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Instruments and techniques

A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system

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Abstract. (1) A preparation is described which allows patch clamp recordings to be made on mammalian central nervous system (CNS) neurones in situ. (2) A vibrating tissue slicer was used to cut thin slices in which individual neurones could be identified visually. Localized cleaning of cell somata with physiological saline freed the cell membrane, allowing the formation of a high resistance seal between the membrane and the patch pipette. (3) The various configurations of the patch clamp technique were used to demonstrate recording of membrane potential, whole cell currents and single channel currents from neurones and isolated patches. (4) The patch clamp technique was used to record from neurones filled with fluorescent dyes. Staining was achieved by filling cells during recording or by previous retrograde labelling. (5) Thin slice cleaning and patch clamp techniques were shown to be applicable to the spinal cord and almost any brain region and to various species. These techniques are also applicable to animals of a wide variety of postnatal ages, from newborn to adult.

Key words: Patch clamp – Brain slice – Central nervous system

Introduction

The patch clamp technique offers an increased resolution in the recording of membrane currents. It allows both the observation of currents through single channels and whole-cell recordings from small cells (Hamill et al. 1981). Because this technique requires the cell membrane to be directly accessible to the recording pipette, it has been largely restricted to isolated cells, disconnected from their native surroundings by enzymatic or mechanical treatment. The requirement for cell isolation previously imposed restrictions on the applicability of the patch clamp technique, especially in the investigation of channels mediating synaptic transmission in the central nervous system (CNS). A few laboratories have previously succeeded in making patch clamp recordings in sliced tissue preparations. Barnes and Werblin (1986) have recorded whole-cell currents from amacrine cells in slices of tiger salamander retina. In slices of mammalian tissue, use of patch clamp techniques has been restricted to a preparation of the hippocampus, in which the slices were split by incubation in proteolytic enzymes (Gray and Johnston 1985). Here we describe procedures for making patch clamp recordings from synaptically connected mammalian neurones, without the use of enzyme treatment. The method is applicable to many different regions of the CNS at various developmental stages. By using thin slices, individual central neurones can be visually identified (Yamamoto 1975; Takahashi 1978; Llinas and Sugimori 1980). Using only physiological saline, such neurones can be partially exposed, allowing tight-seal patch clamp recordings. The methods of exposing single neurones in thin slices from spinal cord and brain are described, and the applicability of the different configurations of the patch clamp technique to these preparations is illustrated.

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Preparation of thin slices for patch clamp measurements

Sectioning of brain or spinal cord tissue

Sectioning of brain or spinal cord tissue for thin slices is generally similar to cutting conventional brain slices with a vibrating slicer (for review see Alger et al. 1984). The specific procedures which have been used for making thin slices are described below.

Preparation of tissue for slicing. The animal is decapitated and the appropriate part of the brain or spinal cord is removed. This procedure should not take more than 1–1.5 min. Keeping the tissue cold throughout sectioning is particularly important. This presumably minimizes damage from anoxia and improves the texture of the tissue for slicing. For this purpose the tissue is submerged in ice-cold physiological saline (see Solutions) whenever possible. If, with large animals (e.g. more than two month old rats) more time is required for removal of the brain, ice-cold physiological saline should also be poured over the brain as soon as the skull is open.

After removal, so that the tissue becomes cold throughout, the brain is incubated at 0–4°C for at least 3 min before slicing. Bisected rat brains have been stored in this state for up to an hour without apparent deterioration. Thus different regions of one brain can be sliced if needed.

Slicing. A standard vibrating microslicer (Microslicer DTK-1000, Dosaka Co.) is used to cut slices of 100–140 μm thickness. The maximum thickness of slices is limited by optical considerations as the transmitted light must pass through the tissue. Slices of 120 μm are thin enough for good

visibility when looking directly through the oculars of the microscope and thick enough to retain healthy cells with intact synaptic connections. Thicker slices (300 μm) can be used if a highly light sensitive television camera is attached to the phototube of the microscope, (see Optical setup).

Mechanical stability of the tissue is essential for making thin slices. For this purpose a larger block of tissue containing the region of interest is cut by hand. A surface of this block, trimmed parallel to the desired orientation of the slices, is glued to the stage of the slicer (e.g. for parasagittal slices the brain can simply be glued on the midline). Firm, instant attachment can be achieved by using a thin film of cyanoacrylate glue. The slicing chamber is then immediately filled with physiological saline and surrounded with ice while slicing. Tissues which are too small to be glued directly (e.g. new born rat spinal cord) can be first embedded in agar (Takahashi 1978). The agar (2% dissolved in physiological saline) is cooled to below 40° C before including the tissue. Careful application of ice-cold physiological saline then facilitates cooling and solidifying of the agar. A block of agar, cut to contain the tissue at the correct orientation, can then be glued to the stage of the slicer and immersed in ice-cold physiological saline, as above.

After slicing roughly down to the required level, at least 1–2 thin slices must generally be discarded before slices of a uniform thickness are obtained. The slicing procedure is continuously monitored through a dissecting microscope and the forward speed of slicing adjusted so that the tissue is never pushed by the blade. When slicing upper brain regions white matter must be cut very slowly (~ 10 mm/min), whereas the softer grey matter allows a somewhat higher speed (~ 20 mm/min). The spinal cord and brain stem are however much tougher and it is necessary to move the blade forward extremely slowly (1–4 mm/min) to avoid pushing the tissue. The cutting blade is set to vibrate at the highest available frequency (~ 8 Hz).

Incubation of slices. After sectioning, each slice is immediately placed in oxygenated physiological saline at 37° C, where it remains until use. In order to ensure efficient oxygenation and continuous movement of the solution, the slices are bubbled directly from below. A method using simple disposable accessories which are easily cleaned or replaced and can hold up to about 12 slices is illustrated in Fig. 1 a, b. Slices are transferred into and out of the holding chamber using a cut and fire polished Pasteur pipette. The condition of the tissue is optimal over the first 3 or 4 h after which some dead cells may appear on the surface of the slices. Nevertheless the many healthy cells which remain can be selected visually and stable recordings are still attained 8–12 h after slicing.

Mechanical fixation for recording. One slice at a time is placed in the glass bottomed recording chamber and held in place with a grid of parallel nylon threads (Fig. 1 c). The U-shaped frame of the grid is made from 0.5 mm platinum wire flattened with a vice (Konnerth et al. 1987). Fine nylon stockings provide a convenient material for making the threads. A hole is made in the stocking so that a "ladder" forms. This results in a parallel array of compound threads which can then be tightly stretched over a ring (about 2 cm diameter) and clamped in place. Under a dissecting microscope one of these compound threads is then separated into individual fine fibres which are arranged on the ring to form

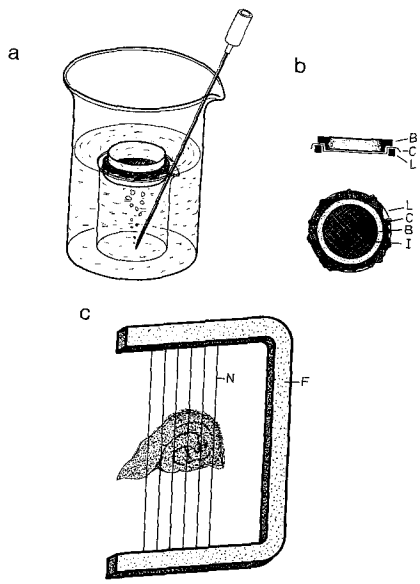

Fig. 1 a–c. Accessories for maintenance and mechanical fixation of thin slices. **a** Maintenance of thin slices following sectioning. A holding chamber is placed on top of a 50 ml beaker which stands inside a 250 ml beaker. Both beakers are filled with physiological saline to the level of the top of the holding chamber. A bubbler inserted through the spout of the inner beaker oxygenates the slices directly from below and causes gentle, continuous movement of the holding chamber. The whole ensemble is placed in a water bath at 37° C and must be covered to prevent evaporation. **b** Construction of the holding chamber. A suitable chamber is made by breaking the top and bottom out of a small (10 × 35 mm) plastic Petri dish, forming two rings. The ring formed from the lid (*L*) is made so that, when inverted, it fits tightly onto the lip of the base (*B*). A piece of fine cotton mesh (*C*) is stretched over the ring made from the base (*B*) and can be clamped in place by the ring made from the lid (*L*). The slices can then be placed inside (*I*). **c** Grid for mechanical fixation of the slice during recording. The thin slice is fixed on the bottom of the recording chamber by a grid of nylon threads (*N*) glued to a platinum frame (*F*). (See Mechanical fixation for details)

parallel threads about 0.4 mm apart. The platinum frame, having been coated with a very thin film of cyanoacrylate glue, is then balanced across the parallel threads, where it is left until the glue dries. Such a grid, placed over the slice, holds it firmly in position on the bottom of the recording chamber (Fig. 1 c). The dimensions of the frame and the distance between the fibres can be varied according to the size of the preparation and recording chamber. A frame of 7 × 9 × 7 mm has been suitable for all the types of slices referred to in this paper. Grids can be reused, usually lasting for at least 20 or 30 experiments.

