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Merging Structure and Function: Combination of In Vivo Extracellular and Intracellular Electrophysiological Recordings with Neuroanatomical Techniques

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Abstract: In order to understand the involvement of activity of single neurons in the context of the activity generated in small or larger neuronal networks, electrophysiological methods and morphological techniques need to be combined. In this chapter I describe a combination of methods designed to enable researchers to record the electrophysiological activity of single neurons in the intact brain, to analyze the interactions of these identified neurons with the surrounding neuronal network, and to investigate afterward the neuroanatomical characteristics of the recorded neurons.

Keywords: extracellular and intracellular recording, reconstruction, immunohistochemistry

I. INTRODUCTION

The deciphering of the function of neurons and neuronal networks in the intact brain is one of the major challenges in neuroscience. The electrophysiological properties of individual neurons and neuronal networks have been studied in great detail for many decades. However, to understand the role of various types of neurons in the genesis and/or the modulation of neural network activity as it is reflected in complex EEG patterns, it is necessary to record single-neuron activity simultaneously with the behavior of these single cells within the context of activity of the neuronal network in which they are embedded. In addition, the combination of intracellular and extracellular electrophysiological recordings with the subsequent morphological and neurochemical identification of the electrophysiologically characterized neurons provides additional information that is essential to understand the structural-functional relationship of neuronal networks.

Combined electrophysiological/network/morphological identification is necessary for the construction of realistic neuronal network simulation models. Such models need to take into account information about the complete physiological and anatomical properties: genomic and neurochemical content, receptor expression and other molecular information, knowledge about the patterns of dendrite and axon arborizations, information about the input (number, type, and origin of synapses terminating on the soma and on the dendritic trees), the output (number of axon terminals and the dendritic domain of postsynaptic targets), and finally information about the types and numbers of synaptically connected partners of different cell types. The degree of convergence and divergence of input/output among

different neuronal populations is a crucial element in neuronal network simulations. Therefore, research has explored these questions, yet mostly in vitro. In spite of the obvious advantages of using slice preparations (e.g., controlled administration of pharmacological reagents, multiple intracellular recording, etc.), the in vitro approach has the substantial disadvantage that the neuronal network is artificially truncated. Measuring neuronal and network activity in the in vitro hippocampal slice preparation illustrates this point. In the intact hippocampus, the sizes, densities, and trajectories of the axonal arborizations of inhibitory and excitatory neurons are very different from each other (Li *et al.*, 1993; Sik *et al.*, 1993; Freund and Buzsaki, 1996). Since in a slice far more excitatory axons are truncated than inhibitory ones, the consequence of slicing is that the excitation–inhibition balance becomes unnaturally modified in the in vitro slice compared with the intact situation. It has been estimated that in the in vitro hippocampal slice preparation as much as 80–90% of excitatory terminals arising from an excitatory pyramidal cell may be truncated (Li *et al.*, 1993; Sik *et al.*, 1993), whereas inhibitory axon arborization remains relatively unaffected (e.g., "O-LM", basket cells, etc.; Sik *et al.*, 1995). Several types of inhibitory neurons can be seriously truncated though [e.g., "backprojection" inhibitory neurons (Sik *et al.*, 1994, 1995)]. Because of this artificial condition, neuronal network properties have to be investigated in vitro with great caution. To overcome this problem and to reliably investigate network properties of neurons, the most recent generation of studies has been designed to obtain information about the function of morphologically identified neurons in the intact brain. Several studies have been published in which intracellular recording of neurons was first obtained in anesthetized animals followed by labeling and subsequent neurochemical analysis of the same neurons (Sik *et al.*, 1994, 1995, 1997).

Compared to the recording in in vitro slice preparations, the major challenge in the in vivo electrophysiological experiment is that the researcher has to perform the recordings without visual control. Furthermore, the brain in living animals is by no means as static as in slices, which makes it very difficult to conduct sustained intracellular recordings and to successfully fill the recorded cell and all its processes with a marker substance once the recordings have been completed. Therefore, it may not be surprising that the number of analyzed cells in research articles reporting in vivo intracellular recording is substantially lower than in articles using in vitro approach (Li *et al.*, 1992; Sik *et al.*, 1993, 1994, 1995, 1997).

In order to characterize the neuroanatomical and neurochemical properties of the electrophysiologically characterized cells, a combination of electrophysiology and various neuroanatomical techniques is required. The simplest technique used in the past to localize the position of the extracellular recording was to simply dip the electrode tip prior to recording in a dye such as methyl blue or fast green, which left in the brain tissue a mark of the position of the electrode (Grossman and Hampton, 1968; Simons, 1978; Takato and Goldring, 1979; Thomas and Wilson, 1965). The resolution

of this method was obviously inadequate to determine the location of the electrode with great precision. When single-cell recording methods emerged, a reliable technique that allowed the researcher to successfully visualize the electrophysiologically recorded cell was highly sought after. At first, the new marker horseradish peroxidase (HRP), being at that time popular in neuroanatomical tract-tracing (Heimer and Robards, 1981), found a second "killer application" as an intracellular marker in neurophysiology. HRP clearly delineates the cell's geometry and it supplies as a bonus an electron-dense label that highlights the labeled neuron and its processes in ultrathin sections studied in the electron microscope (Chang *et al.*, 1981; Jankowska *et al.*, 1976; Kita and Kitai, 1986; Kitai *et al.*, 1976; Snow *et al.*, 1976; Tamamaki *et al.*, 1987; Tepper *et al.*, 1987). However, intracellular injection of HRP enables only a partial analysis of the axonal arborization of neurons because not all axons become filled in their entire trajectory. Fluorescent dyes like Procion yellow (Hassin, 1979; Kaneko, 1970; Kelly and Van Essen, 1974) and the highly fluorescent Lucifer yellow were also used with success (Stewart, 1978, 1981; Takato and Goldring, 1979). Although Lucifer yellow produces better details as a marker compared to HRP, it has a major disadvantage as well: as all fluorescent dyes it loses its light emission capability after a relatively short period of UV illumination (the so-called "bleaching" effect). Therefore, the full reconstruction of the axonal arborization of intracellularly labeled neurons has been difficult, impossible, or dependent on the ability of the researcher to apply the complicated technique of diaminobenzidine photoconversion (Sandell and Masland, 1988). Thus, complete reconstruction of a neuron requires the intracellular injection of a molecule with a low molecular weight, providing fast and complete diffusion into small neuronal appendages, combined with a visualization method that produces a stable, optical, and electron-dense end product. Sensitive intracellular recording followed by anatomical identification of the cell today is based on a widely used biotin-containing low-molecular-weight product called biocytin (Horikawa and Armstrong, 1988). Biocytin appeared to be superior to HRP in completely staining the dendritic trees and axonal arborizations in their finest details, both in vitro (Horikawa and Armstrong, 1988; Kawaguchi *et al.*, 1989) and in vivo (Kawaguchi *et al.*, 1990; Sik *et al.*, 1995, 1997). Recording of the electrophysiological activity of single neurons followed by injection of biocytin and completed with the analysis of the neuron's interactions with the neuronal network is used to study the complex question of neuronal function.

