**(B) Preparation of hippocampal slice cultures**

- (i) Isolate both hippocampi and flatten them on the iced platform. Make a base by sectioning the extremities at one-third to keep only the central regions.
- (ii) Glue the hippocampi on the stage of the vibrating slicer, standing on either of the previous cuts (see **Fig. 3c**). We typically cut 250- to 350-μm slices. Carefully transfer the slices to the sterile incubation chamber containing slicing medium.
- (iii) Put each Millicells in a Petri dish (diameter 35 mm) and add 1 ml culture medium under the Millicells. Plate two to three slices on each Millicells and remove the excess of slicing medium from the surface of the Millicells membrane.
- (iv) Transfer the Petri dishes into the incubator at 37 °C (5% CO<sub>2</sub>).
- (v) Add Ara-C the day after (5 μM final).
- (vi) Change the medium every 2–3 d.

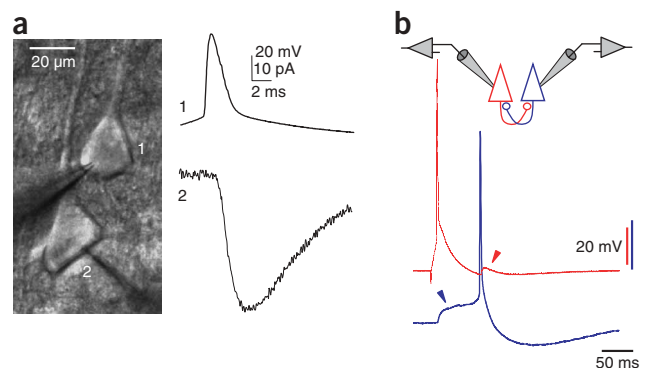
**Slice transfer and visualization ● TIMING ~10–20 min**

**6| Selection of the acute slice/slice culture.** Each slice should be inspected to select those that contain many healthy neurons in a given field examined with the ×40 or ×60 objectives. Healthy neurons display round and weakly contrasted outlines in DIC-infrared video microscopy (see **Supplementary Video 1** online). If necessary, optics should be optimized to visualize primary dendrites and axon initial segments<sup>83</sup>. Neurons with angular and highly contrasted outlines are dying cells. Recording from these cells must be avoided.

**▲ CRITICAL STEP** For L5–L5 connections, we usually select pairs of neurons that are vertically aligned with a distance <40 μm (**Fig. 4a**). This configuration is not critical for hippocampal slice cultures because adjacent neurons are not necessarily interconnected. To optimize local connections, dendrites and axon initial segments should run parallel to the surface of the slice. In these conditions, arborizations of recorded L5 neurons are generally damaged only minimally. This step is less critical in the case of slice cultures because most of the connections are reestablished *in vitro*.

**Paired recording and identification of connections ● TIMING ~15–30 min**

**7| Simultaneous recording from pairs of neurons.** In the ideal case, the presynaptic cell must be recorded in current clamp and the postsynaptic cell in voltage clamp. Because the direction of the connection is unknown *a priori*, the setup should be arranged in such a way that it is possible to easily switch from current clamp to voltage-clamp modes. Small steps



**Figure 4 |** Glutamatergic transmission between pairs of cortical L5 pyramidal neurons. (a) DIC-infrared image of two recorded neurons (left) and their synaptic coupling: an action potential evoked in neuron 1 elicits an EPSC in neuron 2 (right). (b) Reciprocal connection between two other L5 neurons recorded in current clamp. Note the EPSPs evoked reciprocally in each neuron (arrowheads).

