Tissue culture of post natal mammalian neurons

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Morphological and electrical characteristics of postnatal hippocampal neurons in culture: the presence of bicuculline- and strychnine-sensitive IPSPs

K. Fatima-Shad, P. H. Barry

Abstract. A modified method was developed for tissue-culturing postnatal hippocampal neurons using simple mechanical trituration for cell isolation and not including any hydrolysing enzymes, nerve growth factors or antiproliferating agents. The morphological properties of such neurons were characterized with light and interference polarizing microscopy, which revealed the appearance of growth cones from peripheral neurons and the presence of different types of neurons, including bipolar, stellate and pyramidal-like cells (i.e., pyramidal and dentate gyrus granule cells), which could be related to their putative counterparts in intact brain. The whole-cell configuration of the patch-clamp method was used for electrophysiological recordings of inhibitory synapses between these dissociated cultured neurons from the early postnatal rat hippocampus. This study indicated the presence of tetrodotoxin (TTX)-sensitive and TTX-resistant inhibitory postsynaptic potential (IPSPs) and inhibitory postsynaptic currents (IPSCs) in current-clamp and voltage clamp modes respectively. The coincident reversal potentials for IPSCs and for $GABA_A$ and glycine-evoked currents, and the sensitivity of the IPSCs to bicuculline or strychnine, indicated that these IPSCs were Cl^--dependent and mediated by either $GABA_A$ or glycine receptors. Inhibitory postsynaptic currents recorded under voltage-clamp conditions decayed with a time course that could be fitted by a single exponential with a value of 26 ms. An average quantal content of 2.5 was responsible for a typical GABA and glycine-activated IPSC and a single quantum for GABAergic input was inferred to activate about 160, and for glycinergic, about 200 Cl^-, channels.

Keywords: Patch-clamp, tissue-culture, IPSP, GABA, glycine

Introduction

A high degree of neuronal differentiation, including extensive synapse formation, can be expressed in tissue cultures of the central nervous system (see e.g., Nelson 1975). The visual resolution and experimental accessibility of dissociated cells in cultures of brain and spinal cord make such cultures attractive material for the study of central synaptic mechanisms (Fischbach and Dichter, 1974; Godfrey et al., 1975). The technical advantages of cell cultures are particularly striking in contrast to the complexity and inaccessibility of the intact nervous system, but the information gained from these simplified central nervous system preparations must be interpreted with due regard to their limitations.

The early development of the nervous system is a continuation of cellular events initiated during gastrulation and involves a gradual restriction in the developmental potential of individual cells. In all developing nervous systems, cell

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differentiation depends on a series of signals that ultimately control the transcription of specific genes. When cells undergo cell-autonomous differentiation, these signals are initiated by inheritance of asymmetrically distributed cytoplasmic determinants and are perpetuated by an internal cascade of interactions between nuclear factors that regulate transcription. With cells whose fate is more plastic, the critical signals are derived from the environment and indirectly control the expression or activity of nuclear transcription factors. In short, the age of the dispersed cell and the incubational environment and the media are very critical for the induction of a healthy neuronal culture. One-day-old rat pups were used to tissue-culture hippocampal neurons, since the pyramidal layer from Ammon's horn grows rapidly between days E22 to P1 (Bayer, 1980), and the correct incubational environment and the media were a matter of 'trial and error'. Cell-to-cell interactions play a critical role in all stages of vertebrate neural development, from neural induction to the choice of neurotransmitter (e.g., see Landis, 1980). Once the identity of individual cells within the nervous system is established, axonal extension begins and complex but precise connections between these cells begin to form.

The first indication that GABA could be an important inhibitory transmitter in the hippocampus came from the work of Biscoe and Straughan (1966), who observed GABA-induced depression of spontaneous and glutamate-evoked firing of hippocampal cells in vivo. Glycine was first proposed to act as a neurotransmitter in mammalian spinal cord (Werman et al., 1968) and recently a number of laboratories, including our own, have found that glycine receptors are also present in the hippocampus (Fatima-Shad and Barry, 1992, in postnatal tissue-cultured neurons; Ito and Cherubini, 1991, in neonatal slices; Shirasaki et al., 1991, in dissociated cell preparations). Hence, inhibitory postsynaptic potentials (IPSPs) observed in hippocampal neurons in slices or in culture during early postnatal days could be due to the action of GABA or glycine receptor-activated channels. Both GABA and glycine have been shown to be the major inhibitory neurotransmitters in mammalian central neurons, and in the case of $GABA_A$ and glycine receptors, they produce an increase in chloride permeability of the neuronal membrane (Barker and Ransom, 1978; Bormann et al., 1987; Fatima-Shad and Barry, 1992, 1993).

Inhibitory postsynaptic currents (IPSCs) have been observed previously in many different in vivo and in vitro neuronal preparations using conventional voltage clamp techniques, and were first described by Alger and Nicoll (1979). In vitro spontaneous depolarizing potentials were observed in rat and mouse CA1 pyramidal and granule cells when the electrodes contained Cl^- solutions (Alger and Nicoll, 1980; Collingridge et al., 1984; Biscoe and Duchen, 1985), and hyperpolarizing potentials were observed in CA3 pyramidal cells of guinea pig during intracellular recordings, when the microelectrode was filled with K-acetate (Miles and Wong, 1984). These potentials do not appear to be the result of random quantum release from a single terminal, since they occur in clusters, with a non-random interval distribution (Alger and Nicoll, 1980).