II. IN VIVO EXTRACELLULAR RECORDING

Various extracellular recording methods have been developed over the past decades with the purpose to monitor the electrical activity of neural networks. All these methods require that a low-resistance conductor should

be placed in the region of interest. One of the simplest methods is to use a metal wire (Hubel, 1957). Because neurons of the same electrophysiological class generate similar action potentials, the only way to identify a given neuron from extracellularly recorded spikes is to move the electrode tip closer to its cell body (minimum distance is 20 μm in cortex) than to any other neurons. Neuron separation by this method is guaranteed by the differential proximity of the recording tip and one neuron, relative to the other neurons (see for details the chapter by Duque and Zaborszky in this volume). The substantially larger amplitude spikes, relative to the background "noise," guarantee neuron isolation. To record from another neuron, another electrode is needed. Because electrical recording from neurons is invasive, monitoring from larger numbers of neurons with the one-electrode–one-neuron approach inevitably increases tissue damage. Thus, improved methods are needed for the simultaneous recording of closely spaced neuronal populations with minimal damage to the hard wiring of the brain network.

The recent advent of localized multisite extracellular recording techniques has dramatically increased the yield of isolated neurons (Gray *et al.*, 1995; McNaughton *et al.*, 1983; Wilson and McNaughton, 1993). With only one recording site, signals from many neurons with similar size and orientation and which are at the same distance from the tip will provide the same magnitude signal, making single-cell isolation difficult. The use of two or more recording sites allows the triangulation of distances because the amplitude of the recorded spike is a function of the distance between the neuron and the electrode (Henze *et al.*, 2000; see for details the chapter by Nadasdy *et al.* in this volume). Wire tetrodes have numerous advantages over sharp-tip single electrodes, including larger yield of units, low-impedance recording tips, and mechanical stability. Because the recording tip needs not to be placed in the immediate vicinity of the neuron, long-term recordings in behaving animals are possible. Microelectromechanical system-based recording devices can reduce the technical limitations inherent in wire electrodes because with the same amount of tissue displacement the number of monitoring sites can be substantially increased (Bartho *et al.*, 2004; Buzsaki, 2004; Csicsvari *et al.*, 2003; Wise and Najafi, 1991). Whereas silicon probes have the advantages of tetrode recording principles, they are substantially smaller in size. Furthermore, multiple sites can be arranged over a longer distance, thus allowing the simultaneous recording of neuronal activity in various cortical layers (Buzsaki and Kandel, 1998). Currently available multishank probes can record from as many as hundred well-separated neurons.

Cortical pyramidal cells generate extracellular currents that flow mostly parallel with their somatodendritic axis. These extracellular features allow the separation of signals related to individual neurons. In practice, only a small fraction of all possible neurons can be reliably separated with the currently available probes and spike-sorting algorithms. Data processing and viewing algorithms are freely available. Neurophysiological and behavioral data can be explored by NeuroScope (http://neuroscope.sourceforge.net)

(Buzsaki *et al.*, 2004). Spike-sorting is performed in two steps, first automatically using KlustaKwik (http://klustakwik.sourceforge.net) (Harris *et al.*, 2000) and then manually using Klusters (http://klusters. sourceforge.net) (Hazan *et al.*, 2004). A further advantage of silicon probe monitoring of electrical activity is that the closely spaced recording sites have a known one or two dimensions. The multiple site approach allows the simultaneous monitoring of the extracellular flow of ions with high spatial resolution. From the measured voltages the current flow can be calculated, and the extracellular resistivity can be used to calculate the current density. Such current-source density measurements provide valuable information for identifying synaptic pathways and neuronal compartments responsible for generating the locally measured current. For example, if a spatial distribution of current-source density of a spontaneous field pattern matches that of the evoked currents by thalamic but not by callosal inputs, the firm conclusion can be drawn that the spontaneous pattern is generated by thalamic afferents (Bragin *et al.*, 1995; Buzsáki *et al.*, 2003; Nadasdy *et al.*, 1998).

A critical step in the reconstruction of a functional circuit is the identification of the anatomical nature of the recorded and spike-sorted units. This identification is possible only with a combined program comparing extracellular and intracellular spike shape and spike dynamics of morphologically identified neurons. The method requires several steps and dedicated experiments. The examples below are taken from such experiments in vivo in the hippocampus, but the method is compatible with neocortical areas or other structures as well, i.e., there seems to be no limit for the identification of extracellular spikes in behaving animals.

III. IN VIVO INTRACELLULAR RECORDING AND SINGLE-CELL LABELING

Extracellular recording of local fields and/or large numbers of neurons provides information about the cooperative activity of neuronal assemblies, a type of parameter that restricts the patterns of firing of individual cells. If one is interested in the intrinsic properties of individual cells, the inputs that an individual cell receives, the fluctuations of membrane potential, and so forth, then intracellular recording needs to be performed.

