Video Article

Whole-cell Patch-clamp Recordings from Morphologically- and Neurochemically-identified Hippocampal Interneurons

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Abstract

GABAergic inhibitory interneurons play a central role within neuronal circuits of the brain. Interneurons comprise a small subset of the neuronal population (10-20%), but show a high level of physiological, morphological, and neurochemical heterogeneity, reflecting their diverse functions. Therefore, investigation of interneurons provides important insights into the organization principles and function of neuronal circuits. This, however, requires an integrated physiological and neuroanatomical approach for the selection and identification of individual interneuron types. Whole-cell patch-clamp recording from acute brain slices of transgenic animals, expressing fluorescent proteins under the promoters of interneuron-specific markers, provides an efficient method to target and electrophysiologically characterize intrinsic and synaptic properties of specific interneuron types. Combined with intracellular dye labeling, this approach can be extended with post-hoc morphological and immunocytochemical analysis, enabling systematic identification of recorded neurons. These methods can be tailored to suit a broad range of scientific questions regarding functional properties of diverse types of cortical neurons.

Video Link

The video component of this article can be found at https://www.jove.com/video/51706/

Introduction

Hippocampal neuronal circuits have long been the subject of intense scrutiny, with respect to both anatomy and physiology, due to their essential role in learning and memory as well as spatial navigation in both humans and rodents. Equally, the prominent, but simple laminar organization of the hippocampus makes this region a favored subject of studies addressing structural and functional properties of cortical networks.

Hippocampal circuits are comprised of excitatory principal cells (>80%) and a smaller (10-20%), but highly diverse cohort of inhibitory interneurons[1-3]. Interneurons release γ-aminobutyric acid (GABA) from their axon terminals which acts at fast ionotropic $GABA_A$ receptors ($GABA_A$Rs) and slow metabotropic $GABA_B$ receptors ($GABA_B$Rs)[4]. These inhibitory mechanisms counterbalance excitation and regulate the excitability of principal cells, and thus their timing and pattern of discharge. However, GABA released from interneurons acts not only on principal cells, but also on the interneurons themselves[5,6]. Pre and postsynaptic receptors mediate feedback regulation and inhibitory mutual interactions among the various types of interneuron. These inhibitory mechanisms in interneuron networks are believed to be central to the generation and shaping of population activity patterns, in particular oscillations at different frequencies[7].

Whole-cell patch-clamp recording is a well-established method for the examination of intrinsic properties and synaptic interactions of neurons. However, due to the high diversity of interneuron types, investigation of inhibitory interneurons requires rigorous identification of the recorded cells. As hippocampal interneuron types are characterized by distinct morphological features and neurochemical marker expression, combined anatomical and immunocytochemical examination can provide a means to determine precise interneuron identity[6,8,9].

In the present paper we describe an experimental approach in which whole-cell patch-clamp recordings from single neurons or synaptically-coupled pairs are combined with intracellular labeling, followed by *post-hoc* morphological and immunocytochemical analysis, allowing for the characterization of slow $GABA_B$ receptor mediated inhibitory effects in identified interneurons. As an example, we focus on one major type of interneuron, a subset of the so called “basket cells” (BC), which innervates the soma and proximal dendrites of its postsynaptic targets and is characterized by a “fast spiking” (FS) discharge pattern, an axon densely covering the cell body layer, and expression of the calcium-binding protein parvalbumin (PV)[10,11]. These interneurons display large postsynaptic inhibitory currents, as well as prominent presynaptic modulation of their synaptic output, in response to $GABA_B$R activation[12]. The combination of techniques described here can be applied equally well to investigate intrinsic or synaptic mechanisms in a variety of other identified neuron types.

Protocol

Ethics Statement: All procedures and animal maintenance were performed in accordance with Institutional guidelines, the German Animal Welfare Act, the European Council Directive 86/609/EEC regarding the protection of animals, and guidelines from local authorities (Berlin, T-0215/11)

1. Preparation of Acute-hippocampal Slices

1. Take a transgenic rat (17 to 24 day old), expressing the fluorescent Venus/YFP protein under the vGAT promoter, which labels the majority of cortical inhibitory interneurons[13]. Decapitate the rat. Rapidly dissect the brain (<40 sec) into semifrozen, carbogenated (95% O_2/5% CO_2) sucrose-based artificial cerebrospinal fluid (sucrose-ACSF, **Figure 1A)**.
2. Assess the dissected rat brain for Venus/YFP fluorescence with a 505 nm LED lamp and 515 emission filter, mounted on a pair of goggles.
3. Remove the frontal third of the cortex and cerebellum; then separate the hemispheres, all with a scalpel. Remove the dorsal surface of the cortex to provide a flat surface to glue the brain down, as previously described[14].
4. Cut transverse slices (300 µm) of the hippocampal formation on a vibratome, the hemispheres should be surrounded with semifrozen, carbogenated sucrose-ACSF (**Figure 1B**)[14]. Remove additional regions of rostral cortex, midbrain and brainstem. Transfer each slice to a submerged holding chamber containing sucrose-ACSF, which is carbogenated and warmed to 35 ºC.
5. Leave the slices to recover at 35 ºC for 30 min from the time of the last slice entering the warmed ACSF. Do this in order to reactivate metabolic processes and facilitate the resealing of cut neuronal processes. Then transfer to room temperature for storage (**Figure 1C**).

2. Fabrication and Filling of Recording Pipettes

1. Pull patch pipettes from glass capillaries, so that a pipette resistance of 2-4 MΩ is achieved when filled with filtered (syringe filter, pore size: 0.2 µm) intracellular solution containing 0.1% biocytin (for intracellular labeling). Keep the intracellular solution chilled on ice to prevent degradation of its constituents.
2. Fill patch pipettes for identification of postsynaptic currents with a solution containing a physiologically relevant low Cl^- concentration ($E_{R(Cl-)}$= -61 mV; see solution list).
3. For paired recordings to identify the presynaptic receptor mediated responses, fill patch pipettes with intracellular solution with low Ca^{2+} buffer capacity to prevent interference with transmitter release presynaptically, as well as 4-fold higher Cl^- concentration ($E_{R(Cl-)}$= -20 mV) to improve signal-to-noise of observed IPSCs[5] allowing accurate assessment of pharmacological responsiveness. Note that changing Cl^- concentration can alter IPSC kinetics[15].