of current (−10 pA, 10–20 ms) or voltage (−10 mV, 10–20 ms) must be injected on each channel to monitor the access resistance. The correct opening of the postsynaptic membrane should be checked carefully. The access resistance should be <20 MΩ and remain stable during the recording. Because uncontrolled changes in temperature may affect the timing and the properties of synaptic transmission<sup>95,96</sup>, temperature in the chamber is maintained at 34 °C with the controller. The holding potential of the postsynaptic neuron should be adjusted if GABA<sub>A</sub>-receptor-mediated inhibitory synaptic transmission is studied. In practice, the holding potential should be clearly different from the reversal potential of GABA<sub>A</sub> currents. With the internal chloride concentration indicated here (22 mM), the reversal potential is approximately −60 mV and the cell should be held at −50/−55 mV to record outward postsynaptic currents. However, for better resolution, we recommend in this specific case use of a high concentration of chloride ions in the postsynaptic pipette (130 mM, the reversal potential being near 0 mV<sup>44</sup>). In these conditions, the postsynaptic neuron can be held at −70 mV and the identity of the GABAergic connection probed with blockers of GABA<sub>A</sub> receptors.

**▲ CRITICAL STEP** The gain of the signal must be adequately amplified (10× for the presynaptic voltage and 100× or 500× for the postsynaptic signals) to avoid stepwise signals due to digitization of small signals. This precaution is required to resolve small postsynaptic currents or potentials.

**? TROUBLESHOOTING**

**8|** *Adjust the mode of stimulation.* Once a pair of neurons is recorded in whole-cell configuration, the presence of an eventual synaptic connection must be assessed using a brief (5–15 ms) depolarizing pulse injected in the presynaptic cell. These pulses should reliably generate a single presynaptic AP. Usually, we test the presence of a connection between two L5 neurons with a brief train of five pulses that produces a burst of five APs at 20–50 Hz evoked in the presynaptic neuron (cell 1) every 10 s. In these conditions, the time interval is sufficiently long to allow the replenishment of the pool of readily releasable vesicles in the case of a high-release probability synapse. In the favorable case, inward response will be evoked in the postsynaptic cell by spikes in the presynaptic cell (**Fig. 4a**).

**▲ CRITICAL STEP** For some specific synaptic types, the stimulation frequency must be carefully adjusted because release failures may occur at very low stimulus frequency in the case of granule cell–CA3 connection (0.01 Hz (ref. 62)) or at mild frequency at hippocampal CA3–CA3 synapses (1 Hz (ref. 97)).

**? TROUBLESHOOTING**

**9|** *Acquire data.* For experienced researchers, identification of a synaptic connection (even a very weak one) is unequivocal. However, beginners sometimes confuse connections with spontaneous currents or fail to detect weak synapses in the recording noise. One may effectively fail to detect a connection during the time of the experiment, especially if the synaptic connection is very weak (<10 pA or <0.5 mV). Therefore, for each dual recording, at least 50 consecutive trials should be systematically acquired.

**▲ CRITICAL STEP** In order to detect weak unitary connections, noise recording of the postsynaptic cell must be minimized and at least 50 consecutive trials must be acquired.

**? TROUBLESHOOTING**

**10|** *Criteria for monosynaptic connection.* Synaptic contacts between two neurons are considered monosynaptic when the latency between the presynaptic AP and the onset of the response in the postsynaptic neuron is usually short (~1–5 ms for local connections and 3–10 ms for long-distance connections), exhibits little fluctuation (1–2 ms), and when postsynaptic responses are consistently elicited by each presynaptic AP, even with high frequencies of discharge. Polysynaptic EPSC/Ps are suppressed in an all-or-none way when the presynaptic release probability is reduced by decreasing the external ratio of calcium-to-magnesium concentration whereas monosynaptic responses display a progressive decline following this manipulation<sup>63</sup>. Finally, monosynaptic responses display monotonic rising and decay whereas polysynaptic responses are contaminated by several ‘notches’ on their rising phase.

**? TROUBLESHOOTING**

**11|** *Check the eventual presence of a reciprocal connection.* Basically, the operations described above should be replicated in the other direction (from cell 2 to cell 1). An example of bidirectional connexion is illustrated in **Figure 4b**.