For in vivo intracellular recording, glass pipettes with very small tip diameters (<0.5 μm) called sharp electrodes are used most frequently. Recently, patch-pipettes have been used in vivo in order to record the electrical activity of single neurons (Ferster and Jagadeesh, 1992; Margrie *et al.*, 2002). More details on in vivo patch recording are presented in this volume in the chapter contributed by Petersen.

Sharp electrodes are manufactured using glass pipettes of different diameters with the aid of an electrode puller. Since in vivo recording often requires reaching deeper brain structures, the geometry of the electrode is different from the one used in vitro: the shanks of the pipettes must be

sufficiently long. However, long shank electrodes may be too flexible. To reach deeper brain regions with a high degree of precision, it is necessary that the electrode does not drift from the planned path. This is difficult to achieve with flexible electrodes. Therefore, usually for in vivo intracellular recording, glass electrodes with rather thick glass walls are being used. An alternative is to use an even more rigid electrode made of quartz. The disadvantage using quartz is that this material requires a special and expensive electrode puller (e.g., Shutter P2000), where the glass is melted by laser light instead of a tungsten-heating element.

Once the electrode is pulled it is filled up with conductive electrolyte. If no special recording condition is required, usually 0.5 M potassium acetate solution is used for this purpose. Since the tip of the electrode is very small, the filling of the pipette is a process that has to be done with the necessary care and precaution. To support homogeneous filling, glass electrodes with inner filament are used, which, by the capillary effect, drastically increases the probability of a successful filling process of the pipette. Basic rule is that the recording pipette needs to be filled with electrolyte without any discontinuity (for example, air bubbles should be absent). This is not an easy task: simply placing the fluid inside the pipette from the back will certainly produce a certain amount of air bubbles, rendering the pipettes useless. To avoid the situation of ending up with a batch of sharp yet useless pipettes because of poor filling, the following steps are recommended: (1) place small droplets of conductive electrolyte at the open end of the pipette. Hold this still for a couple of minutes until the liquid fills the extreme tip of the pipette completely, (2) use a small diameter filling tube that is being inserted into the recording pipette down to its neck and then slowly and carefully fill the neck and shank up with the electrolyte, and (3) tap gently on the side of the pipette if bubbles are still present. This manipulation can eliminate discontinuities altogether; alternatively, one can use a very fine tungsten or other rigid wire to reach inside the pipette and try to remove any air bubble.

If the goal of the experiment is to anatomically identify the recorded neurons, some dye needs to be injected into the cells. For this purpose, a tracer needs to be incorporated in the electrolyte. This tracer can be a fluorescent dye like Lucifer yellow, or it can be a nonfluorescent substance like the widely used small biotinylated molecules (biocytin, neurobiotin), or even a mixture of several dyes. Dye injection can be achieved through the application of high pressure (Sik *et al.*, 1993; Tamamaki and Nojyo, 1993) or by the application of an electrical current (i.e., if the dye is polarized).

Most widely used intracellular labeling material is biocytin. This substance is a biotin–lysine complex of low molecular weight containing about 65% biotin, which retains a high affinity for avidin. Because of the high affinity of biotin to avidin, the conventional avidin–biotin complex method is conveniently used to reveal the recorded cell. The biotin-containing tracer can be injected either with positive current (i.e., biocytin) (Horikawa and Armstrong, 1988) or with both positive and negative current pulses (i.e., neurobiotin) (Kita and Armstrong, 1991).

IV. COMBINED TECHNIQUES

The combination of the described in vivo electrophysiological and anatomical methods allows both the functional and the structural characterization of neurons. First, the intrinsic electrophysiological properties of the neurons are investigated using intracellular recording methods (Fig. 6.1) in a condition where all the synaptic connections are intact and the extracellular environment is undisturbed. Second, the activity of each neuron is correlated with the neural network activity that is recorded by the extracellular electrode. Even though in many cases a rough classification of the recorded neuron can be achieved by analysis of the electrophysiological traces (excitatory, inhibitory neuron, etc.), the exact characterization requires labeling of the neuron (Fig. 6.2). Via the injection of a tracer the recorded cell can be visualized and identified neurochemically (Fig. 6.3). Because of its low molecular weight, biocytin diffuses easily into small structures. This characteristic makes the detection of complete axonal and dendritic arborization possible (Fig. 6.4). Using an additional immunoreaction and processing for electron microscopy, even the targets or afferents of the investigated neurons can be studied at the ultrastructural level (Sik *et al.*, 1995).

The power of the combined methods is demonstrated below in a hippocampal inhibitory (basket) cell, but naturally the same method can be adapted to any neuronal type. With the appreciation of the pivotal function of inhibitory cells in the orchestration of neural activity many questions need to be answered; for example, how inhibitory and excitatory inputs change the membrane potential of the neuron, how different types of inhibitory neurons fire, how different subtypes of inhibitory cells participate in network oscillations, how inhibitory cells are connected to excitatory versus inhibitory neurons, what is the size of an area that a single inhibitory neuron can innervate, how many excitatory and inhibitory neurons are innervated by a single inhibitory cell, etc. After analyzing the spontaneous activity of the basket cell during theta and non-theta network oscillations (not shown), we analyzed the response of the neuron to positive and negative current injections. The recorded inhibitory cell was firing rhythmically when theta activity was present in the extracellular field potential (Fig. 6.5B). The neuron showed firing frequency accommodation when positive current was injected intracellularly and found no sign of sag (I_h) current (Fig. 6.5A).