Most of the spontaneous IPSPs of hippocampal cells may be generated by action potential activity in interneurons, since 0.5–1 μM tetrodotoxin (TTX) suppresses spontaneous IPSPs (Alger and Nicoll, 1980) and 0.2 mM Cd^{2+} greatly reduces their amplitude and frequency (Collingridge et al., 1984).

In this study we used the whole-cell recording configuration of the patch-clamp technique to investigate spontaneous IPSCs in postnatal tissue-cultured mammalian hippocampal neurons. The purpose of the study was twofold. Firstly, it was to demonstrate that the cells (and their neuronal connections) in our preparation were not significantly abnormal as a result of the traumas suffered during tissue culturing. Secondly, it was to provide preliminary baseline data for further characterization of inhibitory neuronal transmission.

Methods

Tissue-culturing hippocampal neurons

The ideal tissue culture environment chosen was mainly based on the selection of certain appropriate conditions such as substrate, carbon dioxide phase, temperature, pH, osmolality, growth-promoting factors essential for neuronal cell lines and cell attachment factor.

The optimal basic substrate that we found for our cells was Essential Modified Earl's Medium (ICN/Flow Labs) with sodium bicarbonate as a buffering agent, which required a carbon dioxide-rich atmosphere for optimal buffering, as the pK_a of sodium bicarbonate is 6.3 at 37°C. This medium had to be supplemented with 10% fetal calf serum (CSL), 1% Fungizone (ICN/Flow Labs), 2% penicillin and streptomycin solution (CSL) and 1% L-glutamine (ICN/Flow Labs) and from now on is referred to as 'EMEMPlus'. Because of the type of vessels we used (i.e., petri plates), we required a controlled atmosphere with a high humidity and elevated CO_2 tension (5%), which was readily obtained by incubating the plates in a Heraeus incubator, which has excellent regulation of carbon dioxide and humidity. Generally, the temperature was kept at 36.5°C within a range of ±0.5 degrees, although it seemed that consistency was more important than accuracy.

Although most cell types will grow within the pH range 6.5–7.5, the medium for our cells needed to be kept within the range 7.0–7.4 otherwise growth soon ceased and the culture started to die. Although cell culture media do have some inherent buffering capacity due to the presence of phosphates and amino-acids for our long-term cultures, the additional buffering of either sodium bicarbonate or HEPES was required. We preferred to use sodium bicarbonate-buffered media mainly because of its nutritional benefit and lack of toxicity. Osmolality is especially important.

The osmolality of the cell-dissociating solution 'Puck's saline' after adding 0.6% glucose, 1.5% sucrose and 10 mM of HEPES (pH 7.4) was adjusted to an osmolality of 330 mOsm and checked with an osmometer. Although growth-promoting factors were not usually used in the culture media, it became clear later on that the cells grew faster if replated on to plates that were a couple of weeks old and that contained only non-neuronal or glial cells. It seems likely that these glial or non-neuronal cells may have some growth-promoting factor in them (as reported earlier by Barde et al., 1978; Lindsay, 1979) which is immunologically distinct from nerve growth factor (NGF). In order to mimic the normal matrix of glycoproteins and proteoglycans, we used synthetic macromolecules like Poly-L-lysine (Sigma). Poly-L-lysine was used as a cell-attachment factor, and it seemed to work well in promoting neurite extension in postnatal hippocampal cultures. However, it was not nearly as successful as replating our cells on 2-week-old non-neuronal plates.

The correct quantities of the appropriate solutions were always aliquoted into small sampling containers sometime before tissue culturing and then kept in the fridge or freezer, as appropriate. On the day of tissue-culture, the UV light in the laminar flow hood was turned on for 30 min beforehand. After sterilization of the laminar flow hood, the EMEMPlus was prepared under the sterile hood. The EMEMPlus was then incubated at 37°C in an atmosphere of humidified air with 5% CO_2. Five 35-mm plastic plates were coated with the special attachment factor, poly-L-lysine from Sigma at a concentration of 3.5 $\mu g/cm^2$. After 5 min, the plates were rinsed twice by double-osmosis autoclaved water and were then left in the laminar flow hood for air drying, for about 1–2 h. After the plates were dried, they were covered with their lids and were left inside the laminar flow hood away from the dissecting area. Sterile packs of autoclave tissue papers, filter papers, and tooth picks were then opened inside the laminar flow hood. The dissecting stage was then arranged on a wooden block, covered with tissue paper and a filter paper on top of it, with autoclave surgical instruments on one side.

Five 1-day-old rat pups were quickly decapitated one at a time (each time placing a new filter paper on the dissecting stage). The hippocampi were removed using tooth picks and placed in ice-cold Puck's saline (a special dissecting medium, which had been aliquoted beforehand in a 3 ml measure and kept in a refrigerator, since the sodium bicarbonate of the solution starts to lose its buffering properties below 8°C). The hippocampal cells were mechanically dissociated by gentle trituration through a narrow-bored fire-polished Pasteur pipette with 30–40 passages in 3 ml cold Puck's saline (osmolality 330 and pH 7.4). It was very important for the Puck's saline to be very cold at the beginning of the dissection and also for the hippocampi to be removed very quickly in order to retain viability of the maximal number of cells, checked by their exclusion of Trypan Blue (discussed later). Usually 4×10^5 viable cells were distributed into poly-L-lysinated air-dried 35-mm plastic plates containing freshly prepared and controlled (for pH and temperature) 2 ml modified EMEMplus. The plates were then incubated at 37°C in an atmosphere of humidified air with 5% CO_2. The day after tissue-culture preparation, the medium in the plates was exchanged for fresh EMEMPlus and its volume reduced to approximately 1 ml per dish. It was thought that reducing the volume of the culture medium might increase the concentration of commonly accepted autocrine factors released into it. It was found to be beneficial to carefully pipette out the superficial layer of cell medium contaminated with blood cells on the day after culture induction and then after every 3–4 days. Cells were used for standard patch-clamp experiments from 2–3 days of preparation onwards.