3. Whole Cell Patch-clamp Recording from FS-INs

1. Carbogenate the ACSF and feed through the perfusion system to the recording chamber, by means of a peristaltic pump (which also removes ACSF from the recording chamber through a suction line, **Figure 2A**). Turn on all equipment on the setup in preparation for recording.
2. Transfer a slice to the recording chamber and hold in place with a platinum ring strung with single fibers of silk. Position the slice so that the *stratum (str.) pyramidale* of CA1 runs vertically through the field of view, allowing access with 2 pipettes to both the *str. radiatum* and *str. oriens* simultaneously (**Figures 2C** and **4A**).
3. Place the chamber into the setup and start perfusion of carbogenated and warmed (32-34 ºC) recording ACSF at a flow rate of 5-10 ml/min.
4. Assess slice quality under IR-DIC optics at 40X objective magnification, and visualize with a CCD camera viewed on a display. Assume good slice quality if a large number of round, moderately contrasted CA1 pyramidal cells (CA1 PC) can be seen in *str. pyramidale* at depths of 20-30 µm below a smooth and lightly cratered surface (**Figure 2C)**. Poor quality slices contain large numbers of highly contrasted, shrunken or swollen cells, with an uneven slice surface.
5. Identify putative FS interneurons under epifluorescence illumination as those expressing Venus/YFP (**Figure 2B**), with large multipolar somata in or near the *str. pyramidale*. Select cells reasonably deep within the slice (50-100 µm, **Figure 2C**) in order to better preserve their morphological integrity.
6. Mount the recording electrode in the pipette holder on the headstage; then apply a low, positive pressure (20-30 mBar) through the tube line. Lower the pipette to the surface of the slice, slightly offset to the center of the selected neuron.
7. Obtain whole-cell recording configuration as described previously[14,16] and see also **Figures 2D** and **2E**:
    1. Target a cell: Increase the pressure to 70-80 mBar and rapidly lower the pipette through the slice to just above the soma of the selected cell (**Figure 2D**, top).
    2. Approach the cell: Press the pipette against the cell membrane to produce a “dimple” on it (**Figure 2D**, top). Perform this step swiftly, in order to prevent biocytin labeling of neighboring cells.
    3. Create a giga-ohm seal: Release the pressure and simultaneously apply a 20 mV voltage command to the pipette. A giga-ohm seal (1-50 GΩ; **Figure 2D**, bottom and **Figure 2E** middle) typically develops rapidly. Once sealed, apply the expected resting membrane potential (typically between -70 and -60 mV) as a voltage command.
    4. Break through the patch: Once sealed, rupture the membrane patch with a short pulse of negative pressure; thereby achieving the whole-cell configuration (**Figure 2E**, bottom).
8. Compensate whole-cell capacitance and series resistance (R_S). R_s is normally 5-20 MΩ and stable for up to 120 min. Abandon cells if membrane potential (V_M) on break-through is more depolarized than -50 mV; R_S is initially greater than 30 MΩ; or R_S changes by more than 20% over the course of the recording.
9. Identify FS-INs by their response (in current-clamp mode) to a family of hyper- to depolarizing current pulses (-250 to +250 pA, **Figure 2F**, top). FS-INs have relatively depolarized V_M (typically -50 to -60 mV), short membrane time-constant (<20 msec) and respond to a 500 pA

depolarizing current injection with a train of action potentials (APs) at frequencies >100 Hz [11] (**Figure 2F**, bottom), which are markedly different from those in CA1 PCs (**Figure 2F**, middle).

4. Extracellular Electrical Stimulation to Evoke $GABA_BR$-mediated Responses

1. To observe synaptically evoked responses, position an extracellular stimulation electrode (a patch pipette filled with 2 M NaCl; Resistance: 0.1-0.3 MΩ) in the slice at the border of *str. radiatum* and *str. lacunosum-moleculare.* Position the electrode 200-300 µm lateral to the soma to prevent direct electrical stimulation of the cell and minimize stimulation artifacts (**Figure 3A**).
2. Once the stimulation electrode is positioned, obtain whole-cell recording of the chosen cell and assess the physiological phenotype in current-clamp mode as in section 3.9 (**Figure 3B)**.
3. With the neuron recorded in voltage-clamp (V_M -65 mV), deliver electrical stimulation of presynaptic axons at 50 V (~500 µA effective stimulus) every 20 sec, using an isolated constant-voltage stimulator. Use single stimuli (100 µsec duration, **Figure 3C**, top) to observe $GABA_BR$ mediated IPSCs, and interleave with trains of multiple stimuli (at 200 Hz) to produce greater transmitter release.
4. Bath apply ionotropic glutamate receptor antagonists (AMPA receptor: DNQX [10 µM]; NMDA receptor: d-AP5 [50 µM]) to reveal the isolated monosynaptic IPSC (**Figure 3C**, middle upper). Further isolate the $GABA_BR$-mediated IPSC with application of a $GABA_AR$ blocker (gabazine [10 µM]; **Figure 3C** middle lower).
5. Confirm the resultant slow-outward current (**Figure 3C** lower, expanded) as being $GABA_BR$-mediated by the subsequent application of CGP-55,845 [5 µM] (**Figure 3C** lower, underlain in grey)

5. Paired Recordings of Synaptically Coupled FS-IN and CA1 PCs

1. Assess $GABA_BR$-mediated presynaptic control of inhibitory synaptic transmission with simultaneous recordings, performed between synaptically-coupled IN and PC pairs as described below.
2. First, establish a whole-cell recording of a presynaptic interneuron (as in section 3) and confirm the FS phenotype (**Figure 4A**).
3. Then patch a neighboring CA1 PC (20-100 µm distance, **Figure 4A**) and apply brief suprathreshold depolarizing current pulses (1 msec duration, 1-5 nA amplitude) to the presynaptic IN (held in current-clamp mode) to elicit APs. If a synaptic connection is present, APs in the IN result in IPSCs in the CA1 PC, held in voltage-clamp (compensate R_S to about 80%).
4. If necessary, fill a new recording electrode and record from further CA1 PCs until a connection is found.
5. Once a connection is established, elicit pairs of APs in the presynaptic FS-IN to assess both the unitary synaptic response and dynamic behavior. Use a typical paired-pulse protocol of 2 depolarizing stimuli with a 50 msec interval (**Figure 4B**).
6. Collect control traces in baseline conditions. Then, apply the selective $GABA_BR$ agonist baclofen (10 µM) to the perfusing ACSF, thus activating $GABA_BRs$, followed by the antagonist CGP-55,845 (5 µM), to fully block the receptor mediated effects. Collect ~50 traces during steady state of each drug condition (**Figure 4B and C**).
7. Once the recording is complete, seal the somatic membrane by forming an outside-out patch: Slowly withdraw the pipette from the cell body in V-clamp and as the R_S increases, reduce the V_M to -40 mV. Do this to facilitate the formation of the outside-out patch; then remove the pipette from the bath.