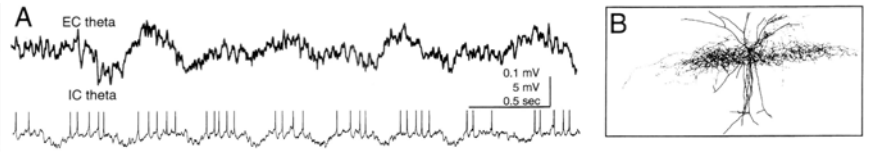

Figure 6.1. (A) Simultaneous recording of extracellular theta activity in the pyramidal cell layer of area CA1 of the rat hippocampus (EC theta) and intracellular activity of a basket neuron (IC theta). (B) Reconstruction of the dendritic and axonal arborization of the basket cell. (Reprinted with permission from Ylinen *et al.*, 1995.)

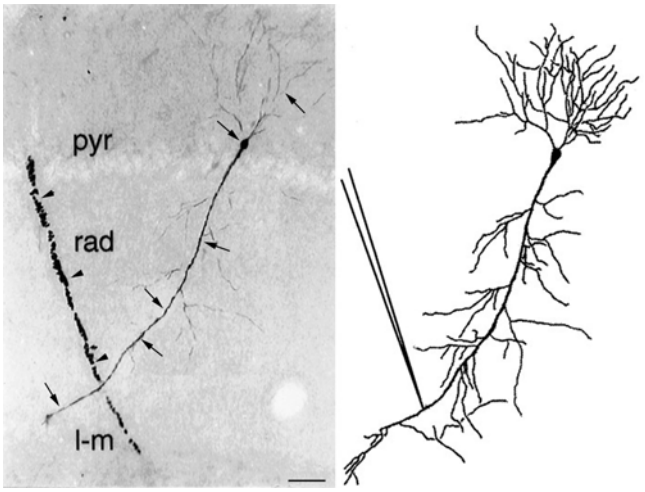

Figure 6.2. Photograph of the recording micropipette track (arrowheads) and the biocytin-filled neuron (arrows) and its camera lucida reconstruction. An intracellular recording was made from the apical shaft of a pyramidal cell at the border of stratum radiatum (*rad*) and lacunosum-moleculare (*l-m*) in area CA1 of the rat hippocampus. The electrode was moved beyond the dendrite during the experiment. The pipette track is filled with reaction product caused by peroxidase activity in red blood cells. *Abbreviation*: pyr, pyramidal layer. Scale bar: 50 μm. (Reprinted with permission from Kamondi *et al.*, 1998.)

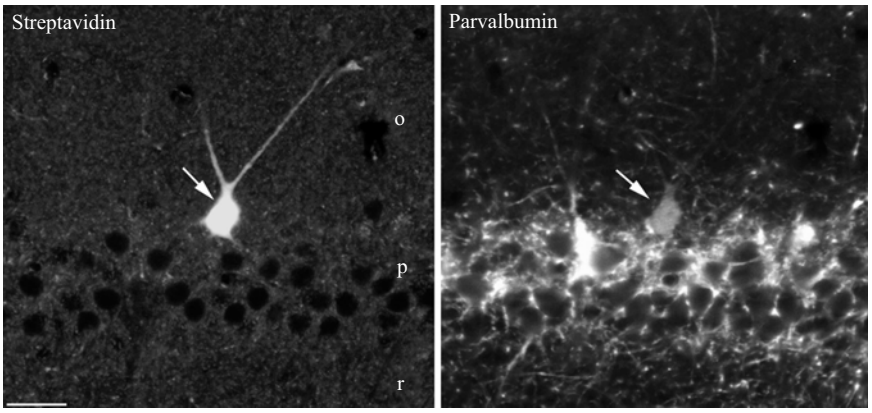

Figure 6.3. Immunohistochemical identification of the filled cell. The electrophysiological property of the neuron was recorded: the cell fired close to the top of theta oscillation and also during ripple activity (not shown). After recording and subsequent filling of the neuron, the visualization was achieved using fluorochrome-conjugated (Alexa 546) streptavidin (left panel). The picture was taken using a confocal laser microscope. The parvalbumin expression of the cell (arrow) was demonstrated by immunofluorescence using a different fluorochrome (Alexa 488) (right panel). The cell was later reconstructed and classified as basket cell (Dumont and Sik, 2006). *Abbreviations*: o, stratum oriens; p, stratum pyramidale; r, stratum radiatum. Scale bar: 25 μm.