Optical setup. An upright microscope (Zeiss "Standard 14") with Nomarski optics is used to see the upper surface of the cells for cleaning (Takahashi 1978). Two changes have been made to the microscope which make recording easier. The focussing mechanism has been altered to move the objective instead of the stage. A hinge has been added to the microscope frame so that the top half of the microscope can be tipped back making it much easier to place the patch pipettes in position in the bath. The objective is an Achromat 40 × water immersion lens with a working distance of 1.6 mm. For improved visibility when using thicker slices, a black and white television camera (CFM 2/6, Kappa Messtechnik,

Gleichen, FRG) was attached to the microscope via an adapted phototube (Zeiss). All procedures were then observed on a television screen (Panasonic video monitor, WV5410/G).

Solutions. The standard extracellular solution, which we will refer to as physiological saline, contains (in mM): NaCl 125, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 26, NaH_2PO_4 1.25, glucose 25, [pH 7.4 when bubbled with carbogen (95% O_2, 5% CO_2)]. It is preferable to perfuse slices continuously during recording, however at room temperature the cells can tolerate periods (up to 30 min) without perfusion.

Various pipette solutions have been used for whole-cell recording and for recording from outside-out patches. The standard internal solution was (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 10, ATP 2, HEPES 10 (pH 7.3). The exact solution used for each experiment is given in the appropriate figure legend.

All drugs were obtained from Sigma except for Diazepam which was obtained from Dr. H. Möhler (Hoffmann-La Roche, Basel, Switzerland) and Pentobarbitone (Ceva, Paris, France).

Cleaning of the slice to expose the membrane of a neurone in situ

In order to form high resistance (GΩ) seals the patch pipette must have an unimpeded access to the cell membrane (Hamill et al. 1981). A simple technique was developed which allows local exposure of the upper membrane of cells, even if they are located deeply within the slice.

After sectioning, slices appear healthy with very little evidence of dead tissue, even on the cut surface. Frequently, cells located at the upper surface of the slice require no cleaning. This is particularly common in slices from young animals (1–7 days postnatal). Cells located deeper in the slice are covered with tissue and cell debris which must be removed. The different steps of the "cleaning" procedure are shown schematically in Fig. 2a–c and illustrated in more detail for hippocampal, spinal and visual cortex slices (Figs. 3 and 4).

The "cleaning" pipette consists of a patch pipette, broken to a tip diameter of 5–20 μm, depending on the cell size and the nature of the tissue. The pipette, filled with physiological saline, is placed in a patch pipette holder (similar to that used for recording) and lowered under microscopic control. The pipette tip is then brought onto the surface of the slice, near the cell to be exposed (Fig. 2a). The tissue above the cell is disrupted by a stream of physiological saline applied by blowing through the mouth piece of the pipette holder (Fig. 2b). Resulting debris is removed by careful suction (Fig. 2c). The removal of the tissue covering the neurone is monitored by continuously focussing up and down above the cell surface (Fig. 3a–d). The cleaned cell looks smooth and bright in Nomarski optics (Fig. 3d, 4a, b, d). A cell lying near the surface of the tissue can be cleaned in about 10 s and even for deeper cells the whole process of cleaning should take less than a minute, usually requiring about two cycles of blowing and sucking.

In layered tissues, in which cells are densely packed [e.g. hippocampal granule or pyramidal cells (Fig. 3d)], several cells can be exposed in one cleaning process. More isolated neurones are generally cleaned one at a time (Fig. 4a–d).

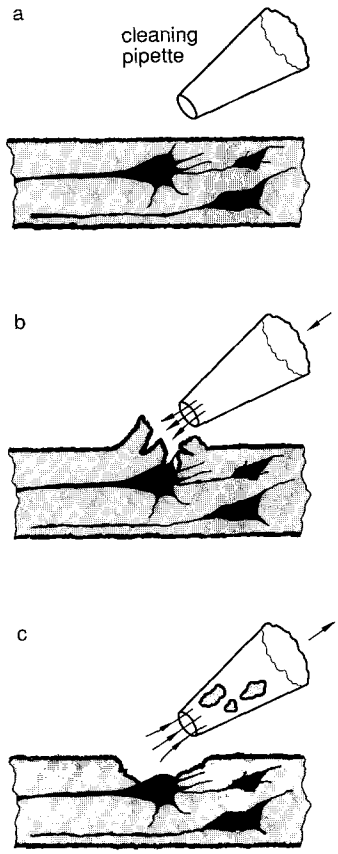

Fig. 2a–c. Schematic diagram of the exposure of neurones. In the scheme neurones are represented by black structures surrounded by glia and neuropil shown in grey. **a** The cleaning pipette is shown positioned close to the slice surface above the soma of a neurone. **b** Tissue which covers the cell soma is softened by a stream of physiological saline blown through the cleaning pipette. **c** Resulting debris is removed by gentle suction through the cleaning pipette.

Patch clamp recordings from neurones in thin slices

Patch clamp recordings from cleaned neurones in slices are obtained with a success rate similar to experiments using isolated cells (Hamill et al. 1981). When not otherwise stated, patch pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, FRG, outer diameter 2.0 mm, wall thickness 0.3–0.5 mm). Due to the small working distance (1.6 mm) of the water immersion objective, the pipette must approach the neurones at a low angle (~ 15° to the horizontal plane). The capacitance resulting from deep immersion of the pipette in the bath can be largely reduced by coating with Sylgard far up the shank (8–12 mm).

After touching the soma with the heat polished pipette tip, gentle suction leads to a continuously improving seal until a high resistance (> 10 GΩ) is reached. Discontinuity in the seal formation may indicate inadequate cleaning of the cell surface. Further cleaning sometimes allows formation of a GΩ seal on a second attempt. All three recording configurations of the patch clamp technique (i.e. whole-cell, cell-attached and cell-free) can be used to study membrane currents and potentials in CNS-neurones in situ. In the following paragraphs we present examples of whole-cell and single-channel recordings from hippocampal and spinal neurones, made with an EPC7 patch clamp amplifier (List, Darmstadt, FRG).