Cell numbers were determined by using a haemocytometer. Unequal cell distribution in the sample and the improper filling of chambers were the main sources of error for haemocytometer counts. The cell suspension was always mixed thoroughly before sampling and the cells were permitted to settle down before a sample was taken. In order to fill the haemocytometer chamber properly by capillary action, the cover slip, chamber and the pipette used to fill the chamber were very thoroughly cleaned (first with distilled water, then with absolute alcohol), and then wiped dry.

There were nine 1-mm^2 squares in the Neubauer chamber. With a $\times$ 10 eyepiece, one square (1 mm^2) would approximately fill the microscope field and the cells that touched the middle line (of the triple lines) to the left and top of the square were counted, but those located to the right and bottom were not.

Cell viability was determined using Trypan Blue (only taken up by non-viable cells); 3 drops of 0.3% Trypan Blue were added to a test tube with 0.5 ml of a well-mixed suspension of hippocampal cells. After mixing, and being allowed to stand for a few minutes, a sample was then collected from this mixture and placed onto a haemocytometer. The percentage of viable cells was then determined as the percentage of unstained cells within the sample.

Hippocampal cells were viewed, before and after incubation, at $\times$400 with an inverted tissue-culture microscope (model IMT; Olympus, Tokyo, Japan) using phase-contrast optics. Although it was difficult to differentiate between cell types in the medium prior to incubation, neuronal cells could still be distinguished from non-neuronal cells by their shiny appearance. However, on the day following plating, almost all the plates and especially the replated plates showed the growth of the three major morphological classes of neurons (i.e., pyramidal-like, bipolar and stellate). The pyramidal-like neurons comprised the majority of neurons in our cultures, and had a triangular-shaped soma, a single dominant dendrite-like process emerging from the apex of the soma and several basal dendrite-like processes from the other apices of the cell. Besides these abovementioned neurons, smaller rounded neurons without any visible apical pole were also seen. The latter cells, which could be granular

neurons, were only seen infrequently. Careful examination at higher magnification revealed the presence of many spines along the whole dendritic tree, including the primary dendrites. Pyramidal-like neurons of smaller size than the main group, with several thin branches arising from their primary dendrites, were sometimes visualized. These pyramidal-shaped neurons could be pyramidal basket cells. The majority of the neurons, which could be recognized, were polymorphic and either spindle-like bipolar or stellate. It has been suggested that GABAergic cells represented 11% of the total population of hippocampal neurons (Woodson et al., 1989). Their morphology was quite heterogeneous and included most types of short axon neurons, including basket, stellate and horizontal cells (Ribak et al., 1978; Woodson et al., 1989).

Most of the neurons in culture could survive for more than 2 weeks in the incubator and were still viable enough at the end of this time to fire off action potentials. We have used the patch-clamp technique (Hamill et al., 1981) to study these cells and have investigated the properties of pyramidal neurons (typical size: 10×7 μm) along with other non-principal cells of similar size.

Electrophysiology

The pipette resistance during the formation of a gigaohm seal was monitored by repetitively applying a small (5 mV) voltage pulse to the pipette. Fire-polished glass pipettes with a resistance in the order of 2–6 MΩ and a pipette tip diameter of approximately 1 μm were generally used. Electrical pulses applied to the pipette could be observed clearly during the process of seal formation.

Cells from freshly plated, as well as from replated dishes, could be used from the second day of preparation for standard patch-clamp experiments. Initially a cell-attached patch with a gigaohm seal was formed. From this configuration, either an inside-out excised patch configuration was obtained by carefully withdrawing the pipette from the cell membrane or alternatively, a whole-cell configuration was achieved by rupturing the patch using a pulse of suction or voltage or both. We used the whole-cell configuration for studying bicuculline- and strychnine-sensitive inhibitory postsynaptic currents in these cultured hippocampal neurons.

The solutions used were mammalian Ringer (in mmol/l): NaCl 132, KCl 10, $CaCl_2$ 0.5, $MgCl_2$ 1, Na-HEPES 5 (pH 7.4) without any agonist in the bath, and a normal pipette solution containing (in mmol/l) KCl 140, $MgCl_2$ 1.5, $CaCl_2$ 1, EGTA 11, HEPES 11, (pH 7.3). Inhibitory postsynaptic currents (IPSCs) were measured in symmetrical Cl^- concentrations with K^+ being replaced by Cs^+ in both bath and pipette solutions. For the observation of miniature inhibitory post synaptic currents (mIPSCs), 1 μM TTX was added to the bath and, for identifying records as GABA- or glycine-mediated synaptic potentials, 10 μM bicuculline or 10 nM strychnine were added to the bath respectively. Records were digitized at 10 kHz and filtered (low-pass) at 2 kHz (–3 dB).