6. Analysis of Electrophysiological Properties

1. NOTE: A multitude of different software packages are available for the acquisition of electrophysiological data. Here, WinWCP, a Windows program in the free Strathclyde Electrophysiology Software package is used, which allows recording of up to 16 analog input channels and output of 10 digital signals.
2. Low-pass filter all data at 5-10 kHz and sample at 20 kHz.
3. Analyze physiological data with an off-line analysis suite.
   NOTE: Stimfit, an open source software package which includes a Python shell, is used in this instance; however other alternatives can easily be used instead.
   1. Analyze passive membrane properties of recorded neurons, acquired in current clamp, from resting membrane potential.
   2. Measure the mean resting membrane potential from the baseline of recorded responses from the beginning of the recording.
   3. Calculate input resistance, using Ohm's law, from the voltage response to the smallest hyperpolarizing current pulses (≤ -50 pA). To improve signal to noise ratio, average multiple traces. Note: our examples are typically averages of 10-50 individual sweeps.
4. Estimate the apparent membrane time constant by fitting a monoexponential curve to the decay of the responses to the smallest hyperpolarizing current pulses.
5. Analyze action potential waveform to determine threshold, amplitude (threshold to peak) and duration (width measured at half height) elicited by threshold level depolarizing current pulses.
6. Analyze $GABA_BR$ mediated IPSCs from voltage clamp recordings. Filter traces off-line at 500 Hz (Gaussian filter) and assess the peak amplitude and latency of the $GABA_BR$ mediated response (in averages of at least 10 traces).
7. Detect the effect of $GABA_BRs$ on the inhibitory output of INs as a change in peak amplitude of the $GABA_AR$-mediated IPSCs measured between peak and preceding baseline. Calculate the mean amplitude from ≥50 traces for the control period and the steady-state of all pharmacological epochs.

7. Visualization and Immunocytochemistry of FS-Ins

1. Following the recordings, fix the slices by immersion in 4% paraformaldehyde with 0.1 M phosphate buffer (PB, pH=7.35) O/N at 4 °C.
2. If necessary slices can be transferred to PB and stored for up to ~1 week before processing.
3. Wash slices liberally in fresh PB and subsequently in 0.025 M PB with 0.9% NaCl (PBS, pH=7.35).

4. To reduce non-specific antibody binding, block the slices for 1 hr at RT in a solution containing 10% normal goat serum (NGS), 0.3% Triton-X100 (a detergent to permeabilize membranes) and 0.05% NaN_3, made up in PBS.
5. To label for PV expression, use an anti-PV monoclonal mouse antibody diluted in a solution containing 5% NGS, 0.3% Triton-X100, 0.05% NaN_3, in PBS. Incubate primary antibodies for 2-3 days at 4 °C[12]. Rinse slices thoroughly in PBS.
6. Apply fluorescent anti-mouse secondary antibodies (*e.g.* Alexafluor-546) along with the biotin binding-protein streptavidin, conjugated to a fluorochrome (*e.g.*, Alexafluor-647); and incubate in a solution containing 3% NGS, 0.1% Triton-X100, 0.05% NaN_3, diluted in PBS and incubate O/N at 4 °C.
7. Liberally rinse slices 2-3x with PBS followed by 2-3 rinses in PB. Mount the slices on glass slides. Use a 300 µm agar spacer to prevent the slice from collapsing. Cover-slip slices with a fluorescent mounting medium and seal with nail-varnish.

8. Imaging and Reconstruction of Visualized FS-Ins

1. Visualize the slices using a scanning confocal microscope, with the fluorochome reporter excited with the appropriate laser line (diode laser 635 nm for Alexafluor-647; Helium-Neon 543 nm for Alexafluor-546 labeling for PV and Argon 488 or 515 nm for Venus/YFP).
2. Take images at an appropriate Z-resolution (typically 0.5-1 µm steps, using a 20X objective) to produce a Z-stack of the whole cell. Multiple stacks are normally required to image the whole cell, which can be digitally stitched off-line using FIJI/ImageJ software (**Figure 5A**).
3. Reconstruct the cell from the stitched image stack using a semi-automatic tracing method (Simple Neurite Tracer plugin in FIJI/ImageJ software package[17], **Figure C)**.
4. Finally, assess the PV-immunoreactivity of the interneuron with a high numerical aperture objective lens (60X silicon-immersion, N.A.=1.3). Make images of the soma, proximal dendrites and proximal axon, or alternatively of axon terminals if somatic washout of PV is too strong. Cells are deemed immunoreactive for PV if immuno-labelling is seen to align with the biocytin-labeled structures (**Figure 5B**).

Representative Results

Provided that slice quality is appreciably good, recording from both CA1 PCs and FS-INs can be achieved with minimal difficulty. The transgenic rat line expressing Venus / YFP under the vGAT promoter[13] does not unequivocally identify FS-INs, or indeed BCs. However recordings from INs in and around *str. pyramidale*, where the density of FS-INs is typically high[1], results in a high probability of selecting FS-INs (**Figure 2B**). FS-INs can be distinguished by their characteristic physiological properties different from those of both CA1 PCs and RS-INs. They have a relatively depolarized resting membrane potential (-58.9 ± 1.5 mV, 15 cells, vs. -62.6 ± 1.1 mV in CA1 PCs, 26 cells), low input resistance (92 ± 12 MΩ vs. 103 ± 14 MΩ in PCs) and fast apparent membrane time constant (15.4 ± 2.6 msec vs. 22.0 ± 2.7 msec in PCs), resulting in rapid voltage response to hyperpolarizing current pulses (**Figure 2F**). FS-INs discharged very brief action potentials (0.38 ± 0.01 msec) of relatively low amplitude (82.8 ± 1.0 mV) followed by very prominent fast afterhyperpolarization (mean amplitude 22.6 ± 2.9 mV). In response to large depolarizing current pulses (250 pA), FS-INs fired at high frequencies (82 ± 10 Hz; Range: 34-128 Hz; **Figure 2D,** left).