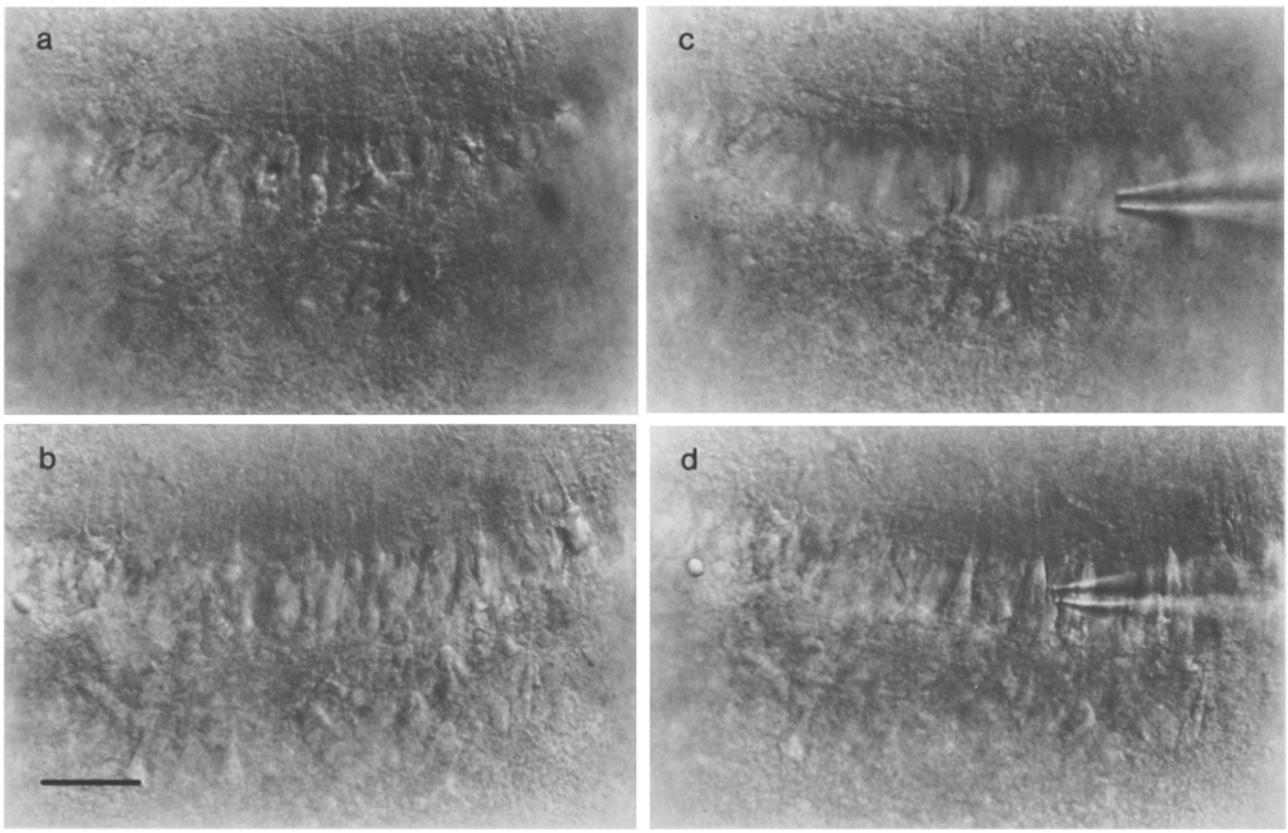

Fig. 3a–d. Exposure of densely packed cell somata in the CA1 layer of the hippocampus. Thin slices were obtained from a 21 day old rat. All photographs show the same view at one of two different levels of focus. **a, b** Before cleaning: The surface of the slices (**a**) is relatively featureless but by focussing deeper (**b**) cell bodies and dendrites can be more clearly distinguished. **c, d** After cleaning: Focussing on the surface (**c**), the crater formed by the cleaning procedure can be seen. Focussing deeper (**d**) the "clean" membranes of the now exposed cell bodies are visible. In panels **c** and **d**, the cleaning electrode is visible on the *right hand side*. *Calibration bar* 20 μm

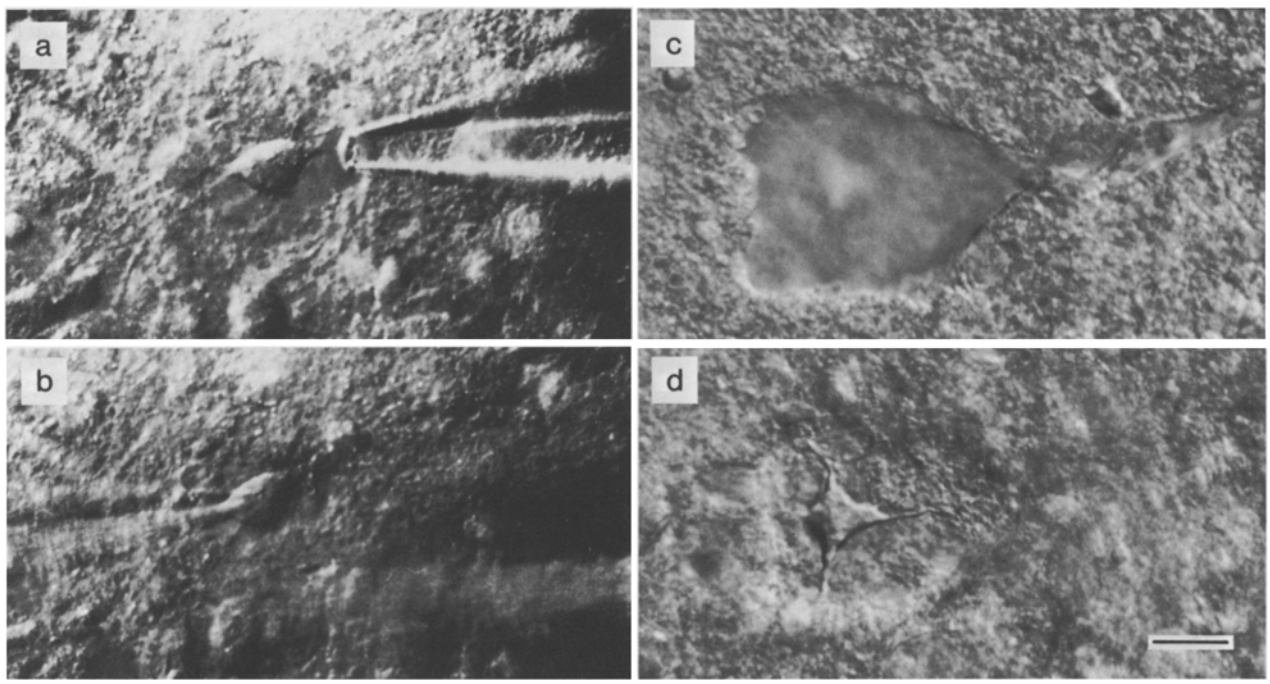

Fig. 4a–d. Exposure of separately located cell somata in thin slices of spinal cord and visual cortex. **a, b** A motoneurone in a thin slice of spinal cord from a 4 day old rat. **a** Exposed cell surface with the cleaning pipette (tip diameter about 10 μm). **b** The motoneurone with an attached patch pipette (*shadow on left hand side*). **c, d** Exposure of a layer 5 neurone in a thin slice of visual cortex from a 20 day old rat. **c** Slice surface after cleaning. Note the crater like hole formed by cleaning down to a cell deep within the slice. **d** On focussing into the bottom of the "crater" the cleaned cell soma with proximal processes can be seen. The *calibration bar* shown in panel **d** represents 20 μm for panels **a, b** and 10 μm for panels **c, d**. Despite the apparent isolation of such cells, synaptic currents were always observed

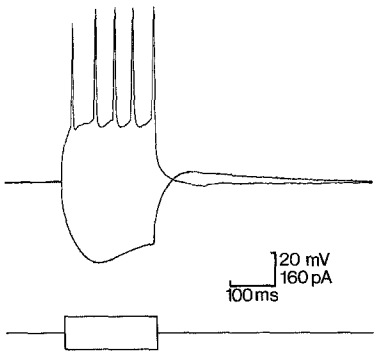

Fig. 5. Voltage recordings from a rat hippocampal CA1 pyramidal cell. The *upper panel* shows two superimposed whole-cell recordings of voltage responses evoked by a depolarizing and a hyperpolarizing current pulse (± 80 pA, as indicated in the *lower panel*). The depolarizing pulse resulted in a train of action potentials. The sag evident in the hyperpolarizing response is presumably due to activation of the K^+ inward rectifier. Membrane potential −80 mV; pipette solution (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11, HEPES 11 (pH 7.3). Voltage traces represent the digitized output (2 kHz sampling rate) of the voltage monitor of the patch clamp amplifier in the current clamp mode. Data was filtered (low-pass) at 2 kHz (−3 dB). In this and all following figures, depolarizing potentials are represented by an upward deflection of the trace

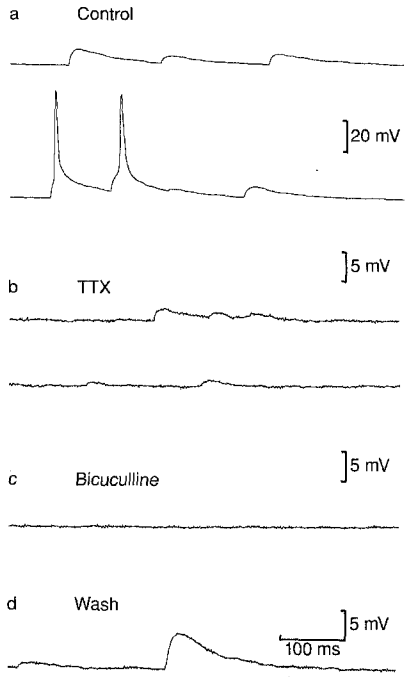

Fig. 6a–d. Voltage recordings of spontaneous postsynaptic potentials in a rat hippocampal CA1 pyramidal cell. **a** In normal physiological saline frequent, large potentials were observed. When spontaneous synaptic potentials crossed the threshold, action potentials resulted (*lower trace*). **b** After bath application of tetrodotoxin (TTX, 1 μM) only small (miniature) potentials remained (shown at higher gain). **c** Bath application of bicuculline (10 μM) completely abolished these potentials, identifying them as GABA-mediated synaptic potentials. **d** After washing for 20 min the effects of bicuculline and TTX were largely reversible. Pipette solution (in mM): KCl 140, $MgCl_2$ 2, $CaCl_2$ 2, ATP 2, EGTA 10, HEPES 10 (pH 7.3). The resting membrane potential (−70 mV, measured in whole-cell mode), remained stable throughout recording, without input of holding current. The recordings represent the digitized (2 kHz) output of the voltage monitor, filtered (low pass) at 3 kHz (−3 dB)

All the following experiments were performed at room temperature (21–24° C) on thin slices from brains of 17–21 day old rats or from spinal cords of 4 day old rats. Similarly stable recordings have been made at temperatures up to 35° C.