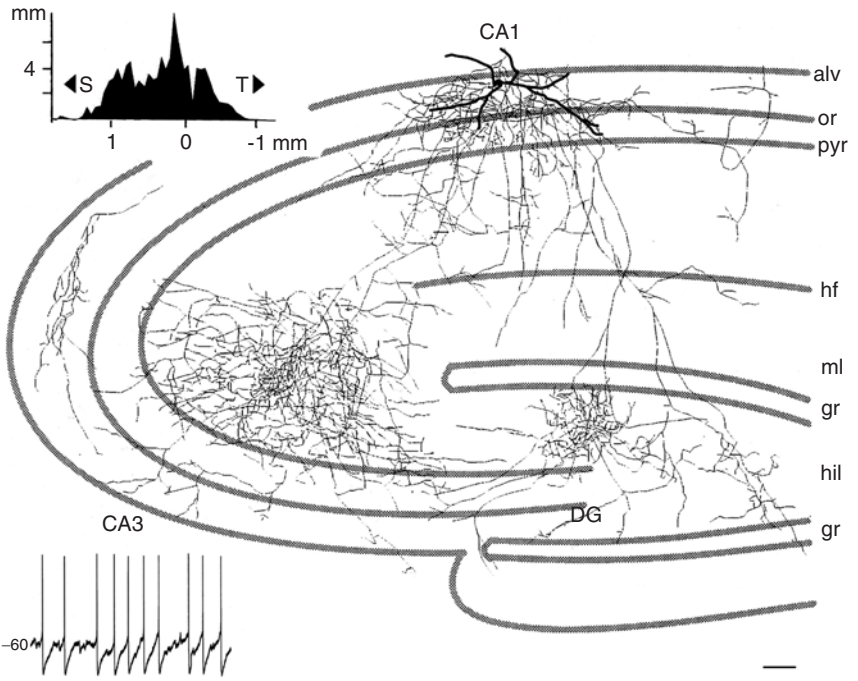

Figure 6.4. Reconstruction of an intracellularly recorded and subsequently filled feedback neuron in area CA1 of the rat hippocampus. After "developing" the filled neuron using the DAB–Ni method, the complete dendritic and axonal arborization of the neuron could be reconstructed. The cell body of the neuron is located in the alveus; axon collaterals are present in areas CA3, CA1, and in the hilus of dentate gyrus (DG). The upper left inset indicates the summated length of axon collateral along the septotemporal axis. The lower left inset shows the spontaneous activity of the neuron at resting membrane potential. *Abbreviations*: S, septal direction; T, temporal direction; alv, alveus; or, stratum oriens; pyr, stratum pyramidale; rad, stratum radiatum; hf, hippocampal fissure; ml, molecular layer; gr, granule cell layer; hil, hilus. Scale bar: 100 μm. (Reprinted from Sik *et al.*, 1994.)

Figure 6.5. Complete electrophysiological, neurochemical, and neuroanatomical characterization of a hippocampal basket cell. (A) Response of the neuron to depolarizing and hyperpolarizing current injection. (B) Simultaneous recording of intracellular activity of the basket neuron and extracellular activity during theta oscillation. (C) Biocytin-labeled basket cell. (D) Fluorescent parvalbumin immunolabeling of the same neurons (arrow). (E) Parvalbumin-containing target of the filled basket cell. In the inset, the white arrow indicates a putative synaptic contact between the biocytin-filled terminal and a parvalbumin-immunoreactive neuron. (F) Correlated electron microscopic analysis of the same bouton shows a symmetric synapse (arrow in the inset) on the cell body. (G) Partial reconstruction of the labeled basket cell indicating other parvalbumin-immunoreactive targets (large circles). Inset shows the position of the neuron in the CA1 region of the hippocampus. (H) Distribution of pyramidal cell and parvalbumin-immunoreactive inhibitory neuron targets of the intracellularly filled basket cell in the septotemporal direction. Overall, 99 boutons in contact with 64 parvalbumin-positive cells were counted. Graph in the

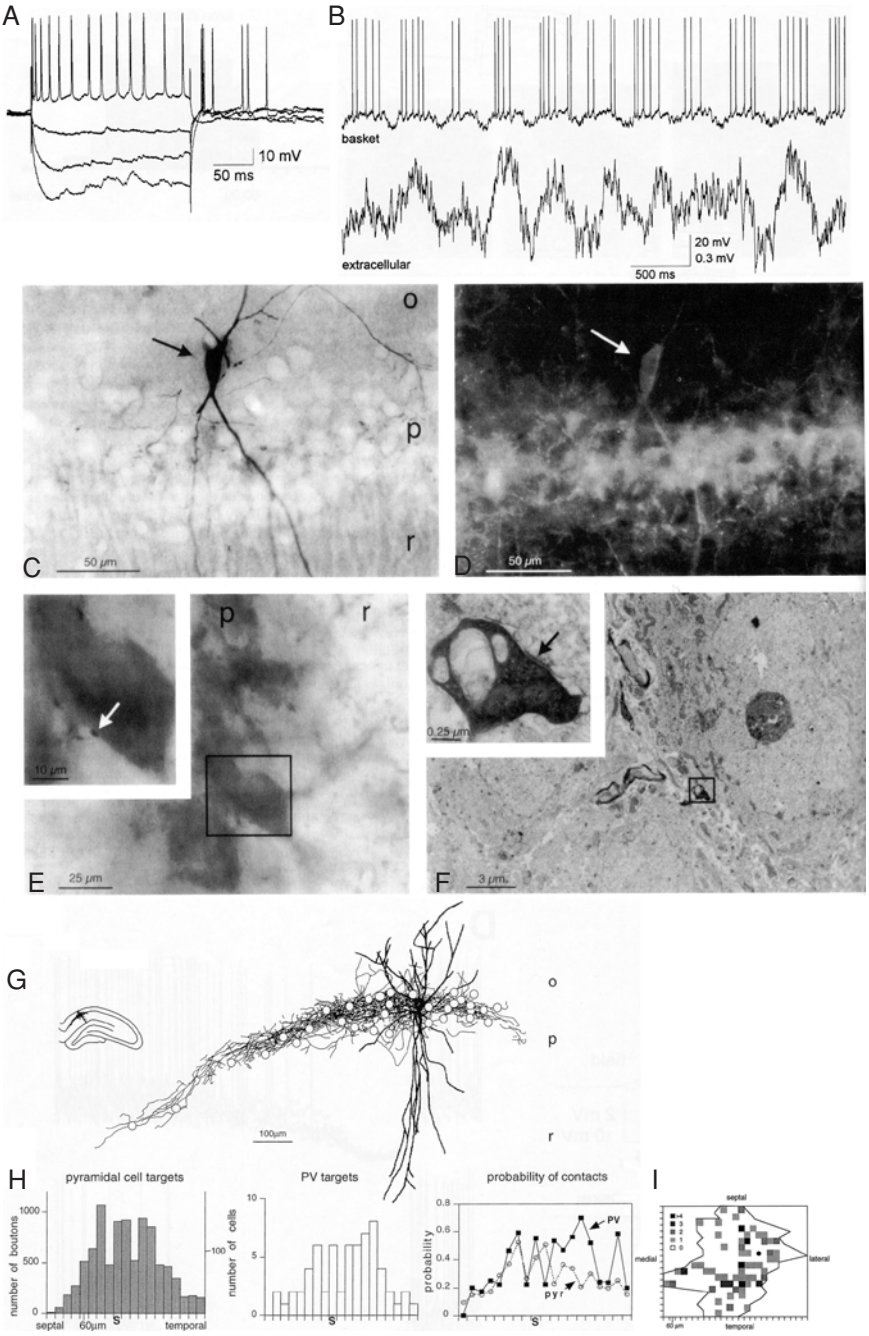

Figure 6.5. (*Cont.*) middle shows the probability of pyramidal and parvalbumin-immunoreactive inhibitory cells innervated by the filled neuron. (I) The 2D distribution of the interneuron–interneuron contacts is shown in H. *Abbreviations*: pyr, pyramidal; PV, parvalbumin; S, position of the soma. (Adapted, with permission, from Wang and Buzsaki, 1996, and Sik *et al.*, 1995.)