To assess $GABA_BR$-mediated postsynaptic currents in FS-INs, pharmacologically isolated synaptic responses were elicited by extracellular stimulation of inhibitory fibers at the *str. radiatum/lacunosum-moleculare* border. **Figure 3** shows an example of a FS-IN, in which sequential blockade of components of the compound synaptic response isolates the monosynaptic $GABA_BR$-mediated slow IPSC. Synaptic responses were elicited by single stimuli or trains of 3-5 stimuli (50 V intensity, 0.1 msec duration, delivered at 200 Hz) to the *str. lacunosum-moleculare/ radiatum* border (**Figure 3A**). The initial compound response including both excitatory and inhibitory components (**Figure 3C, top**) was strongly reduced by bath application of AMPA and NMDA receptor antagonists (DNQX, 10 µM and AP-5, 50 µM, respectively: **Figure 3C, middle**). The residual monosynaptic IPSC comprised an early fast inward current (a putative inward Cl^- mediated current at the holding potential -65 mV, with a reversal potential of approximately -60 mV, in these experiments) and a slower outward current (mediated by a putative K^+ conductance with a reversal potential close to 100 mV). Application of the selective $GABA_AR$ antagonist gabazine (SR-95531, 10 µM) abolished the fast IPSC, leaving an isolated the slow $GABA_BR$-mediated IPSC (**Figure 3C,** bottom, black trace); which was observed in response to single stimulation, but more clearly in response to short trains of stimuli. This response was confirmed as a $GABA_BR$-activated K^+ conductance, as it was blocked by the potent and selective antagonist CGP-55,845 (CGP, 5 µM, **Figure 3C,** bottom, gray trace). FS BCs typically show large amplitude $GABA_BR$-mediated IPSCs, such as shown in our recent publication[12].

To assess presynaptic regulation of FS-IN inhibitory output by $GABA_BRs$, paired recordings were performed from synaptically-coupled FS-IN and CA1 PCs. Synaptic connectivity between FS BCs and CA1 PCs is relatively high, as shown previously[1,3]. In these recordings, as shown previously, coupling probability is over 50% between closely located cell pairs (≤50 µm; **Figure 4A**)[5]. However, this depends strongly on the interneuron type examined. Connectivity was tested with either a long depolarizing current injection (100 msec, ≥ 500 pA) or a train of short depolarizing pulses (1 msec duration, 1-5 nA, up to 10 pulses delivered at 20 Hz) eliciting a single AP each. APs in the presynaptic interneurons elicited fast $GABA_AR$-mediated IPSCs with short latency, rapid rise and decay in synaptically-coupled PCs. When paired-pulses (2 pulses at 20 Hz) were applied, the synapse showed short-term depression (Paired-pulse ratio < 1)[1]. In the example cell shown, bath application of the $GABA_BR$ agonist baclofen (2-10 µM) resulted in a substantial reduction in the amplitude of the first IPSC (**Figures 4B** and **4C**). Subsequent bath application of the antagonist CGP-55,845 invariably resulted in a recovery of the IPSC (5 out of 5 cells[12]; **Figures 4B** and **4C**).

Once a recording had been completed, an outside-out patch successfully formed, the slices were fixed overnight and subsequently processed to visualize and analyze the morphology of the recorded cells and determine their neurochemical marker content. **Figure 5A** illustrates the morphology of one representative FS BC in a projection of combined image stacks obtained on a confocal microscope. The cell's expression of PV was confirmed in immunocytochemical labeling that gave a clear immuno-signal over the cell body and proximal dendrites of the recorded and biocytin-labeled cell (**Figure 5B**). Three-dimensional reconstruction of the cell was performed from stitched image stacks using the Simple Neurite Tracer plugin in FIJI software (**Figure 5C**)[17]. The axon showed a high density of collaterals in and near the *str. pyramidale* with "baskets" formed around the somata of CA1 PCs (**Figure 5A, inset**), putatively identifying this cell as a BC. Furthermore, the localization of the soma near to *str. pyramidale* and the radially-oriented dendrites spanning all layers of the CA1 correspond well to the typical morphological feature of FS BCs[10].

In summary, in whole-cell recordings obtained from identified FS PV+ BCs, we have demonstrated that these cells express large amplitude $GABA_BR$-mediated slow IPSCs and their synaptic output is also markedly inhibited by the activation of presynaptic $GABA_BRs$.

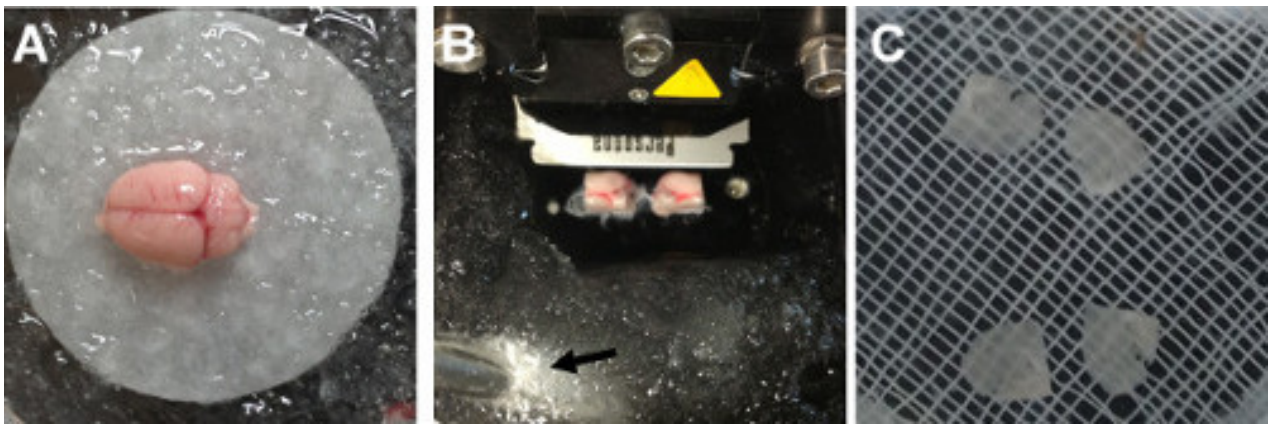

Figure 1. Preparation of acute hippocampal slices. (A) A freshly dissected rat brain. Note the ice surrounding the brain, maintaining the temperature of the whole brain near 0 °C. **(B)** Cutting of 300 µm hippocampal slices on a Leica VT1200s vibratome. The hemispheres are aligned so that the cortical surface is cut first. Note the large amount of slushy ice surrounding the hemispheres and the constant carbogenation of the icy ACSF (arrow). **(C)** After cutting the brain slices are moved to warmed sucrose-ACSF. The slices were turned over and trimmed to remove the forebrain and midbrain, leaving only the hippocampus and overlying cortex.