**Basic post hoc analysis ● TIMING ~ 30–90 min**

**12|** *Post hoc evaluation of the presence of a synaptic contact.* In the favorable case, a synaptic connection is clearly identifiable during the experiment. However, a systematic exploration of the postsynaptic signal must be performed in most cases because the signal-to-noise ratio is too small. The simplest solution consists in averaging the postsynaptic response after aligning the pre- and postsynaptic signals with the reference to the presynaptic spike. This procedure can be automated in most analysis software with the sharp detection of the peak (or onset) of the presynaptic AP. The smallest EPSCs are usually in the 2–5 pA range (~0.1 mV for an excitatory postsynaptic potential (EPSP)).



**▲ CRITICAL STEP** Examination of each sequence before averaging is necessary in order to not include spontaneous currents, failures of presynaptic spike triggering and eventual spike doublets. All these uncontrolled events may alter the averaged postsynaptic response and must be discarded from the averages.

**? TROUBLESHOOTING**

**13| Analysis of individual currents.** Individual currents can be analyzed to determine the stability of the postsynaptic response amplitude and analyze the quantal distribution, study the paired-pulse ratio, long-term changes or latency fluctuations. Additional information on the analysis of synaptic currents may be found in other protocols<sup>98</sup>.

**● TIMING**

- Steps 1–5, slicing and recovery of slices: 90 min
- Step 6, slice transfer and visualization: 10–20 min
- Steps 7–11, paired recording and identification of connection: 15–30 min
- Steps 12 and 13, basic *post hoc* analysis: 30–90 min

**? TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**. Basic troubleshooting information on potential sources of error and solutions in patch-clamp recordings or problems with the quality of the slice cultures can be found in other protocols<sup>89,91–93</sup>.

**TABLE 1 |** Troubleshooting table.

Problem	Possible reason	Solution
No postsynaptic response	No physical connection because local axon collaterals are severed. This may happen because the recorded cells are too near to surface of the slice, the neurons are too distant or the slicing plane is not correct	Select two other cells at 50–70 μm from the surface of the slice, aligned vertically and not farther than ~40–50 μm distant. Re-examine the orientation of neurons in the slice and select a region (or re-slice another cortex) until you find a cortical region where the dendrite and axon run parallel to the slice surface
	Holding potential of the postsynaptic neuron is equal to the reversal potential of the postsynaptic receptor-mediated response. This problem is particularly critical when studying inhibitory synaptic connections mediated by GABA <sub>A</sub> receptors ( $E_{Cl} = -70$ mV in control conditions)	Depolarize the postsynaptic cell (to -55 mV) to enhance the driving force for GABA <sub>A</sub> -receptor-mediated responses, or record the postsynaptic neuron with a pipette solution containing a high concentration of chloride ions (140 mM)
	Presynaptic axonal failures due to the activation of A-type K <sup>+</sup> current <sup>74</sup>	Depolarize the presynaptic cell to -55/-60 mV to inactivate the axonal A-current
	Presynaptically silent synapse because release probability is very low (see ref. 62, their Fig. 1)	Increase the rate of stimulation with short bursts of presynaptic spikes at 20–50 Hz  Alternatively, try reducing the rate of stimulation to 0.025 Hz (ref. 104)
	Postsynaptically silent synapse because AMPA receptors are not expressed at the synapse <sup>105</sup>	Use older animals (neocortical slices) or older cultures because the percentage of silent synapses decreases during development <sup>106</sup> . Alternatively, one may look for a postsynaptic NMDA response, with an appropriate protocol, taking into account the biophysical properties of these receptors <sup>107,108</sup>
Connection with slow kinetics and small amplitude	The postsynaptic recording pipette is not correctly open and the fast signals are filtered by the high resistance at the tip of the electrode	Check the transients in the recordings and gently blow or apply a slight suction to fully rupture the membrane under the electrode
Connection hardly detectable	The signal-to-noise ratio is too low	Reduce the recording noise on the setup and check for possible loop in the earthing <sup>94</sup>  Work with younger animals: recordings of immature neurons, which have a higher input resistance, and a lower capacitance allow better voltage-clamp control and a higher signal-to-noise ratio