The filled cell contained parvalbumin (PV) (Fig. 6.5C,D) and innervated other PV-immunoreactive neurons (Fig. 6.5E). The putative synaptic contacts identified under the light microscope were further analyzed using the electron microscope (Fig. 6.5F). Indeed, the filled basket cells formed symmetric (inhibitory) synaptic contacts on other PV-containing neurons besides terminating on pyramidal cells. The number of synaptic contacts and synaptic targets were determined (Fig. 6.5G). Overall, 99 boutons in contact with 64 PV-positive cells were counted. The total number of pyramidal cell targets (~1500) was estimated by counting the number of boutons of the filled basket cell in each 60-μm-thick Vibratome® section, and assuming that a basket cell formed 9–10 boutons on a single pyramidal cell (Halasy *et al.*, 1996). The probability of contacts formed on PV-containing versus pyramidal cells was calculated by dividing the number of contacted PV or pyramidal cells by the total number of PV or pyramidal cells in the area innervated by the axon collaterals. The probability of postsynaptic contacts, however, decreased with the distance between the cell pairs (Fig. 6.5H,I). Thus, with the sequential application of the aforementioned methods the complete electrophysiological and neuroanatomical characterization of single neuron was achieved.

A computer simulation based on the obtained quantitative data demonstrates that inhibitory synaptic transmission could provide a suitable mechanism for synchronized oscillations in a sparsely connected network of inhibitory cells. This network can, through subthreshold oscillations in excitatory cell populations, synchronize discharges of spatially distributed excitatory neurons (Wang and Buzsaki, 1996).

V. SUMMARY OF ADVANTAGES AND LIMITATIONS

A. Advantages

Combination of in vivo intracellular recordings with morphological methods can provide crucial and detailed information about the structure and function of neurons. The quantitative data of connections of a single intact neuron are indispensable for further meaningful computer simulation of neural networks.

B. Limitations

In spite of the great advantages of the combination of in vivo intracellular or extracellular recording with neurochemical and morphological characterization of the neurons, several limitations still remain.

- Since the recording and subsequent filling of the recorded neurons are performed blindly, this is a very time-consuming process. Tedious work

may result in only a handful of analyzed cells. Thus, if the experiment requires dozens of cells to be analyzed, it is advisable to use alternative methods such as the in vitro preparation or in vivo juxtacellular labeling.
- Anesthetics can alter the firing pattern of the neurons; therefore, careful judgment of the chosen drug is a prerequisite for reliable electrophysiological analysis. To overcome this problem, "juxtacellular" labeling was adapted to unanesthetized, drug-free animals by taking advantage of the head-restrained recording technique (Lee *et al.*, 2004, 2005).
- When the sharp electrode penetrates the cell, neurons often discharge artificially (the so-called "injury discharge"). If the membrane does not seal perfectly around the intercellular electrode, it can induce a higher activity rate than that in normal circumstances, resulting in erroneous identification of the neural activity.
- Precise application of pharmacological reagents into a small area is difficult or impossible.

Unless one asks specific questions that can be addressed only by intracellular recording (like membrane oscillations, EPSP, or IPSP measurement in an identified cell, etc.), this powerful but time-consuming approach can be substituted by juxtacellular labeling. Despite these limitations, we feel confident that the recording of electrophysiological activity of single neurons and analysis of their interactions with the neuronal network, in conjunction with the acquisition of accurate structural data on the synaptic architecture, provide sufficient data for realistic experimental modeling of neuronal function.

APPENDIX: DETAILED METHODS

A. Anesthetics

Different anesthetics can be used depending on the planned length of the recording session, whether the animal has to survive, etc. In acute terminal experiments (when the animal is sacrificed following the recording), urethane is the preferred anesthetic because the anesthetic effect lasts many hours. If the animal needs to survive longer than 12 h after the experiment, anesthesia by a cocktail of ketamine and xylazine is recommended.

- *Urethane*: Prepare urethane stock solution (5 g urethane in 10 ml of 0.9% NaCl) and inject i.p. (1.3–1.4 g/kg for rat);
- *Ketamine/xylazine*: Inject i.p. 75 mg/kg ketamine and 10 mg/kg xylazine (rat). If ketamine/xylazine is used in some cases, it has to be readministered during the experiment. Use ketamine only (20 mg/kg) whenever the animal shows signs of awakening. It can be injected i.p. or intravenously into the tail vein. This later provides more precision of the dosage but is harder to execute.

- *Combination of urethane and ketamine/xylazine*: Use 1.25 g/kg urethane and supplement the drug with 20 mg/kg ketamine and 2 mg/kg xylazine as needed.

B. Surgery and Implantation of Stimulating and Extracellular Recording Electrodes

- Shave off the hair from the area overlying the part of the brain studied, i.e., over the skull or the vertebrae.
- Place the animal into a sturdy stereotaxic apparatus. An antivibration table is highly recommended to make the intracellular recordings more stable and longer lasting.
- Use an animal thermoregulation device to keep the body temperature constant. A low body temperature dramatically decreases the activity of neurons.
- If the recordings will take prolonged periods, protect the animal's eyes with commercially available eye drops or with paraffin oil, etc. against dehydration of the cornea.
- Cut the skin with a scalpel and drill a small hole over the region where the recording will be performed. The hole should be as small as possible but should also provide sufficient space for manipulation. The ideal size is about 1 × 1 mm. If an extracellular recording electrode is placed into the same region through the same bone window, the size of the hole must be larger (1.5 × 1.0 mm).
- If a stimulating electrode is to be used, prepare a small bone window over the desired stimulation region.
- Implant the stimulating electrode and fix it with acrylic cement.
- Maneuver the extracellular recording electrode into position. The position of the electrode can be checked by driving the appropriate input pathway with the stimulating electrode. The extracellular recording electrode should be fixed by acrylic cement; thus, no extra electrode holder (which would occupy space) is necessary.
- Open the dura mater with the very sharp tip of a pointy surgical blade (for example, size 11) or by the tip of a small needle (like 27 g × ½″). Be careful not to cause any bleeding. If a large blood vessel is in the way, drill another hole or enlarge the original hole. Do not let the brain surface dry out, e.g., by putting a drop of 0.9% NaCl into the opening.