Current and voltage traces were continuously displayed on an oscilloscope and events of interest were captured, stored and digitized. The decay of IPSCs could be well fitted by an exponential function with a single time constant. The time constant of decay (τ) of the currents was determined by finding a value of τ that gave the best (least-squares) fit of measured currents to:

$$I(t) = I(0) \exp(-t/\tau)$$

where I(0) represents the peak current. The line through the decay of currents, as shown later in Fig. 7B shows, the best (least-squares) fits of this equation. The selected τ was used with I(0) to generate an exponential curve through the average IPSCs (see Fig. 7A, later). All data were analysed using PNSCROLL software (Barry and Quartararo, 1990), and least-squares regressions and curve fitting were done using *Sigma Plot* (Jandel Scientific, Corte Madera, California, USA). All voltages have been corrected for junction potentials by using the program *JPCalc* (Barry, 1994).

Histology

Cultures were photographed in the living state with a polarizing interference (Jamin-Lebedeff) microscope. The colour in the photographs depends on the filters used and is dependent on both the differing refractive indices in the cytoplasm of the living cell and the extracellular medium, and the (arbitrary) setting of a beam splitter in the condenser lens, the particular setting being selected to obtain maximum contrast and definition. The cells were also photographed with an SC 35 Olympus camera under an Olympus inverted tissue-culture light microscope during different stages of development.

Results

Morphology

The morphological differences in the postnatal tissue-culture hippocampal cells of 1-day-old specific pathogen-free (SPF) rat pups, from which the cell cultures were prepared, is shown by an inverted microscopic comparison of the culture on the day of preparation (Fig. 1A) and after 2 days of incubation (Fig. 1B). Figure 1A shows the appearance of cells about 2 h after dissociation, before they have begun to form processes. At this stage, about 80% of the cells are small and spherical, with a mean diameter of about 8.5 μm. The morphology of hippocampal cells after 2 days in culture is shown using a low-power photomicrograph of living cells in Fig. 1B. The isolated cells, which were attached directly to the substrate, have extended one or two unbranched neurites. Several cells, which have not developed processes, were also present. Note the appearance of the major and minor processes (Fig. 1B) and the prominent growth cone at the tip of the major processes and the swellings along its surface, which presumably could be synaptic boutons (Fischbach and Dichter, 1974).