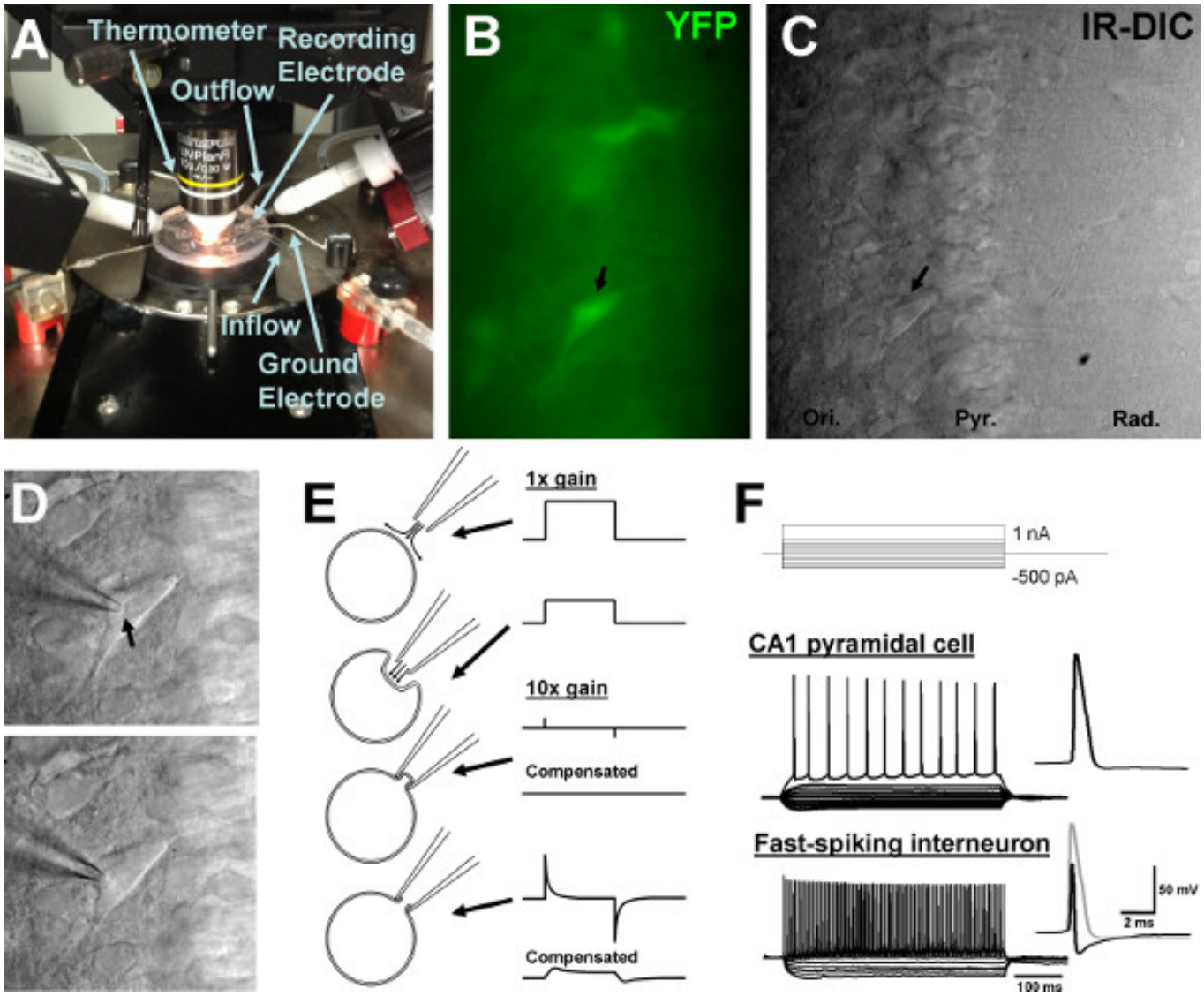

Figure 2. Whole-cell patch-clamp recording from interneurons. **(A)** The recording configuration around the chamber. Note the inflow and outflow are on opposing sides of the chamber to achieve a close to laminar flow. Also visible are the recording electrodes, mounted on the headstages, and the ground electrodes on the two sides, as well as the objective (40X, water-immersion) in the middle. **(B)** Low-power epifluorescent image of the vGAT-Venus/YFP signal in the CA1 of the hippocampus in a slice. **(C)** IR-DIC imaging of the same area. The arrows in B and C indicate a Venus/YFP positive interneuron in the cell body layer. **(D)** High-power IR-DIC images of the soma of the neuron indicated in panel **B**, with an approaching patch pipette forming the dimple on the surface (top) and, subsequently, the cell in the whole-cell configuration (bottom). **(E)** Schematic illustration of the major stages of establishing a whole-cell patch-clamp recording (left side) with cartoon responses to a test voltage-pulse monitoring the resistance at the pipette tip (right side). Top row: Pipette in the bath away from the cell; Upper middle: Dimple formation, accompanied by a reduction in the pulse amplitude, indicating a moderate increase in resistance. Lower middle: Giga-ohm seal formation. Note that the current pulse amplitude is dramatically reduced. Only the fast capacitive currents are visible at the beginning and end of the pulse before pipette capacitance is compensated. Bottom: Whole-cell recording configuration is achieved by breaking through the membrane patch under the pipette tip. Note that the large but relatively slow capacitive currents at the beginning and end of the pulse before series resistance compensation is applied. **(F)** Representative traces of a FS-IN (top) and CA1 PC (bottom), elicited by a family of hyper- to depolarizing current pulses (protocol shown above). Note the very high frequency discharge of the FS-IN compared to the much slower firing of the CA1 PC. Insets on the right: Comparison of the AP waveform from the CA1 PC (top) and the FS-IN (bottom, in grey, overlaid on the PC AP in black), illustrating the difference in the waveform of the AP and the AHP.