C. Intracellular Recording

After lowering the intracellular electrode into the area of interest, cells need to be impaled with the sharp electrode. There are differences in the membrane structure and intracellular ion content among cells that will result in differences of penetrability and survival of the cells following the penetration. As a general procedure, follow the next steps:

- Pull the electrode from 2.0-mm-diameter thick wall glass capillary.
- Fill the electrode with electrolyte (0.5 M potassium acetate) containing 1–3% biocytin.
- Keep the electrodes in a humid chamber (Petri dish with some moistened paper or cotton) to prevent clogging due to drying at the tip of the electrode.
- Insert the intracellular electrode into the region of interest, and cover the bone window with a mixture of paraffin and paraffin oil (1:1, kept warm before the application at ~60°C) to prevent drying of the area and to reduce pulsation of the brain.
- Use quick, small steps (2 μm/step) to advance the intracellular electrode.
- When the recorded potential starts decreasing, it indicates that the electrode is pushed to a membrane. Use one of the following methods to penetrate into the cell: (a) "buzz" [small, short lasting (1–20 ms) electric current injection setting the electrode tip in motion causing small vibration of the tip. Many intracellular amplifiers have this button on their recording unit] and (b) mechanically, by very gently tapping the electrode holder or the motor. This will move the pipette a few micrometers, which may be sufficient to penetrate the cell membrane.
- After penetrating into the cell, apply a negative current to counteract the depolarization caused by ion leakage through the membrane opening through which the pipette tip has entered the cell. Hyperpolarize the cell till it stops firing and keep it at this state for a couple of minutes to allow the cell membrane to seal around the distal pipette shaft.
- Perform the electrophysiological recording. At the end of the session, use positive (or negative if it has beneficial effect) current pulses to inject the dye into the cell (300–500 ms at 1 Hz using 0.5–2.5 nA current). The necessary labeling time differs from cell to cell. A labeling time of 2–30 min is usually sufficient to obtain complete labeling of neurons. Cells with large axonal arborization, and/or long projection may require longer time with higher current.

D. Survival Time

The intracellularly injected dye spreads through the neuron and its processes by active transport mechanisms and by diffusion. If the reconstruction of complete axonal arborization is the goal, this requires a longer transport/diffusion period until the dye has filled all the thin axon collaterals down to their terminal arborizations. Neurons with axons projecting over longer distances require lengthier survival time. Typically, the survival time after the injection of the marker varies between 0 and 12 h. Longer survival is not recommended if biocytin or neurobiotin is used, because enzymes that might be activated due to the trauma may destroy these tracers. If a longer survival time is required, injection with biotinylated dextran amine is recommended instead of biocytin (BDA, MW 3000).

E. Fixation

Choosing the appropriate fixative is important if the intention is to determine the neurochemical features of the recorded neuron or other cells (like target or input cells). Most immunohistochemical staining procedures require fixation with a buffered solution of formaldehyde (depolymerized paraformaldehyde).

- Reanesthetize the animal if necessary.
- Open the thorax and insert a large diameter needle via the left ventricle into the ascending aorta.
- Place a clamp on the descending aorta, between the liver and the lungs.
- Open the right atrium to allow blood and perfusates to flow out.
- Flush the blood using 0.9% NaCl or 0.1 M PBS until the outflowing liquid from the heart is clear, typically 1–3 min.
- Switch the solution to the appropriate fixative (typically 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4).
- Fix the brain for 30 min.
- Remove the brain from the skull. If the brain is too soft, postfixation may be carried out (1 h to overnight).

F. Visualization of the Intracellularly Filled Cells

Depending on the goal, the labeled cells can be visualized with either a fluorescent dye or a permanent marker [like 3,3′-diaminobenzidine (DAB) peroxidase reaction product]. We recommend a fluorescence-based visualization if the aim of the experiment is to further characterize the recorded cells immunohistochemically (see section "Immunolabeling"). If the reconstruction of the cell is the goal, the sections have to be kept in sequential order during the whole process.

- Section the brain using a vibrating microtome (section thickness 30–100 μm).
- Wash the fixative with PB (5×15 min).
- Treat the section with detergent if examination is planned other than electron microscopy (0.5% Triton X-100 in PB for 30 min).
- Wash with Tris-buffered saline (TBS) (3 × 15 min).
- React the sections with either HRP containing avidin–biotin complex (ABC; dilution 1:500 in TBS) or with fluorochrome-conjugated streptavidin (dilution 1:100–1:1000 in TBS depending on the type of the fluorescent dye) (1 h at room temperature for ABC; 5 h overnight at 4 h for fluorescence).
- Wash the reagent (3 × 15 min TBS).
- If fluorescent dye has been used, mount and coverslip with antifading medium and analyze the finding in a fluorescence microscope using the proper filtering. If ABC has been used, visualize the cell using the

following steps:

1. Prepare a DAB–Ni solution: measure 100 ml 0.01 M PB.
2. Add 30 mg 3,3′ diaminobenzidine (DAB) (☠ toxic and carcinogenic, decontaminate spoils and leftovers with chlorine bleach; ☼ light sensitive, cover with aluminum foil).
3. Add 40 mg NH_4Cl.
4. Add 5 ml 0.05 M $NiNH_4SO_4$ (Nickel ammonium sulfate; add dropwise under agitation).
5. Filter this DAB–Ni solution.
6. Remove the last wash, add 1 ml of DAB–Ni solution.
7. Allow the sections to be saturated with DAB–Ni (20 min room temperature, dark).
8. Add 10 μl of H_2O_2 solution (made up fresh by pipetting 10 μl of 30% H_2O_2 solution into 10 ml double distilled water) to start the reaction. A black reaction product will form in all structures that contain ABC (the reactive compound is the peroxidase). This reaction can take 5–40 min to fully develop. Inspect the section regularly in a microscope to monitor the progress of the reaction.