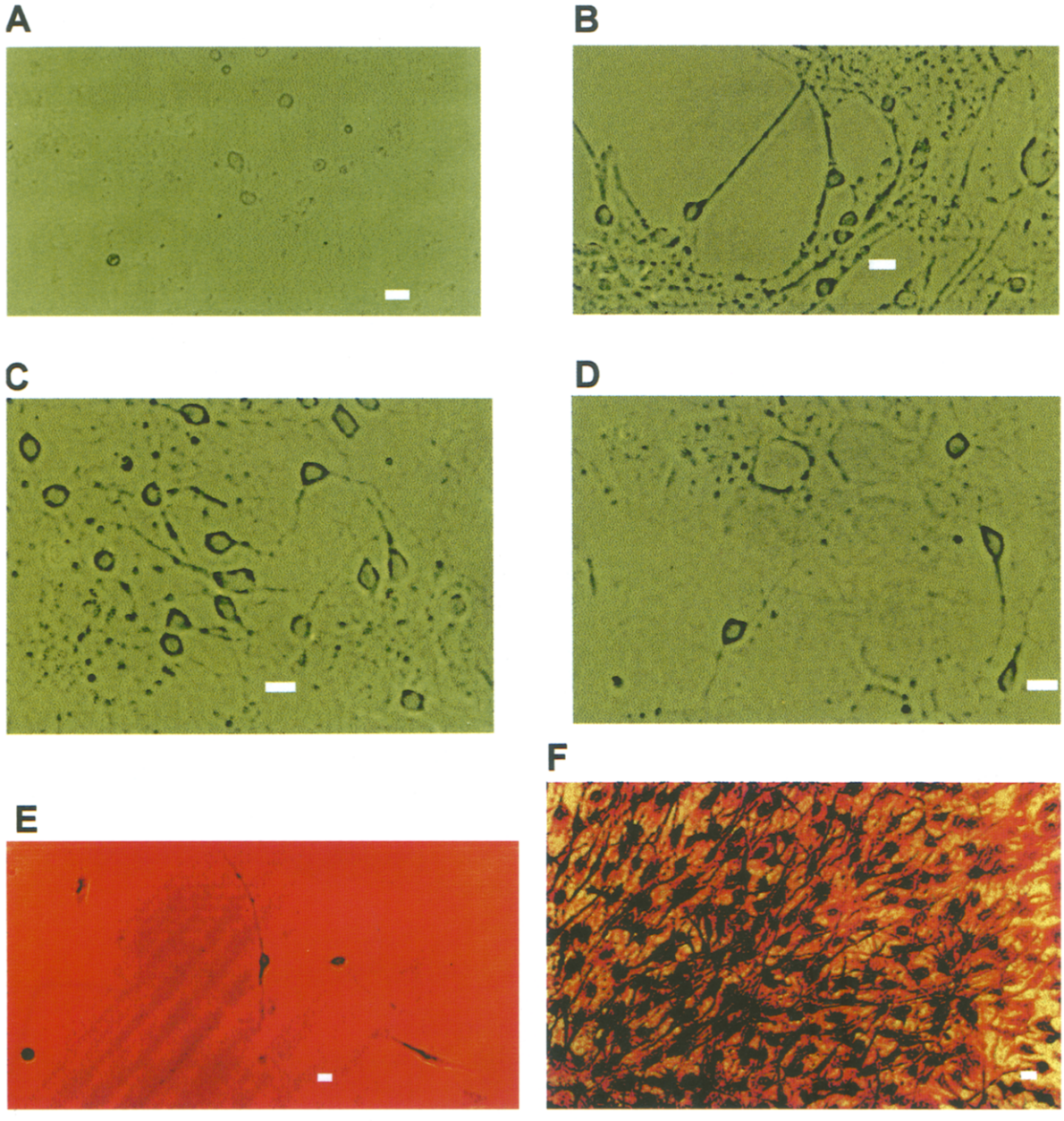


Fig. 1 Hippocampal cells shortly after mechanical dissociation by trituration viewed with light microscopy. (A) The cells were allowed to attach to a poly-L-lysine-treated petri dish and were photographed at × 400, high power, 2 h after dissociation. The cells present were usually small and round in shape. (B) The same hippocampal cells after 2 days in culture, photographed at × 10, low power. Note the swellings on the upper surface of the long processes, which could be synaptic boutons. At this stage, some cells have extended one or two major or minor processes and some cells are still without any neurites. (C) and (D) Cells in culture 3 days after isolation, grown in different conditions; (C) neurons plated on top of 2-week-old cultures; (D) neurons plated on poly-L-lysine-coated petri plates. (E) The surviving neuronal cells with major and minor processes, synaptic boutons and the growth cones, after 1 week in culture. (F) An example of a 2-week-old culture, containing only glial cells, which were used as the substrate for freshly dissociated cells in (C). Scale bar = 10 μm, for all panels.

Most of the cultures developed in our laboratory survived for more than a week or two. The nerve cells continued to extend processes within the first 2 or 3 days in culture. Two cultures were examined in different conditions: cells were plated on a dish that contained non-neuronal cells (Fig. 1C) and cells were plated on a poly-L-lysine-coated petri plate (Fig. 1D). The number of cells with neurites remained fairly constant during the first 2 or 3 days in culture, but an obvious decrease in the number of cells which lacked processes was observed in cells plated on a poly-L-lysine-coated petri plate. Beyond the 3rd day, virtually all of the surviving cells had neurites (Fig. 1E) plated on non-neuronal cells. A similar selective loss of neurons that fail to form processes has been observed in cultures of rat prenatal hippocampal neurons (Banker and Cowan, 1977).