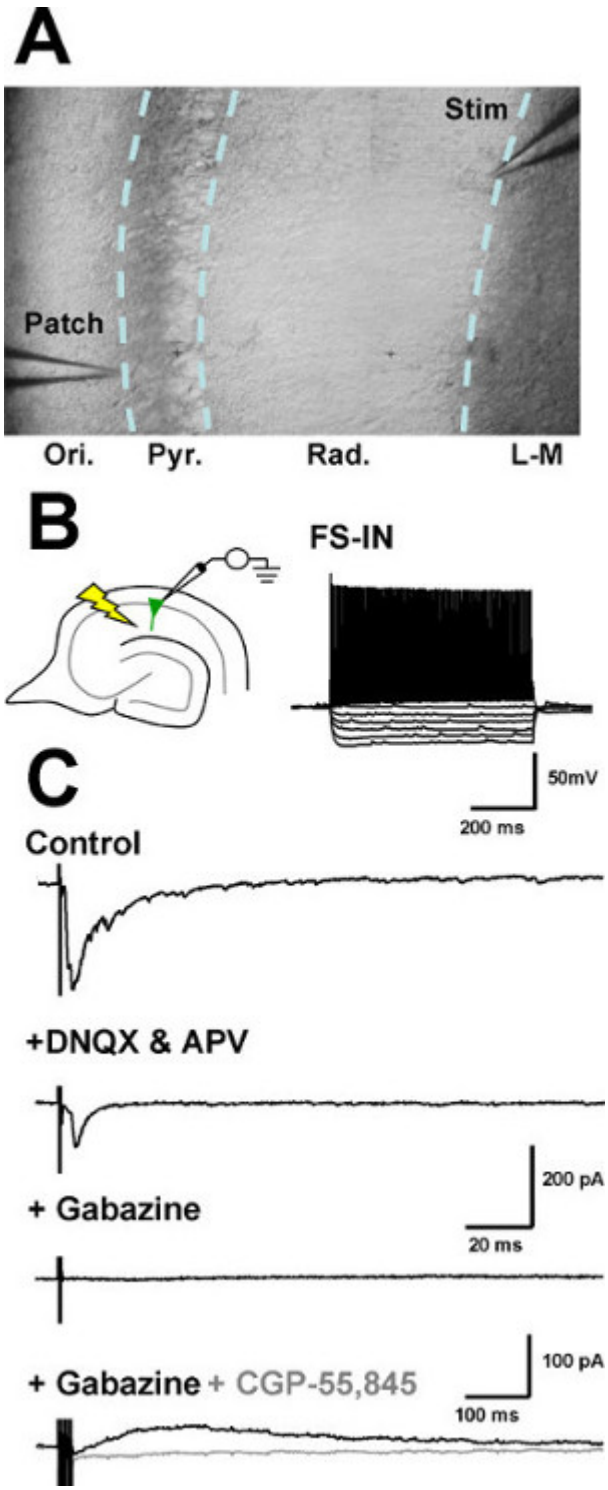

Figure 3. Pharmacological dissection of the compound synaptic response, isolating slow $GABA_BR$-mediated IPSCs in a FS-IN. (A) IR-DIC image of the slice in the recording chamber, with the recording electrode (Patch) placed at the border of the *str. oriens* (Ori.) and *pyramidale* (Pyr.) and the simulation electrode (Stim) at the border of the *str. radiatum* (Rad.) and *lacunosum-moleculare* (L-M). **(B)** Left: Schematic illustration of the recording configuration. Right: Superimposed voltage responses in the FS-IN elicited by a family of hyper- to depolarizing current injections. **(C)** Representative traces recorded from the FS-IN in response to extracellular stimulation. Top: The compound synaptic response produced in normal ACSF elicited by a single stimulus (50 V intensity, 0.1 msec duration). Middle upper: Isolated monosynaptic IPSC after bath application of DNQX (10 μM) and d-AP-5 (50 μM). Middle lower: The fast monosynaptic IPSCs is blocked following the addition of gabazine (10 μM) to the bath. Bottom: A train of 5 stimuli (at 200 Hz) reveals a slow $GABA_BR$ mediated IPSC (black trace) which is blocked by subsequent application of CGP (5 μM) to the bath (superimposed grey trace).