(continued)



**TABLE 1** | Troubleshooting table (continued).

Problem	Possible reason	Solution
	The recording parameters may not be optimal	Temperature and/or rate of stimulation may not be optimal for the recording of a given connection. The postsynaptic receptors may also be desensitized (e.g., AMPA receptors <sup>104</sup> ). Block desensitization pharmacologically
Connection vanishes with time	This is commonly observed at inhibitory connections in the cerebellum because GABA supply is exhausted in the presynaptic compartment <sup>109</sup>	Patch the presynaptic cell using gramicidin or amphotericin, or add GABA in the pipette in whole-cell configuration <sup>12</sup>
	The rate of stimulation is too high for the synapse under analysis, and the releasable pool of vesicles is exhausted <sup>97</sup>	Decrease the rate of stimulation (down to 0.025 Hz)

**ANTICIPATED RESULTS**

If the above-mentioned hints are followed, any electrophysiologist should be able to routinely obtain connected pairs of neurons. Making simultaneous recordings from pairs of neurons is fundamentally similar to whole-cell patch-clamp from single cells. If high-quality, long-lasting patch-clamp cannot be achieved with a very high probability (>90%), paired recording will be extremely challenging, if not impossible. Therefore, a prerequisite is optimization of conditions for obtaining stable and high-quality recordings. When starting dual recordings, it would not be surprising to obtain no connected pairs in the first few days (even if five to eight pairs are recorded per day). We have observed that the rate of connection regularly increases with the experience of the experimentalist. Practically, 2 or 3 months of continuous practice are necessary before reaching a satisfying probability of connections. Other tricks can be used to further enhance the probability of connection. Simultaneous triple<sup>99,100</sup>, quadruple<sup>52,70,101,102</sup> or septuple<sup>103</sup> recordings may enhance the probability of finding connected pairs. The major difficulty here resides in the management of three to seven pipettes simultaneously and the availability of micromanipulators and amplifiers.

The rate of synaptic connections can be as high as ~30% between adjacent pairs of L5 neurons<sup>33</sup>. However, a robust signal-to-noise ratio that allows detailed analysis occurs in only ~10% of the tested pairs because 50% of unitary EPSCs are <12 pA<sup>33</sup>. Similar findings have been observed in hippocampal slice cultures. The median is ~0.8 mV for unitary EPSPs<sup>26</sup>.

In our hands, chemical synaptic transmission can be recorded in stable conditions as long as pre- and postsynaptic neurons are held in good conditions. We have obtained stable recordings from pairs of L5 neurons lasting >6 h (S.B. & D.D., unpublished data). This duration of recording allows many different manipulations on the same connection (extracellular applications of ion channel blockers or postsynaptic receptor antagonists, induction of LTP) without any apparent rundown.

Note: Supplementary information is available via the HTML version of this article.

**ACKNOWLEDGMENTS** We thank M. Seagar, O. El Far, J.M. Goillard, Y. Frégnac and G. Alcaraz for stimulating discussions and F. Dubruc for help with video recordings. This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Agence Nationale de la Recherche (Neuroscience, Neurologie and Psychiatrie, 06-Neuro-014-01 to D.D.), the Fondation pour la Recherche Médicale (to E.C.), the Fondation Française pour la Recherche sur l'Épilepsie (to O.C.), the Ministry of Research (doctoral grant to S.B. & E.C. and ACI Jeunes Chercheurs to D.D.), AFM (to R.C.) and the European Community (LSHM-CT-2004-511995, synaptic scaffolding proteins orchestrating cortical synapse organization during development to D.D.).

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