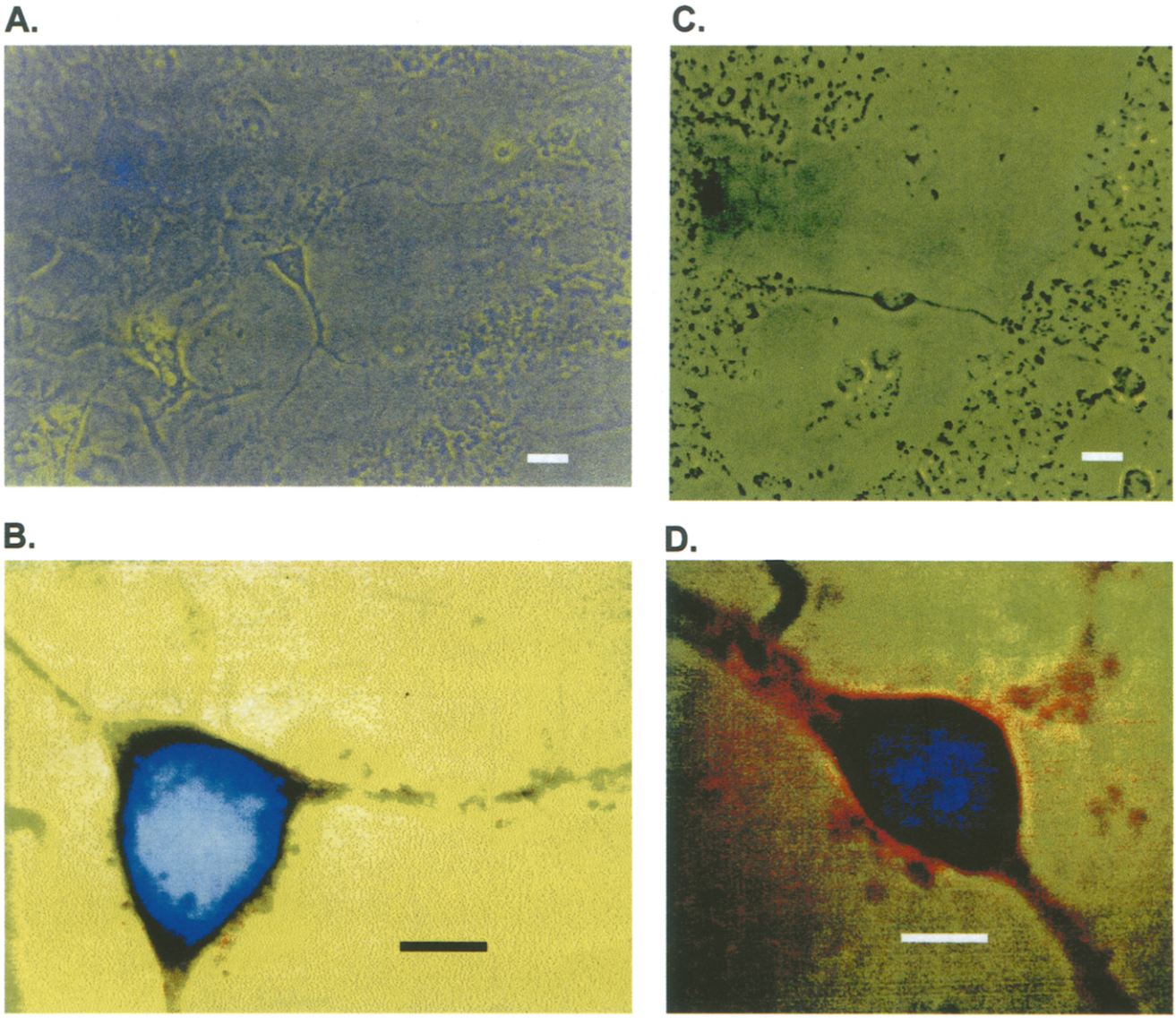


Fig. 2 (A) Light microscopic and (B) polarizing optical photomicrographs of single hippocampal neurons in culture. In (A) note the pyramidal-shaped cell body, the stout apical process which bifurcates near the cell body and the slender basal process. (B) shows another example of a pyramidal neuron with stout apical and slender basal processes; note the appearance of microtubular and golgi complex rudiments in the basal process, indicating that this process may be destined to become the axon (Dotti and Banker, 1991). (C) Light microscopic and (D) polarizing interference photomicrographs of a bipolar cell, another commonly present cell type in this hippocampal neuronal culture. Scale bar = 10 μm, for all panels.

The survival rate was poor in the dish where the cells were plated using the attachment factor poly-L-lysine (Fig. 1D) in comparison to the dish where the cells were replated (Fig. 1C) on a 2-week-old plate in which there were only non-neuronal cells. An example of a non-neuronal cell plate is displayed in Figure 1E, in which a large number of flattened epithelial-like or fibroblast-like cells, together with the occasional presence of a well differentiated astrocyte, are left in the culture plate after more than 2 weeks of incubation. Some neurons in these long-term cultures bear a striking resemblance to in vivo hippocampal pyramidal cells (Fig. 2A). Note the pyramidal-shaped cell body, the single stout apical dendritic process, which bifurcates a few micrometres away from the soma, and a slender basal process. This is the characteristic rudimentary form of pyramidal cells from field CA3 of the hippocampus (Banker and Cowan, 1977). A polarizing interference optical photomicrograph of the pyramidal neuron is exhibited in Figure 2B. Neurons which had two processes of approximately equal length were classified as bipolar cells. Light microscopic and interference photomicrographs displaying bipolar cells are shown in Figures 2C and 2D. An interference photomicrograph of the neuron with the prominent growth cone at the tip of the major processes is shown in Figure 3A. Note the elongation of its growth cone and its final contact with part of another cell with which it will possibly form a synapse. The light microscopic photomicrograph of the cell with a large

process and cone with finger-like extensions (filopodia) are shown in Figure 3B. Another cell type which was also normally present in the culture, mainly inhibitory in nature and functioning as an interneuron, is the basket cell, which is present in Figure 3E (top right) along with other cells. Mattson and Kater (1989) found in hippocampal slices that neurons of each morphological class exhibited intense GABA-like immunoreactivity.

Synapses

Synapses onto a single central neuron are grouped according to function and all three regions of the nerve cell: axon, cell body, and dendrites can be receptive sites for synaptic contact. The most common types of contact therefore are axo-axonic (Fig. 3C), axosomatic (Fig. 3D) and axodendritic (Fig. 3D) where, by convention, the presynaptic element is identified first. Axosomatic synapses are often inhibitory, the synaptic current generated at an axosomatic site having a stronger signal and therefore a greater influence on the outcome at the trigger zone than current from the more remote axodendritic contacts. Axodendritic synapses can occur at the shaft or spine of the dendrite. Different types of synapses can be observed between different types of cells, with axodendral and dendrodendral synapses between pyramidal and basket cells being common in the hippocampal culture grown in our laboratory (Fig. 3E).

Membrane properties of the cultured hippocampal neurones

The electrical properties of the membranes of hippocampal neurons were investigated using the whole-cell recording technique with mammalian Ringer in the bath and a pipette solution with K^+ as the main cation. In current-clamp mode, in the absence of GABA and glycine, the resting potentials of the cultured neurons ranged between –55 to –75 mV, the mean value being –65 ± 10 mV (mean ± SEM; $n = 28$) and the average input resistance for the cells being 2.1 GΩ.

Action potentials

Often oscillatory irregular and rapid current fluctuations were initially observed in the first few minutes when the voltage was held at the resting potential in the whole-cell configuration (Fig. 4A). The larger potential spikes recorded in current-clamp mode with a typical response usually greater than 80 mV (Fig. 4B) must have been due to action potentials, but some of the smaller ones could have been IPSPs given that E_{Cl} was close to zero.