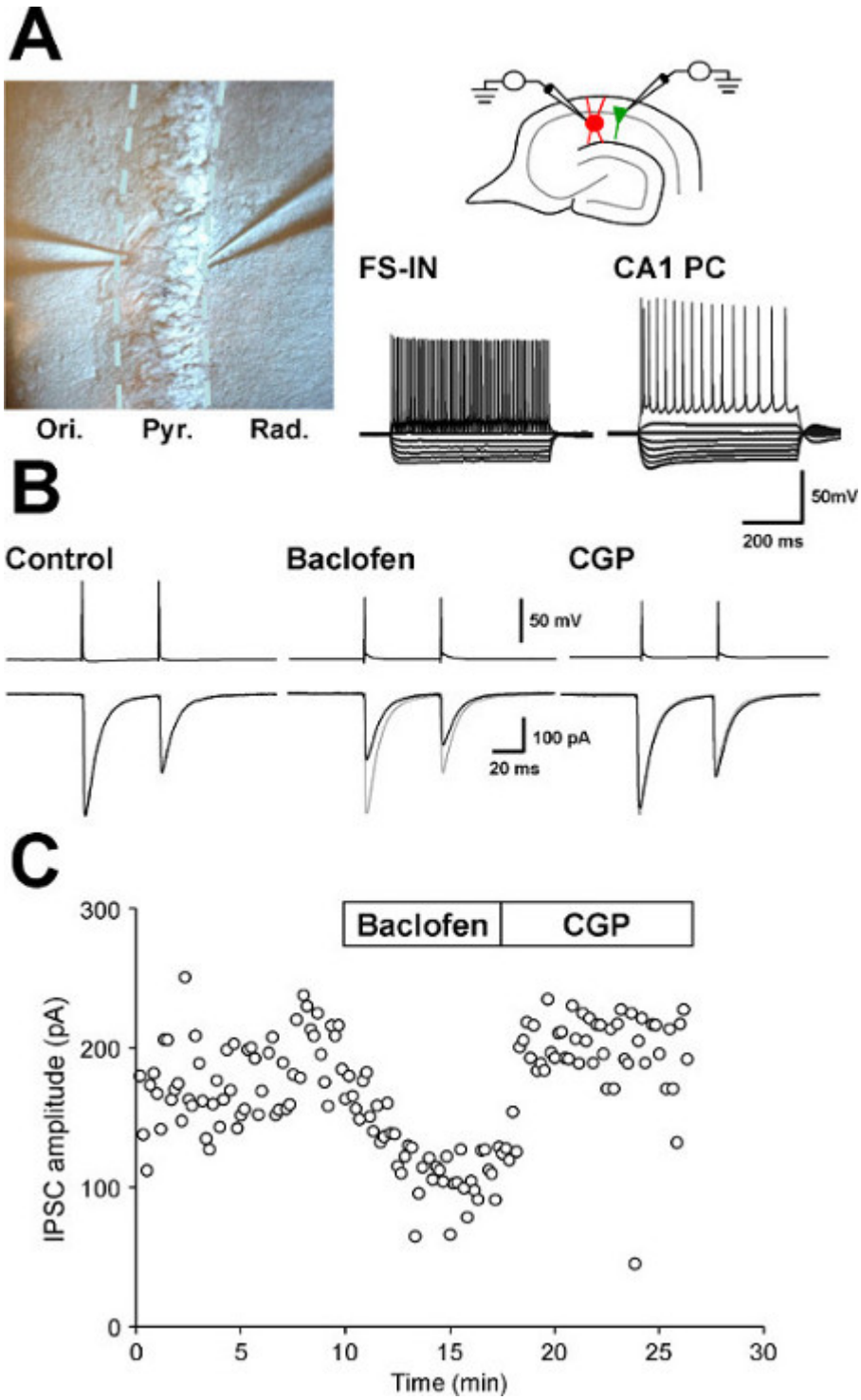

Figure 4. Analysis of presynaptic modulation of inhibitory synaptic transmission in a paired recording from a coupled FS-IN and CA1 PC pair. (A) Left panel: IR-DIC image of the two recording pipettes, the left one patched onto the FS-IN at the border of the *str. oriens* (Ori.) and *pyramidale* (Pyr.) and the right patched onto a CA1 PC closer to the *str. radiatum* (Rad.). Right panel: A schematic of the recording configuration, with representative voltage responses from the FS-IN (left) and CA1 PC (right) to a family of current pulses. **(B)** Representative traces showing the APs elicited in the presynaptic FS-IN (top) and the short-latency unitary IPSC in the CA1 PC (bottom) under control conditions (traces on the left), after bath application of baclofen (10 μM, middle), and subsequent application of CGP (5 μM, right). Note that baclofen reduced the IPSC amplitude by ~50%, whereas CGP resulted in an almost full recovery of the IPSC amplitude. Control traces are shown underlain. **(C)** Time course plot of the IPSC amplitude shows the effect of baclofen and CGP. IPSCs were recorded at 10 sec intervals.

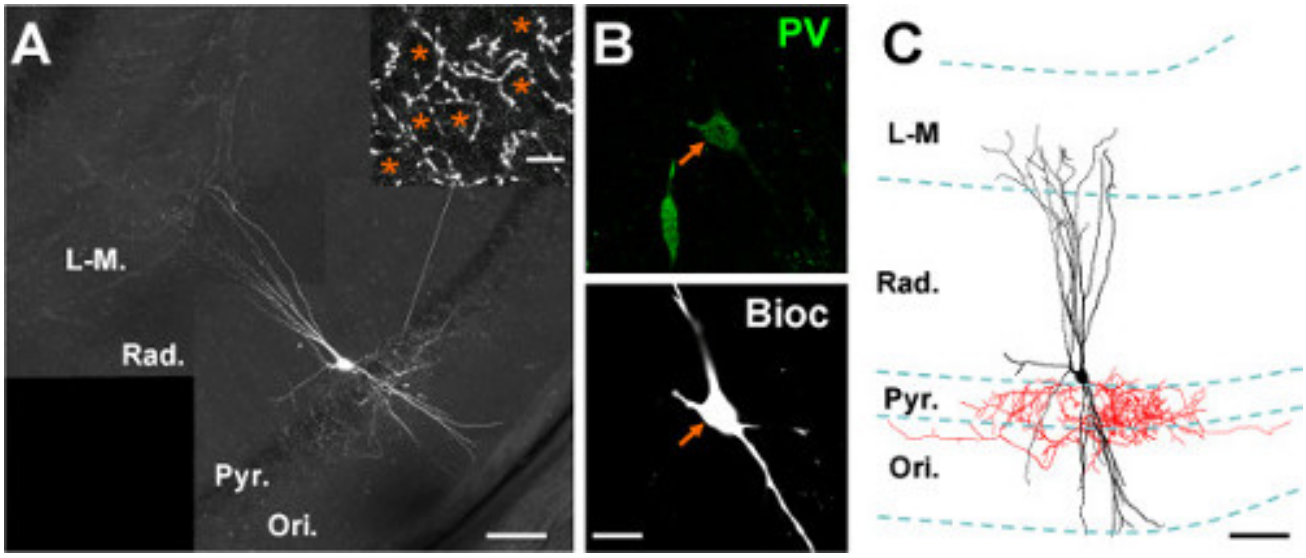

Figure 5. Visualization, reconstruction and immunocytochemical identification of a biocytin-labeled recorded FS-BC. **(A)** A projection of stitched stacks of images of a FS-IN imaged with a 20X objective on a confocal microscope. The somata is located at the border of the *str. radiatum* (Rad.) and *pyramidale* (Pyr.) of the CA1 area, the dendrites run radially and span all layers, whilst the majority of axon is found in and around the cell body layer; as typical for BCs. Scale bar:100 µm. Inset, top right: high magnification projection of a 20 layers, showing typical baskets of axon forming around putative CA1 PC somata (orange asterisks). Scale bar: 10 µm. **(B)** The biocytin-filled cell body of the IN (white pseudocolour, lower panel, arrow) shows immunoreactivity for PV (in green, upper panel). Scale bar: 20 µm. **(C)** Projection of the 3-dimensional reconstruction of the cell. The soma and dendrites are in black and the axon colored red. Layers of CA1 are delineated in blue. Abbreviations: Ori, *str. oriens*; L-M, *str. lacunosum-moleculare*. Scale bar: 100 µm.