- When sufficient reaction product has formed, rinse the sections three times in TBS.
- Mount on glass slides and dry. Do not coverslip if the procedure is followed up with immunolabeling (see below).

G. Immunolabeling

In order to determine the neurochemical content of the recorded cell, an immunohistochemical reaction needs to be performed. Since the DAB or DAB–Ni reaction product masks the immunosignal of the cell, an immunofluorescence technique should be used in this case (Kawaguchi, 1993). Once the cell has been visualized by a streptavidin-conjugated fluorochrome (see above), a regular immunoreaction using fluorescence secondary antibody can be applied. The exact steps can be found in other books like *Immunohistochemistry* (Cuello, 1993). Briefly:

- Wash out the fixative (see above).
- Wash the sections by rinsing 3 × 10 min with 0.05 M TBS.
- Block aspecific immunosignal by incubating 45 min in blocking solution (TBS containing 5% normal goat serum (NGS) or other blocking serum; and 0.5% Triton X-100).
- Treat the sections with fluorochrome-conjugated streptavidin (dilution 1:100–1:1000 in TBS depending on the type of the fluorescent dye) (5h overnight at 4°C) in the dark.
- Wash out the reagent (3 × 15 min TBS).

- Use primary antibody diluted into TBS containing 0.5% NGS (or other serum), 0.01% sodium azide added. Incubate the sections in the incubation medium in the dark (overnight 2 days at 4°C) to allow antibodies to penetrate into the sections. Longer incubation results in better penetration of the antibody. During incubation, place the vials or well plates that contain the sections on a rocking plateau to ensure gentle agitation.
- Wash out the primary antibody (3 × 15 min TBS).
- Incubate the sections (in the dark) in the solution containing the fluorochrome-conjugated secondary antibody (dilution 1:100–1:1000 in TBS for 6 h to overnight). The excitation–emission spectrum of this fluorochrome should be of course different from that used to visualize the labeled cell.
- Wash 3 × 15 min with TBS.
- Mount on slides, allow sections to air-dry.
- Add antifading reagent like Mowiol (has to be at room temperature) and coverslip.
- Seal with nail polish.
- Analyze the neurochemical content of the cell using a fluorescence microscope equipped with the proper excitation–emission filters.
- Store at 4°C temporarily, or at −20°C for the long term.
- Then the permanent visualization of the filled cells using the ABC-DAB protocol is performed as described above.

H. Reconstruction

The entire dendritic and axonal arborization of the recorded neurons can be reconstructed after successful intracellular labeling. If sections are air-dried after mounting on slides, shrinkage in *Z* direction is substantial (about 80–90%). In the case when the real three-dimensional (3D) structure of the neuron is important, embedding of the section in plastic resin is necessary. The following protocol is an example using Durcupan, but other resins can also be used, such as Araldite, Spurr, and so forth.

- Dehydrate the sections in an ascending alcohol series (50%, 70%, 90%, 2 × 100% 10 min each).
- Change to intermediate solution (100% propylene oxide if Durcupan is used) (2 × 10 min). *Note*: Propylene oxide is volatile, toxic, and combustible. Use a fume hood and wear gloves.
- Place the section in pure Durcupan (overnight).
- Mount the sections, coverslip, and cure in an oven at 58°C for 24 h.

The cells can be reconstructed using a drawing tube or a microscope-computer equipped with Neurolucida software. The advantage of using the latter equipment is that the 3D information is preserved in each individual section. When a drawing tube is used, the reconstruction will be manufactured using the 2D projection of each section. Thus, with this procedure the 3D information in individual sections is lost (see further details

in the chapters by Ascoli and Scorcioni and Duque and Zaborszky in this volume).

I. Equipment and Supplies (Some Recommended Equipment Is in Parenthesis, But Other Items Can Be Used)

1. Equipment for Surgery and Recording

Drill (NSK Emax)
Vibration isolation system (Newport VH Isostation)
Stereotaxic apparatus (Kopf Model 920)
Thermoregulator with heating pad (CWE TC 1000)
Operating microscope (Olympus SZ series)
Light source (WPI)
Inchworm motor system (Burleigh)

2. Equipment for Data Acquisition

Digital oscilloscope (Tektronix TDS 2014)
Noise reduction device (Hum bug) or traditional Faraday cage
Amplifier (Axon Multiclamp 700A computer-controlled microelectrode amplifier with Softpanel or Axoclamp-2B)
Analog-digital converter (Axon Digidata 1322A data acquisition system)
Data acquisition software (Axon pClamp 9.0 electrophysiology software)
Isolated pulse stimulator (A-M systems Model 2100)
Differential amplifier (A-M systems Model 3000) for recording EEG

3. Other Equipment

Micropipette puller (Sutter Instruments)

VI. CHEMICALS

Biocytin or Neurobiotin (Vector Laboratories)
Potassium acetate (Sigma)
Paraformaldehyde, glutaraldehyde, Durcupan (Electron Microscopy Sciences)

V. SOLUTIONS

- Phosphate buffer (PB) 0.2 M pH 7.4

  Stock solution A: 0.2 M NaH_2PO_4
  Stock solution B: 0.2 M Na_2HPO_4
  Add solution A to solution B in a 1:4 ratio until the pH reaches 7.4 to give 0.2 M PB.

- Tris-buffered saline (TBS) 0.05 M pH 7.4

  Trizma base 0.05 M
  Trizma acid 0.05 M
  0.9% NaCl

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