Voltage-activated currents

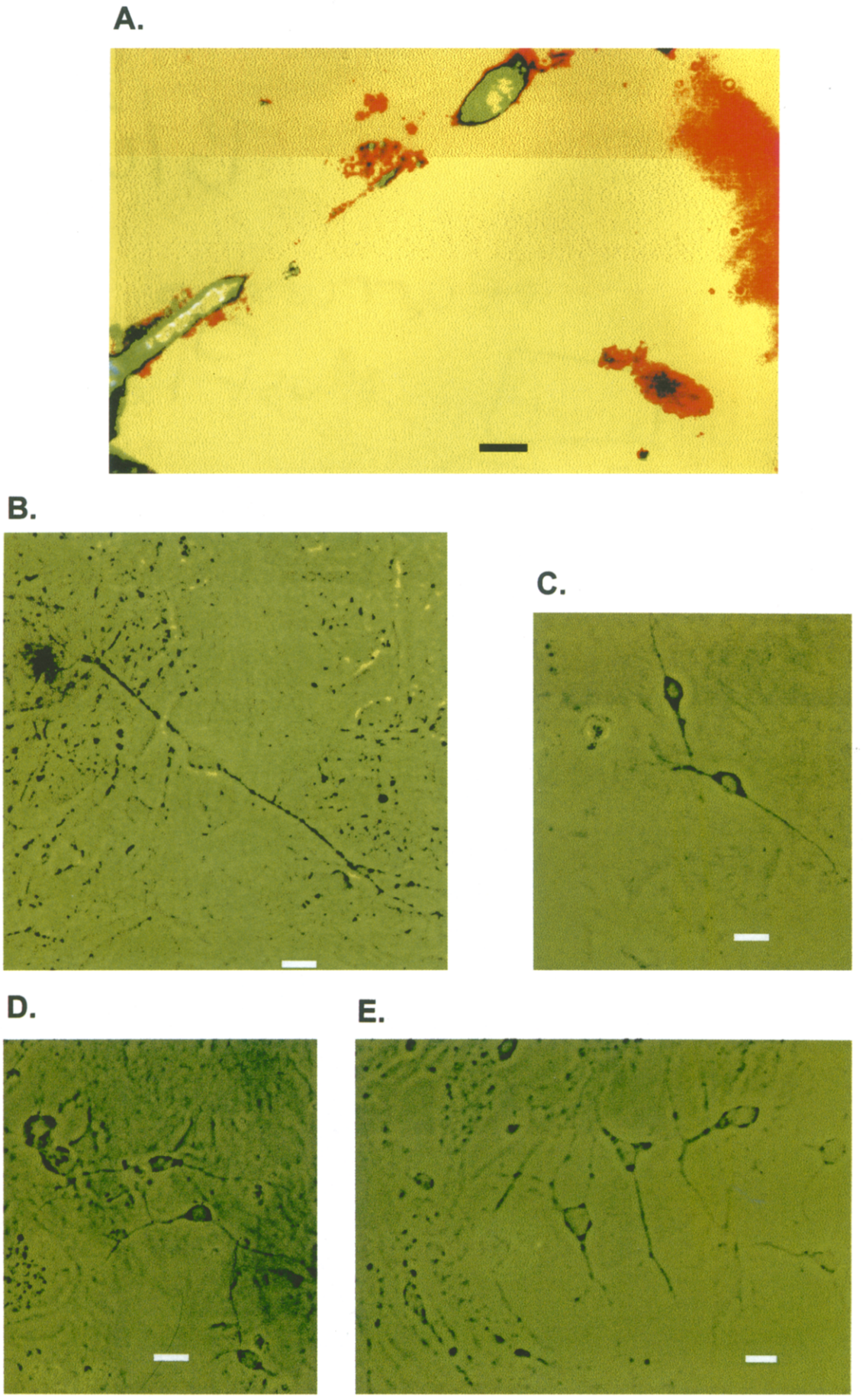
Hippocampal neuronal membranes, like other neuronal membranes, are highly selective to K^+. As mentioned earlier, the average resting membrane potential of these cells was –65 ± 10 mV, close to the K^+ equilibrium potential (E_K), and in normal mammalian Ringer the whole cell current response of these cells reversed close to E_K (= –66 mV; Fig. 4C, circles). Addition of 20 mM TEA to the bath significantly blocked the response leaving a much smaller residual component (Fig. 4C, triangles). Examples of two whole-cell current traces are shown in the boxed inset of Fig. 4C in the absence and presence of 20 mM TEA at 0 mV membrane potential.

Synaptic currents and potentials

Spontaneous IPSCs were observed in cells with values of resting membrane potential more negative than –60 mV and action potential amplitudes greater than 80 mV. They were recorded for a range of membrane potentials and reversed at positive potentials, the actual reversal potential being dependent on the concentration of Cl^- in the pipette. With symmetrical Cl^- concentrations inside (145 mM) and outside (145 mM) the cell membrane and low Ca^{2+} (0.5 mM) in the bath (to block Ca-dependent action potentials), 'IPSCs' of 0.3–1.5 nA were observed (Fig. 5A), before the bath application of 1 μM TTX. It should be pointed out that the magnitudes of these 'IPSCs' were increased because E_{Cl} was close to zero, and more positive than the resting potential and that in this artificial situation these 'IPSCs' would have actually resulted in a large depolarization. These currents must have been generated in response to action potentials in presynaptic cells and were found to vary significantly in amplitude and duration, and often appeared as bursts with IPSCs superimposed on the decay of a preceding event. The long duration of the IPSCs in Fig. 5A suggests that they are not inward action potential currents. However, within 1–2 min after the bath application of 1 μM TTX, the large spike responses disappeared, to be replaced by what appeared to be miniature IPSCs (generally, 0.25–0.5 nA), which were observed at a lower frequency in the same cell (Fig. 5B). They were more regularly spaced with less variability in amplitude, consistent with their being miniature IPSCs, which would have suggested that each one resulted from the spontaneous release of a single quantum of neurotransmitter.