Whole-cell recording

In sliced tissue the passive properties of neurones measured under patch clamp conditions were generally found to be considerably different from those impaled with a microelectrode.

Passive membrane properties were measured in the whole-cell patch configuration for 7 out of 8 consecutive hippocampal granule cells using our most common experimental conditions [3 week old rats (45 g), 24° C]. The resulting values were: membrane potential −67 ± 1.9 mV (range 60–75 mV), input resistance 1.1 ± 0.2 GΩ (range 0.6–2.1 GΩ) and time constant 37.6 ± 3.4 ms (range 23–50 ms). Input resistance and time constants were measured from the responses to ± 10 pA, 200 ms pulses applied at the resting membrane potential. One cell was excluded from the measurement as it had a membrane potential of −40 mV which was > 4 standard deviations from the mean.

A direct comparison with intracellular recording conditions is not possible as the published data refers to older animals and is recorded at higher temperatures. However, the results from one such study are listed below for a general comparison. Crunelli et al. (1983) recorded the passive membrane properties of 54 impaled granule cells in slices from adult rats (200 g), at 37° C. They reported mean values (± SEM) for resting membrane potential: −66 ± 1.8 mV (range 59–75 mV), membrane input resistance: 48.4 ± 2.7 MΩ (range 19–80 MΩ) and membrane time constant: 10.2 ± 0.5 ms (range 7.5–14.3 ms). The mean resting membrane potentials were remarkably similar between the two methods, however the mean input resistance is considerably higher when using the patch clamp technique compared to intracellular recordings. This electrical tightness is also reflected in the longer membrane time constant. The very much higher input resistance, seen under patch clamp conditions, is unlikely to be due only to the differences in age and temperature but is presumably a result of the extremely high resistance of the seal between the patch pipette and the cell membrane (> 10 GΩ). This results in considerably lower membrane noise so that synaptic currents as small as 10 pA are clearly resolvable.

However, despite the advantages of patch clamp techniques, certain remaining limitations should be noted. Voltage clamp recordings from neurones inevitably face the problems of space clamping. This is particularly true in slices where an extensive dendritic tree remains intact (Fig. 13a). In addition, in the whole-cell configuration, the ionic composition of the cell interior is virtually replaced by that of the pipette solution (Pusch and Neher 1988). Thus problems may arise from dilution or washing out of essential intracellular constituents.

Action potentials. Having established whole-cell configuration in the voltage clamp mode, the patch clamp amplifier was set to the current clamp mode for potential recordings.

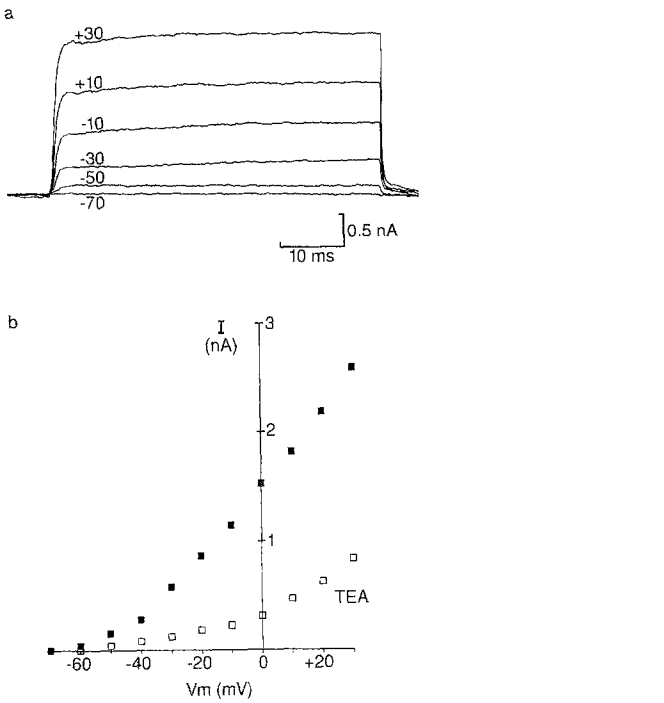

Fig. 7a, b. Whole-cell recordings of voltage-activated currents. Rat hippocampal granule cell. **a** Voltage-activated K^+ outward currents elicited in response to depolarizing pulses from a holding potential of −80 mV to the test potentials indicated on the traces. **b** The resulting current-voltage relation in normal physiological saline (■) and after adding 20 mM tetraethylammonium chloride (TEA) to the bath solution (□). Current amplitudes were measured at the end of each response. Pipette solution (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11, HEPES 10 (pH 7.3). The bath solution was physiological saline to which 1 μM TTX was added in order to block Na^+ currents. The possibility of contamination by Ca^{2+} currents was reduced by recording more than 30 min after establishing whole-cell configuration. Under these conditions, with no ATP in the pipette solution, Ca^{2+} currents tend to disappear, presumably due to washout. Depolarizing pulses were applied at a frequency of 0.2 Hz; leakage and capacitive currents were subtracted on-line by the P/4 procedure (Armstrong and Bezanilla 1974)

Figure 5 shows recordings of action potentials in pyramidal neurones in the CA1 region of rat hippocampus. A depolarizing current pulse (80 pA, 200 ms) elicited a train of action potentials. The peak voltage response to the hyperpolarizing current step (80 pA) indicates that the input resistance of this cell was 0.6 GΩ. In smaller cells (e.g. hippocampal granule cells) even higher input resistances are observed (see above).

Synaptic potentials. Figure 6 shows a recording of spontaneously occurring postsynaptic potentials at the resting membrane potential (−70 mV) in another CA1 pyramidal cell. As the pipette solution contained a high concentration of Cl^- (148 mM), similar to that in the physiological saline (131 mM), opening of Cl^- channels at negative potentials resulted in Cl^- ions flowing out of the cell and thus caused depolarization. When these depolarizing inhibitory postsynaptic potentials (i.p.s.p.s) were large enough to cross the threshold (approximately −50 mV) action potentials resulted (Fig. 6a, lower trace). Addition of tetrodotoxin (TTX) to the bath to block action potentials, resulted in the abolishment of all large (evoked) synaptic potentials

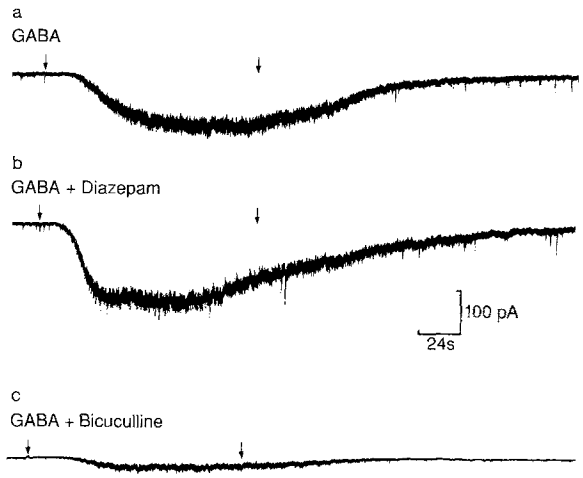

Fig. 8a–c. Whole-cell recordings of GABA-activated currents. Rat hippocampal granule cell. Bath application of **a** 4 μM GABA, **b** 4 μM GABA plus 5 μM Diazepam or **c** 4 μM GABA plus 10 μM (−)Bicuculline methiodide. Drugs were added to physiological saline and applied by slow perfusion (1 ml/min) for 2 min. In *each trace* the *arrows* indicate the start and finish of the drug applications. Between drug applications the preparation was washed with physiological saline for 5 min. Holding potential was −50 mV. Pipette solution (in mM): KCl 140, $MgCl_2$ 2, $CaCl_2$ 2, ATP 2, EGTA 10, HEPES 10 (pH 7.3). The records represent traces taken on a chart recorder with a frequency response of 300 Hz (−3 dB)

(Fig. 6b). Only miniature postsynaptic potentials remained. Addition of the $GABA_A$ receptor antagonist, Bicuculline (Fig. 6c), reversibly abolished all synaptic activity. Thus these potentials represent i.p.s.p.s mediated by $GABA_A$ receptors.

Voltage-activated currents. Figure 7 shows recordings of whole-cell outward K^+ currents elicited by depolarizing voltage steps to various test potentials in a hippocampal granule cell. The current-voltage relation indicates that these outward currents activate at about −60 mV (closed symbols). Addition of 20 mM TEA to the bath solution largely blocked the response leaving a fast inactivating component (open symbols).