Name	Composition (mM)	Use	Notes
Sucrose-ACSF	87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 75 sucrose, 7 $MgCl_2$, 0.5 $CaCl_2$, 1 Na-pyruvate, 1 Na-ascorbate	Preparation and storage of brain slices	pH= 7.4; osmolarity= ~350 mOsm
Recording ACSF	125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, 2 $CaCl_2$, 1 Na-pyruvate, 1 Na-ascorbate	Recording from brain slices	pH= 7.4; osmolarity= 310-315 mOsm
Intracellular solution (1)	125 K-gluconate, 10 KCl, 10 HEPES, 10 EGTA, 2 $MgCl_2$, 2 Na-ATP, 0.3 Na-GTP, 1 Na-phosphocreatine and 0.1% biocytin	Filling electrodes of GABAB IPSC recordings	pH= 7.4; osmolarity= 295-305 mOsm
Intracellular solution (2)	105 K-gluconate, 40 KCl, 10 HEPES, 0.1 EGTA, 2 $MgCl_2$, 2 Na-ATP, 0.3 Na-GTP, 1 Na-phosphocreatine and 0.1% biocytin	Filling electrodes for paired recordings	pH= 7.4; osmolarity= 295-305 mOsm
Phosphate buffer (PB)	100 mM NaH_2PO_4	Rinsing fixed slices	pH= 7.4
Phosphate buffered saline (PBS)	25 mM NaH_2PO_4, 154 mM NaCl (0.9% w/v)	Rinsing fixed slices and antibody incubation	pH= 7.4
Paraformaldehyde fixative	4% w/v Paraformaldehyde, 0.1 mM PB	Fixation of brain slices	pH= 7.4

Table 1. Solutions list.

Discussion

We describe a method which combines electrophysiological and neuroanatomical techniques to functionally characterize morphologically- and neurochemically-identified neurons in vitro; in particular the diverse types of cortical inhibitory INs. Key aspects of the procedure are: (1) pre-selection of potential INs; (2) intracellular recording and neuron visualization; and finally (3) morphological and immunocytochemical analysis of recorded INs. Although this study has addressed PV-INs in particular, the described protocol can be used for similar recordings from any interneuron or other neuronal types, with minimal alteration.

The relatively low number of interneurons in cortical areas makes random selection and recording from these cells highly inefficient. Divergent localization and morphological features have enabled researchers to distinguish and routinely record from some interneuron types. However in slices, identification of interneurons in and near the cell body layers remains difficult, such as with FS-INs. The advent of transgenic mouse lines, expressing the fluorescent proteins in specific interneuron populations offers an elegant solution which makes pre-selection and recording of these neurons much more efficient[2]. Now many transgenic lines, mostly mice, but increasingly also rats[13] are available, facilitating the investigation of interneurons. When using transgenic lines, however, it is essential to establish the extent and the specificity of the reporter expression.

Quality of cellular labeling and morphology, as well as the electrophysiological recording, critically depend on viable, high quality slices; for which the brain needs to be rapidly dissected (ideally 20-40 sec), handled very carefully and continuously chilled. We find that the use of sucrose-ACSF, whereby the overall sodium concentration and thus excitability of neurons is reduced, dramatically improves the quality of the slices and interneuron survival[18]. However, it should be highlighted that many researchers have made use of standard recording ACSF for slice preparation, to great effect[10,11,15]. Morphological integrity depends heavily on the angle at which slices are cut[13]; which varies by region and cell type. However, many interneurons can be reliably recorded from transverse, coronal or sagittal slices. To further minimize severance of dendrites and axon, cells deeper in the tissue should be targeted, albeit sacrificing IR-DIC quality and reliability of giga-ohm seal formation. Under these circumstances recordings from both CA1 pyramidal cells and FS-INs can be reliably obtained.

Labeling and visualization of the neurons is achieved following a typical recording session lasting 30 min or longer. If recordings last less than 30 min, it is necessary to allow this time prior to fixation to enable biocytin to diffuse into distal dendritic and axonal processes, guaranteeing complete filling and thus *post-hoc* visualization of cells.

In whole-cell recordings, maintaining low and stable series resistances is imperative to accurate physiological measurement of synaptic and intrinsic properties, as well as thorough labeling of the neurons. However low-resistance electrodes allow rapid dialysis of the cytoplasm with the intracellular solution contained within the pipette. Supplementing the intracellular solution with ATP, GTP and phosphocreatine certainly helps to maintain the energy supplies and recording quality. However, dialysis of neurochemicals and protein can lead to diminished responses, reduced plasticity[19] and can also hamper neurochemical identification. For instance PV is consistently washed out of neurons over the course of longer experiments. Therefore, examination of distal dendritic or axonal processes may be required to determine immunoreactivity of the recorded neuron. In such cases where such dialysis is a concern, recordings using perforated-patch configuration can be utilized to preserve the intracellular environment[20], however the patch must be broken at the end of recordings or the neuron subsequently “repatched” in the whole-cell configuration to allow biocytin filling.

Pharmacological investigation of recorded neurons is a useful method to assess the functional significance of divergent neuromodulatory mechanisms in shaping the intrinsic and synaptic activity of INs. In the methods described above, pharmacological isolation and paired recordings are used to assess post and presynaptic $GABA_BR$-mediated effects, respectively; however one can easily modify the combination of receptor agonists and antagonists to assess the role of alternative receptor families, for example the cannabinoid system[21].

Histological and immunocytochemical processing of recorded cells is highly reliable, once protocols are established. The immunocytochemistry protocol described here has been tuned with respect to the combination of antibodies used, slice thickness and requirement to identify the neurochemical content. We utilize normal goat serum as all secondary antibodies used are raised in goat. As alternatives, it is also possible to use bovine serum albumin (BSA) or milk powder as blocking agents. It should be noted that this protocol uses phosphate buffered saline (PBS) for antibody incubation, however alternatively one could simply use 0.1 M PB in lieu of PBS. Using a relatively high concentration of detergent (0.3% TritonX-100), does not diminish apparent antigenicity, while allowing penetration of antibodies into the first 100 µm surface layer of the slice, where neurons are routinely recorded. If deeper cells are recorded, or the slices are thicker, it is advisable to resection the slices, using a cryostat or a vibratome, and perform the immunolabeling on the thin (40-70 µm) sections. For 300 µm slices, a long incubation of the antibodies (at 4 ºC to preserve structural integrity of the slices) produces highly reliable immunocytochemical results.

Identification of the neurons relies on the combined information obtained in the recording, the *post-hoc* morphological and the immunocytochemical analysis. In the hippocampus, due to the strict laminar organization, axonal distribution is a good indicator of the postsynaptic targets of interneurons. Severed axon or dendrites, however, can make the identification difficult or impossible. Furthermore, some ambiguity remains, for example in differentiating PV+ BCs and axo-axonic interneurons, due to similarity in the overall axonal localization. A final definitive identification would require electron microscopic analysis of postsynaptic targets[1,3] or further immunocytochemical dissection[22].

Disclosures

The authors declare that they have no competing financial interests.

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