Transmitter-activated currents. Figure 8 illustrates GABA-activated whole-cell currents and the effect of pharmacological modifiers on the GABA response. When added to the bath perfusion, GABA concentrations as low as 1 μM elicited detectable responses in hippocampal neurones, the lower limit of detection being between 10 and 20 pA. The GABA response is shown to be enhanced by Diazepam and suppressed by Bicuculline. Thus putative transmitter substances and pharmacological modifiers can be identified and characterized, in situ, at higher resolution than previously possible, using whole-cell recordings from visually identified neurones.

Single channel recording

Cell-attached. Figure 9 shows a series of three consecutive traces of K^+ single channel currents recorded from a cell-attached patch in a hippocampal granule cell. Following a 60 mV depolarizing voltage step, from an unknown resting potential, outward currents of unitary amplitude but vari-

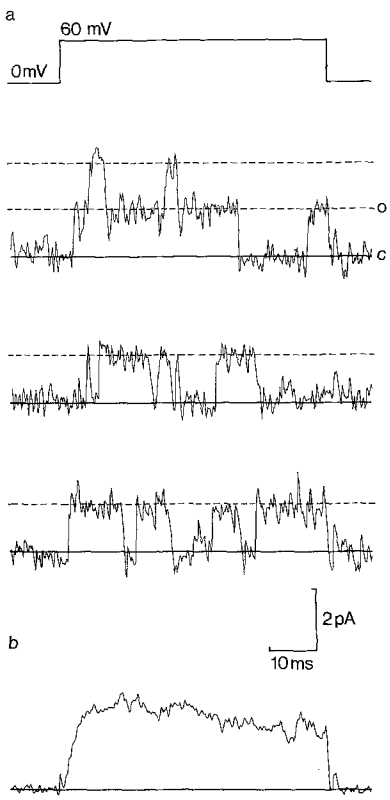

Fig. 9a, b. Single channel current recordings from cell-attached patches. **a** K^+ currents activated by 60 mV depolarizing voltage steps (as indicated in the *top panel*) from the unknown resting membrane potential of a rat hippocampal granule cell. Closed (*c*) and open (*o*) levels are indicated by *continuous* and *broken lines*, respectively. *Broken lines* reflect the average single channel amplitude (1.5 pA). The current traces represent three consecutive records. **b** Averaged record from 16 consecutive responses to depolarizing voltage steps of 60 mV. Voltage steps were given at a frequency of 0.5 Hz. Pipette solution (in mM): NaCl 140, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10 (pH 7.3) and TTX 1 μM. *Calibration bars* refer to both panels. Records were filtered (low pass) at 0.8 kHz (−3 dB) and digitized at 5 kHz. Leakage and capacitive currents were subtracted on-line

able durations are elicited (Fig. 9a). The average of 16 single channel current responses shows a delayed, voltage dependent activation and little inactivation of this current (Fig. 9b). The time course of activation and inactivation are comparable to the whole-cell K^+ currents elicited by similar voltage steps (Fig. 7a).

Inside-out. Figure 10a shows the activation of Ca^{2+} dependent K^+ currents in an inside-out patch isolated from a hippocampal granule cell. The extracellular face of the patch is in contact with the physiological saline inside the pipette. The cytoplasmic side of the patch faces the bath which during control recordings contained a high K^+, low Ca^{2+} solution. Under control conditions, at 0 mV membrane potential the most prominent outward current was mediated by a large conductance K^+ channel which opened briefly at irregular intervals. Changing to a high Ca^{2+} bath solution resulted in very long currents with more than one level being detectable. These currents reflect openings of large Ca^{2+}-activated K^+ channels similar to the "big" K^+ channel described by Marty (1983).

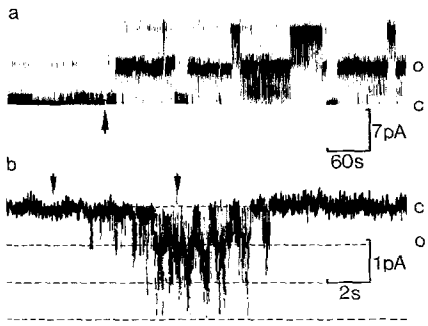

Fig. 10a, b. Single channel current recordings from isolated patches. **a** Inside-out configuration. Control bath solution (washing cytoplasmic side of the membrane) contained (in mM): KCl 140, HEPES 10 (pH 7.3), EGTA 10. Ca^{2+} activated K^+ currents were stimulated by switching to a high Ca^{2+} solution containing: 140 mM KCl, 10 mM HEPES and 40 μM added Ca^{2+} (indicated by the *arrow*). In control solution, activity consisted of occasional brief outward currents (upwards deflections). Within 6 s of changing to the high Ca^{2+} solution long lasting outward currents were activated, frequently showing two distinct levels. The pipette solution (bathing the external side of the membrane) was normal physiological saline. Membrane potential 0 mV. **b** Outside-out configuration. Single channel currents were activated by local application of 10 μM GABA (indicated by *arrows*). Within 2 s of the beginning of GABA application, the inward current increased in a stepwise fashion, showing three integral levels. The levels (indicated by *broken lines*) were determined by measurement of single channel current amplitudes (mean 0.9 pA, holding potential −50 mV). The patch pipette solution contained (in mM): N-methylglucamine 150, HCl 125, $MgCl_2$ 2, $CaCl_2$ 2, ATP 2, EGTA 10, HEPES 10 (pH 7.3). The application and recording pipettes were lifted well above the slice surface (> 1 mm) and the slice was also moved more than 3 mm (out of the visual field) so as to ensure complete isolation of the patch from the neurone. Both panels represent recordings from rat hippocampal granule cells taken on a chart recorder (frequency response 300 Hz). In both panels closed and first open level are indicated by *c* and *o*, respectively

As inside-out patches are excised, formation of vesicles at the tip of the pipette may cause problems (Hamill et al. 1981). This did not occur however, if the patch was excised while still bathing the slice in physiological saline to which no Ca^{2+} was added. The bath solution was changes to the "intracellular" solution [containing (in mM): KCl 140, HEPES 10, EGTA 10, (pH 7.3)] only after successful formation of the inside-out patch. Using this procedure all patches remained open and stable, without further manipulation. Exposure of the slice to the high K^+, low Ca^{2+} solution causes rapid cell deterioration and thus a new slice must be taken for subsequent recordings.

Outside-out. Figure 10b shows the activation of single channel currents in response to application of 10 μM GABA to an outside-out patch, isolated from a hippocampal granule cell. For GABA application a pipette with a tip opening of 30 μm was brought close to the patch (within 50 μm). Gentle positive pressure resulted in the activation of single channel currents superimposing to multiples of a unitary amplitude, indicating the activation of a 20 pS conductance state of the GABAR-channel.

Stimulation of inhibitory and excitatory inputs

In thin slices it is possible to stimulate both, excitatory and inhibitory synaptic inputs. For this purpose a second

stimulating electrode is placed either in an afferent fibre tract or close to a neighbouring interneurone. In some preparations pairs of synaptically connected neurones can be examined by double patch clamp experiments.

Field stimulation of afferent fibres. The major excitatory input to neurones in the granule cell layer of the hippocampus comes from fibres of the perforant path (Lorente de Nó 1934). In order to stimulate this fibre bundle, a stimulation electrode (25 µm diameter Teflon-coated platinum wire) was placed about 200–400 µm away from a recorded cell. Square pulses (180 µs, 1–6 V), delivered by a stimulus isolation unit, evoked synaptic currents in granule cells. These currents were abolished by the addition of 1 µM TTX, indicating that fibre activation and not direct presynaptic terminal stimulation of synapses was responsible for transmitter release.

Figure 11 a, b shows postsynaptic potentials and currents resulting from stimulation of the perforant path. The signals recorded in control solution consisted of inhibitory and excitatory components (Fricke and Prince 1984). Bicuculline (10 µM) reduced the current, indicating the contribution of a GABA receptor-mediated component. The remaining current was largely blocked by kynurenic acid (1 mM), but not affected by APV (2-amino-5-phosphonovalerate). These observations are consistent with previous reports indicating that glutamate receptors of the Quisqualate/Kainate type play a central role at the synapses formed between fibres of the perforant path and granule cells (Crunelli et al. 1983).

Extracellular stimulation of inhibitory interneurones. In spinal cord or hippocampal slices it is possible to stimulate a visually identified interneurone extracellularly, while recording from a cell to which it is synaptically connected. In the spinal cord, after establishing the whole-cell configuration in a motoneurone, stimuli were applied to neighbouring cells until a synaptically connected interneurone was found. Stimulation pulses (150–200 µs, 1–10 V) were delivered from a large patch pipette (5–10 µm tip diameter) placed near the surface of the interneurone.

Figure 11 c shows postsynaptic currents recorded from a spinal motoneurone in response to stimulation of an interneurone (upper panel). The currents were completely abolished by strychnine added to the bathing medium (middle panel) and partly recovered after washing (lower panel).

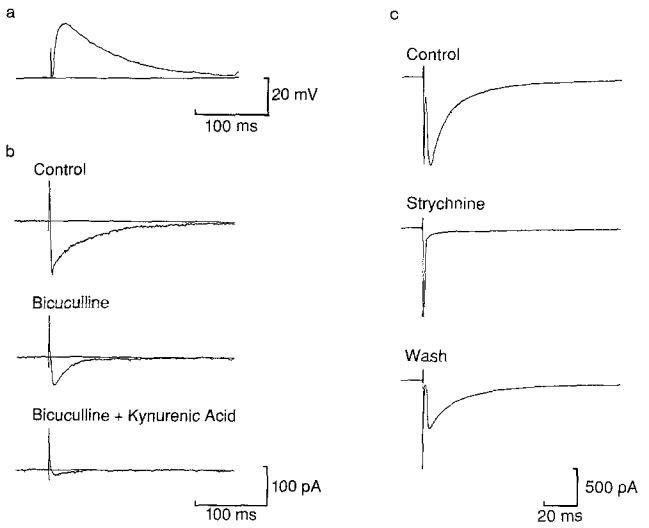

Fig. 11 a–c. Postsynaptic currents and potentials evoked by the extracellular stimulation of afferent fibres and interneurones. **a** Voltage recording of postsynaptic potential in a neurone of the hippocampal granule cell layer evoked by the stimulation of the perforant fibre tract. In this and the following records the stimulation artifact can be seen as a short spike immediately preceding the trace. **b** Current recordings from the same cell. The upper trace shows a postsynaptic current recorded in physiological saline under identical conditions to the voltage recording in **a**. Addition of bicuculline (10 µM) to the perfusion solution decreased the amplitude and duration of the postsynaptic response (*middle trace*) indicating a GABA-mediated component. The remaining current was largely suppressed when glutamate receptors were blocked by the further addition of kynurenic acid (1 mM). Thus the signals in control solution (**a** and *top trace* in **b**) are the result of the activation of combined inhibitory/excitatory inputs. For both **a** and **b** membrane potential was −90 mV. Pipette solution contained (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 1, ATP 2, EGTA 11, HEPES 10 (pH 7.3). Signals were evoked by square pulses (180 µs, 5.2 V, 0.5 Hz) applied through a bipolar stimulation electrode located in the perforant path about 200 µm away from the recorded cell. **c** Postsynaptic currents in a spinal motoneurone evoked by the extracellular stimulation of an interneurone. The high Cl^--content of the pipette solution (same composition as above) resulted in inward currents at the holding potential (−50 mV). The large inward current recorded in normal physiological solution (*upper trace*) was suppressed in the presence of 2 µM strychnine (*middle trace*) and recovered partially (*lower trace*) after 30 min of perfusion with normal saline. Each trace represents an average of 10 consecutive records. Supramaximal stimuli (200 µs, 8 V) were delivered at a frequency of 1 Hz

Thin slice preparations from various brain regions, different developmental stages and different species

The thin slice preparation and cleaning procedure described above were initially developed on slices of rat spinal cord and hippocampus. However, these techniques are also applicable to many other structures from different parts of the brain and from different species. In the following section we briefly describe various thin slice preparations. In each structure we have measured membrane currents following the establishment of GΩ seals in at least one of the possible recording configurations of the patch clamp technique. We describe the slicing procedure and some characteristic aspects of the different preparations.

Other regions of the rat brain

Visual cortex. Parasagittal slices of the visual cortex were cut from the posterior half of one hemisphere of the brain. The block of tissue was glued on the medial surface with the posterior end facing the blade of the microslicer. The exact position and the extent of the visual cortex (areas 17 and 18) depends on the age of the animal. For slices from three week old rats, the slicing chamber was raised until the blade just touched the bottom. The chamber was then lowered by 3 mm. After the initial rough trimming about 10 visual cortex slices could then be cut, bringing the blade to a minimum of 1.5 mm from the parasagittal medial surface, on which the brain was glued. From each slice a piece (approximately 2–3 mm^2) of cortex from the area facing the blade was dissected free. The resulting visual cortex slices show distinct layering under the 40 × objective with layers 2–3 and 4–5 being clearly distinguishable.

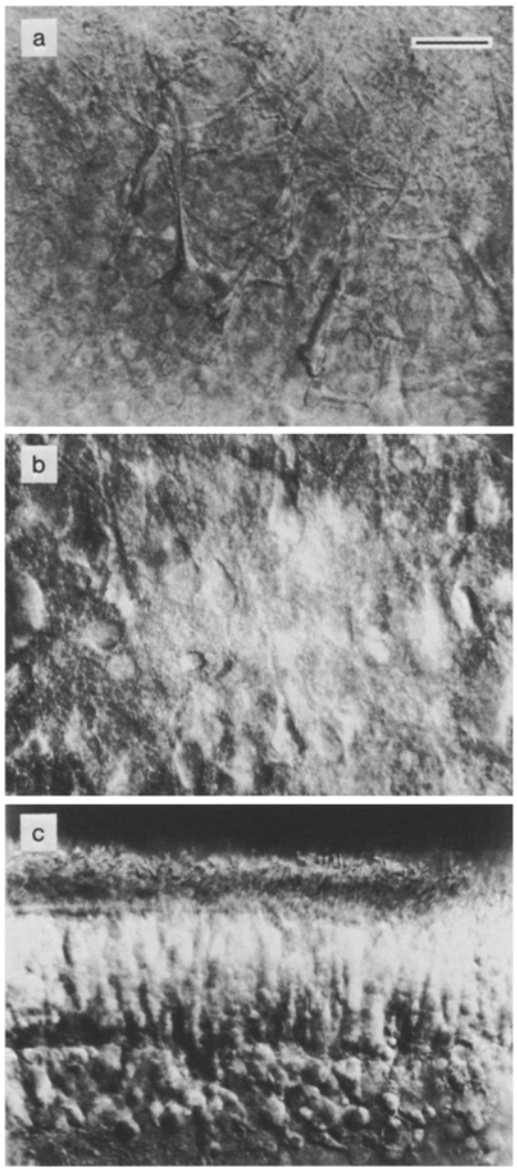

Fig. 12a–c. Living cells in thin slice preparations from various regions of the rat brain. All photographs show uncleaned slices (120 μm thick) from 19 day old rats. The *calibration bar* in **a** refers to all panels. **a** Mitral cells in the olfactory bulb. *Calibration bar* 13 μm. **b** Pyramidal cells in frontal cortex. Note the long parallel apical dendrites lying in the plane of cutting. *Calibration bar* 10 μm. **c** Retinal cell layers. From top to bottom the receptor, bipolar and ganglion cell layers are clearly discernible. *Calibration bar* 10 μm

High resistance seals (> 10 GΩ) were easily obtained in pyramidal and stellate cells in layer 4–5. Single channel currents were recorded in the cell-attached mode and spontaneous synaptic currents in the whole-cell mode. Other cells and configurations have not been attempted so far.

Figure 4d shows a layer 5 neurone located deeply within the slice. Panel cl illustrates the slice surface with the crater produced by the cleaning process.

Cerebellum. Parasagittal cerebellar slices were cut after bisecting the cerebellum longitudinally and gluing one half on the midline. Such slices show very clear layering with individual Purkinje cells being clearly visible even under a 10 × objective. Recordings were easily made on granule cells, Purkinje cells and interneurones. In Purkinje cells, single channel currents have been observed in the cell-attached mode, spontaneous synaptic currents in whole-cell configuration and GABA-mediated single channel currents in outside-out patches.

Olfactory bulb. The olfactory bulbs, being embedded in the nasal cavity, were slightly more difficult to remove from the skull. The rest of the brain was removed quickly and then the remaining skull was submerged in ice-cold physiological saline, while gently levering out the olfactory bulbs with a spatula. Horizontal slices resulted in clearer layering than parasagittal or coronal slices. The distinctive mitral cells were clearly visible (Fig. 12a). The tissue seemed to be very fibrous and though recording from granule cells presented no difficulties, it was more difficult to obtain seals on mitral cells. Nevertheless successful cell-attached patches have also been obtained and single channel currents recorded from mitral cells.

Frontal cortex. To obtain slices from the frontal cortex, the anterior half of one hemisphere of the brain was glued on the medial surface with the anterior end facing the blade. The slices featured large pyramidal cells with long, broad parallel apical dendrites (Fig. 12b). Seals were easily formed on the somata of these cells and cell-attached and whole-cell recordings were made.

Retina. As it takes some time to remove the retina, the dissection was performed as far as possible under ice-cold physiological saline. The eye is removed from the skull and opened by cutting around the border between the sclera and cornea with fine scissors. The retina detached from the sclera readily when the cup was turned inside-out.

Flat retina. Recordings could be made from visually identified ganglion cells when small pieces of retina were laid flat under the grid with the inner eye surface upwards. Although the flat retina has the advantage of synaptic connections being virtually undisturbed it also presents technical difficulties. The whole surface of the retina tends to be covered with the inner limiting membrane and vitreous humor both of which are difficult to remove.

Retinal slice. The retinal slice allows easier cleaning of ganglion cells and makes other cell types accessible. A small piece (~ 3 × 3 mm) of retina was cut with a new scalpel blade, making sure that at least one edge was straight. Parallel to this straight edge, strips of retina were simply cut by hand under the dissecting microscope. The strips were narrower than the total depth of the retina so that they tended to lie sideways on the bottom of the dish. Retinal strips are very sticky and difficult to manipulate precisely. However, by placing 6–8 strips side by side so that they can be covered with the grid, about half will lie with the cut side exposed. Rod, bipolar cell and ganglion cell layers were clearly visible (Fig. 12c). We have recorded from ganglion, amacrine and bipolar cells in the cell-attached and whole-cell configurations.

The proximity of cells in the retina could make it a very good preparation for simultaneous recording of two synaptically connected cells.

Different developmental stages

One particularly useful application of the thin slice technique is the possibility of recording currents in the same type of neurone at different developmental stages. We have recorded from CA1 and/or granule cells in the hippocampus of rats ranging from 2–42 days postnatal. Preparation of slices from older rats (> 25 days) is somewhat more difficult than from younger rats because the brain takes longer to remove and the tissue is tougher. However, with careful cleaning good seals (> 10 GΩ) can still be achieved. Adult tissue is also more prone to anoxia, it is thus especially important to freeze the cutting solution and to use it at as close as possible to zero degrees.

Different species

The thin slice technique is not only applicable to rat tissue but has been successfully applied to mouse and cat brain.

Mouse. Preparing thin slices of adult (4 month) mouse brain presents no difficulties. Compared to adult rat, the brain is smaller and connective tissue presents less of a problem. The hippocampus was cut using exactly the same method as for cutting adult rat hippocampus. The CA1 or granule cells are considerably smaller than in the rat. Whole-cell recordings of synaptic currents were easily achieved.

Cat. The brains of two adult cats were removed after several days of in vivo experiments. The cats were kept alive (anaesthetized and respirated) while the skull and dura were opened. Ice cold physiological saline was then poured on the brain in situ, the cat was killed with an overdose of Pentobarbitone and the brain was scooped out quickly and placed in ice cold solution. The size of the cat brain and the amount of tough white matter present some difficulties.

Cat hippocampus. As in other preparations, a suitable block of tissue was cut, containing the hippocampus. This block was glued to the stage of the slicer such that the side with the least white matter faced the blade, facilitating the slicing procedure. Nevertheless, as white matter is cut, the brain tends to be slightly compressed and the hippocampus is pushed upwards. This means that it is not possible to cut even slices of set thickness. However, by selecting a nominal thickness of 60–80 μm, hippocampal slices with usable portions were obtained.

Cat visual cortex. As so much of the research on visual cortex to date has been performed on cat, it is of interest to note that the thin slice technique can also be applied to this structure. The cat neocortex is considerably easier to cut than cat hippocampus as white matter does not present such an obstacle. Thin slices of visual cortex were cut using a procedure similar to that described for the rat visual cortex. A small block of tissue was cut and glued to the stage of the microslicer with the posterior end facing the blade. Slices were then made by cutting only through the cortex and dissecting it away. This prevented all problems with white matter. We have successfully recorded from synaptically connected cells in layer 4/5, both in the cell attached and whole-cell configurations.

Identification of cell types by fluorescent staining

One advantage of using thin slices as a preparation for patch clamp recording is that neurones can be identified by their shape (Takahashi 1978). One way to characterize them in more detail is to fill the neurone to be studied with fluorescent dye. Two methods were found to be useful: labelling during recording or retrograde labelling prior to the electrophysiological experiment.

Labelling during whole-cell recording. Neurones can be filled during recording and the shape and extent of the dendritic tree of the cell studied can be examined during the experiment.

Rapid loading was achieved by including the dye in the patch pipette solution and establishing the whole-cell configuration. Lucifer Yellow (2 mg/ml) and Texas Red (0.1 mg/ml) diffused particularly well filling even distal dendrites (more than 100 μm from the soma) within 1–3 min. The presence of these dyes had no obvious effect on inhibitory postsynaptic currents recorded in hippocampal or cerebellar cells.

Figure 13a, b show two different Lucifer Yellow filled Purkinje cells at different levels in the slice. The cell pictured in Fig. 13a lay deep in the slice and the fluorescent dye reveals the preservation of an extensive dendritic tree, despite the slicing procedure. Spontaneous synaptic currents were recorded during the filling of this cell. In contrast, the soma of the cell, shown in Fig. 13b, lay on the surface so no cleaning was required and the dendritic tree was largely truncated. Despite the minimal dendritic tree spontaneous synaptic currents were also frequent in this cell.

Figure 13c shows two different neurones in the granule cell layer of the hippocampus each filled with one of the two dyes mentioned above. Two photographs, taken at the same level of focus with different fluoresence filters were later superimposed. In this 2-dimensional view possible points of connection can be seen between the cells. Synaptic currents have already been recorded in response to specific extracellular stimulation of a nearby visually identified neurone both in the spinal cord (Fig. 11c) and hippocampus (not shown). In addition, whole-cell recordings have been made simultaneously from two cells in the CA1 region of the hippocampus. The 2-dimensional view shown here suggests the future possibility of combining these techniques with confocal microscopy and 3-dimensional image reconstruction allowing detailed analysis of the anatomical location of electrophysiologically characterized synapses.

Retrograde labelling. The use of retrograde labelling allows neurones to be identified according to distant anatomical connections. For the purpose of recording from a particular cell type, fluorescent dye was injected 1–3 days prior to recording, in the region where the axons terminate.

Figure 14a, b show low magnification pictures of a spinal cord slice. A fluorescent dye, Evans Blue, was injected in several places in the left hind leg muscle one day before slicing. Only motoneurones in the ventrolateral column on the injected side were stained (Fig. 14b). Under high magnification the Nomarski and fluorescent views can be correlated (Fig. 14c, d). Spontaneous and evoked synaptic currents were not measureably different from those recorded in unstained neurones.

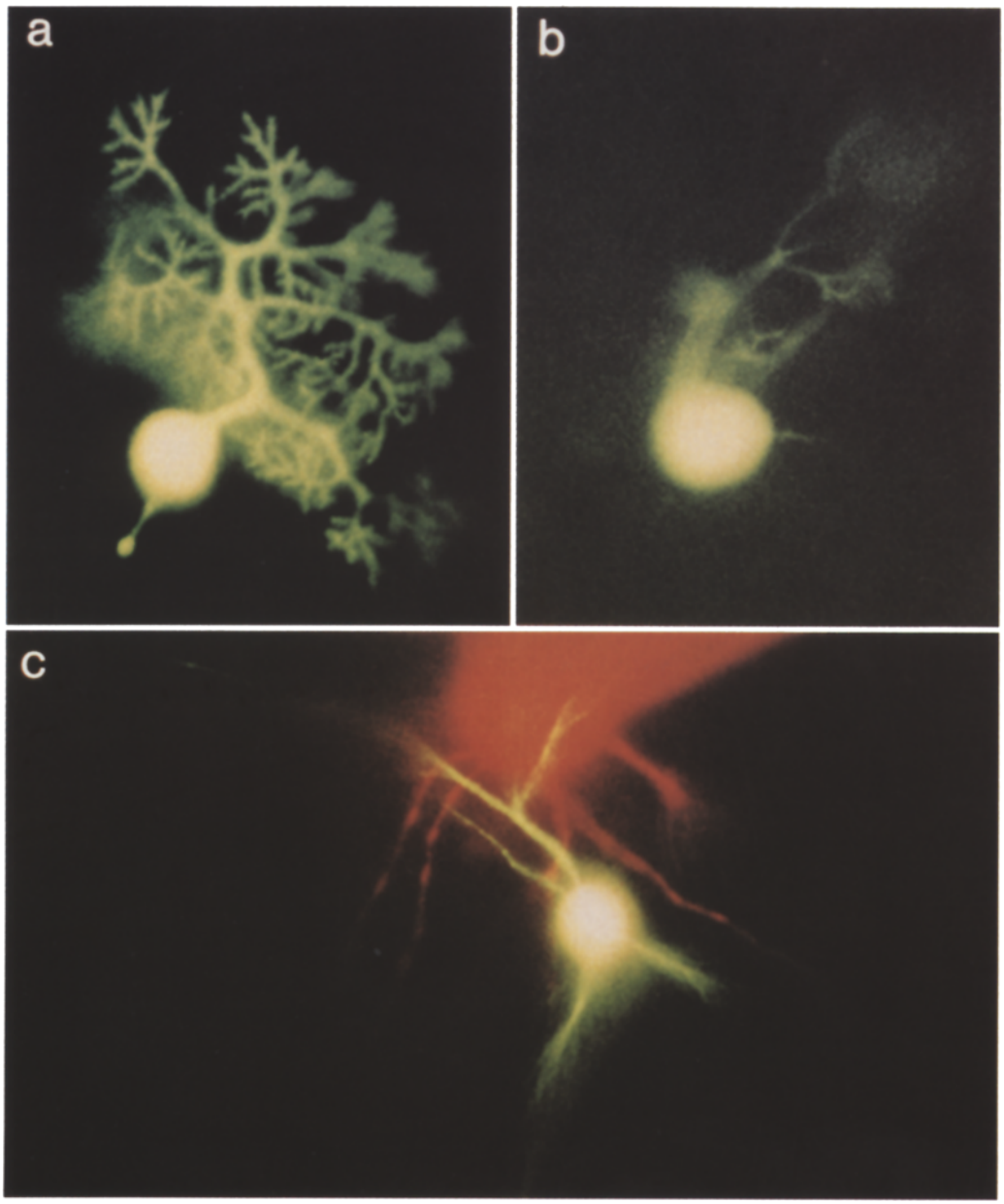

Fig. 13a–c. Fluorescent staining of living cells during electrophysiological recording. All photographs show cells in slices of 19 day old rats. **a** Fluorescence micrograph of a cerebellar Purkinje cell lying deep in the slice. The cell was filled during recording in the whole-cell mode. Pipette solution contained 2 mg/ml Lucifer Yellow added to the internal solution (in mM): KCl 140, $MgCl_2$ 2, $CaCl_2$ 2, ATP 2, EGTA 10, HEPES 10 (pH 7.3). Processes are extensively filled within 1 min after establishing the whole-cell configuration, though further details may become visible over the next 3–5 min. The cell body is approximately 15 μm in diameter. Frequent large synaptic currents were recorded from this cell during filling. **b** A different cerebellar Purkinje cell lying at the surface of the slice. Lucifer Yellow was injected as above. Note the considerable truncation of the dendritic tree. Nevertheless this cell also showed large synaptic currents. **c** Two cells in the granule cell region of the rat hippocampus. A cell lying just outside the cell layer was filled with Lucifer Yellow as above. Another cell lying within the granule cell layer was filled with Texas Red (0.1 mg/ml in the pipette solution). *The large red area at the top* is the result of the presence of the dye-filled pipette (out of focus) still attached to the soma. Separate photographs were taken of the same view at the same level of focus, using appropriate fluorescence filters and were later exactly superimposed. The focus was chosen to allow optimal resolution of processes. Crossing points may indicate synaptic connections. *Calibration*: the total width of the figure represents 300 μm. Micrographs of Texas Red filled neurones were made using a band pass filter 546 nm excitation and a long pass 590 nm for emission. For Lucifer Yellow filled neurones a band pass filter 450–490 nm was used for excitation and another band pass 515–565 nm for emission. This is a standard Zeiss filter combination

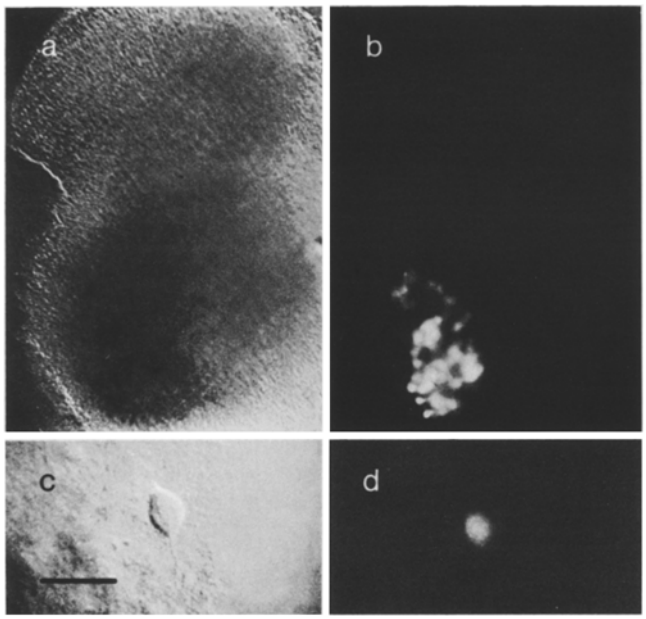

Fig. 14a–d. Identification of cell types by retrograde staining. Spinal cord slices were taken from a 4 day old rat which was injected one day previously with Evan's Blue (0.3 ml, 1 mg/ml in H_2O) in the left hind leg muscle. **a, b** The same view of a spinal cord slice under low power magnification (× 50) with Nomarski (**a**) and fluorescence (**b**) optics. Only neurones in the left ventral horn were labelled. The other half of the spinal cord showed no labelling. **c, d** A motoneurone from the above slice seen with high magnification (× 400) using Nomarski (**c**) and fluorescence (**d**) optics. Filters the same as for Texas Red (Fig. 13c). *Calibration bar* 40 μm

Conclusions

The present paper describes procedures for preparing thin slices of mammalian brain or spinal cord, in which patch clamp recordings from visually identified, synaptically connected neurones can be made. This new method eliminates various problems of other preparations used so far to study ion channels in CNS neurones, particularly channels involved in synaptic transmission. Previously, to obtain cells suitable for patch clamp recording, it was necessary either to grow neurones in culture or to dissociate them freshly using proteolytic enzymes. When using neurones grown in culture, the cell type and developmental stage is difficult or impossible to assess, as is the nature of the synaptic connections formed. On the other hand, in acutely dissociated neurones, in addition to problems of identifying cells, the proteolytic enzymes required for dissociation may drastically alter the membrane proteins studied (McCarren and Alger 1987, Allen et al. 1988) and synaptic connections are completely lost. Although using brain slices avoided these problems, only microelectrode recordings were previously possible, allowing considerably lower resolution of whole-cell currents compared to recordings using the patch clamp technique and providing no possibility for single channel recording. The use of patch clamp techniques in thin slices combines the advantages of the above techniques, allowing recordings from visually identified, synaptically connected neurones at an order of magnitude higher resolution of electrical recording than was previously possible.

The application of patch clamp techniques to thin slices opens several new possibilities in research of CNS physiology. Firstly the improvement in resolution allows study of the mechanism of synaptic transmission in the CNS. Secondly, the fact that the intracellular solution is controlled in patch clamp recordings makes it feasible to study the gating and ionic characteristics of synaptic currents, under defined ionic conditions. These characteristics can then be compared to those of currents activated by putative transmitter substances. Thirdly, by varying the composition of the pipette solution, the role of intracellular messenger systems in central synaptic transmission can now be directly studied, using both whole-cell and isolated patch configurations. Fourthly the position of the recording pipette on different parts of a neurone, such as the soma or dendrites, can be clearly distinguished and thus differences in membrane properties can be studied.

Rapid filling of neurones with fluorescent dyes and the possibility of observing them during recording further broadens the research possibilities. Firstly, local neuronal circuitry can be studied more directly than was previously possible (MacVicar and Dudek 1980, Knowles and Schwartzkroin 1981) by the combination of electrophysiology, staining of neurones with fluorescent dyes and image analysis. Pairs of neurones can be filled with fluorescent dyes during simultaneous whole-cell recording. If such a pair of neurones were synaptically connected, stimulated or spontaneous action potentials recorded in the presynaptic cell would result in synaptic responses which could be measured in the postsynaptic cell (Miles and Wong 1984). Together with recent development of confocal microscopy and its application to neurones (Åslund et al. 1987; Wallén et al. 1988) this would allow detailed investigation of the location and nature of synapses involved. Secondly, early trials suggest that the filling of neurones with calcium sensitive dyes (Fura-2) could be combined with the techniques described above. This could allow the measurement of synaptically activated Ca^{2+} signals in single neurones and their processes (Ross and Werman 1987) together with measurements of ionic conductance changes.

The techniques presented here are also generally applicable to CNS neurones over a wide range of postnatal ages, in all areas of the brain so far attempted and in different species. Thus these methods provide new possibilities for ontogenic and comparative studies of particular types of neurones, in terms not only of synaptic function and connectivity but also of the characteristics of voltage gated ion channels.

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