The voltage responses of spontaneous postsynaptic potentials in postnatal tissue-culture hippocampal neurons were observed in current-clamp mode (Fig. 5C). These must

Fig. 3 (A) Photomicrograph of a pyramidal cell in culture using polarizing interference optics, showing a prominent growth cone at the tip of the major process as an enlargement of the shaft of the axon. (B) Several finger-like extensions (filopodia) of the growth cone are shown in the light microscopic photomicrograph of another cell in a 1-week-old hippocampal culture. (C) and (D) Light microscopic photomicrographs of hippocampal neurons: (C) Two bipolar cells making synaptic contacts between their axons; note the presence of irregularly extended filopodia from the rudiments of the growth cone at the synaptic site; (D) Three cells are making synaptic contacts between each other, presumably between the axon and dendrites of each other. (E) Neuronal cell types in hippocampal cultures forming different types of connections. Pyramidal, stellate and basket cells are apparent here, whereas other cells are still in their initial morphological stages without any defined axons. Scale bar = 10 μm, for all panels.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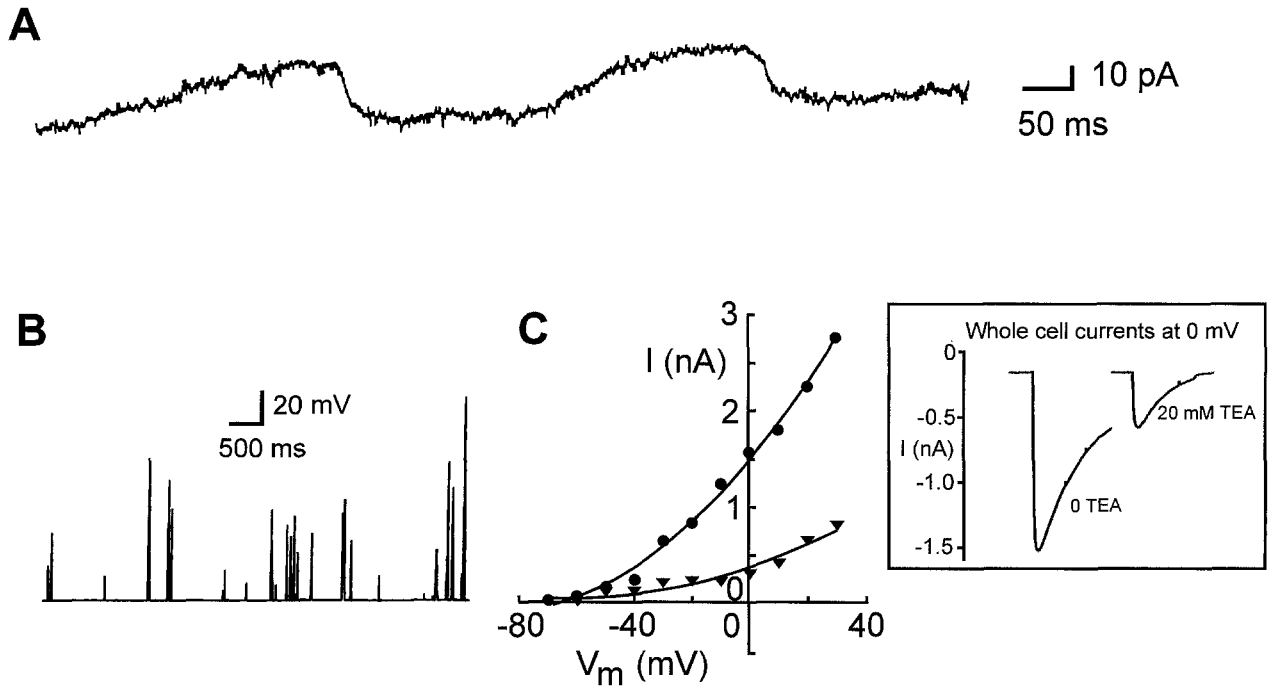

Fig. 4 The passive electrical properties of postnatal tissue-cultured hippocampal neurons measured under patch-clamp conditions were generally found to be very similar to those of adult neurons in vitro. The cells were bathed in mammalian Ringer (in mmol/l): NaCl 132, KCl 10, $CaCl_2$ 0.5, $MgCl_2$ 1, Na-HEPES 5 (pH 7.4) without any agonist. The pipette solution contained KCl 140, $MgCl_2$ 1.5, $CaCl_2$ 1, EGTA 11, HEPES 11, (pH 7.3) and the average membrane potential was –65 ± 10 mV. The records were digitized at 10 kHz and were filtered (low-pass) at 2 kHz (–3 dB). In all the figures, positive potentials are represented by upward deflections of the trace. (A) Spontaneous whole-cell current fluctuations were often seen shortly after the cells were voltage-clamped. Such oscillations normally lasted for a few minutes at the membrane potential (–70 mV). (B) Along with the membrane oscillations spontaneous potential spikes were also seen in the current-clamp mode during the early period after the establishment of the whole-cell configuration for the same cell as in (A). The larger ones must clearly be action potential spikes, but some of the smaller ones could have been IPSPs given that E_{Cl} was close to zero. Because of the large magnitude of the spikes and the low A/D gain necessary to record these spikes, the noise in the baseline apparent on the oscilloscope trace has not been resolved in this record. (C) The current–voltage relationship in the whole-cell configuration in normal mammalian Ringer (circles) and after adding 20 mM TEA to the bath (triangles), in response to depolarizing pulses from a holding potential of –80 mV, with the SEM being less than the symbol size for all the points. The boxed inset shows typical traces in the absence and presence of 20 mM TEA. Since the reversal potential of the currents was close to E_K (≈ –66 mV), this indicated that the underlying channels were selective to K^+ in this preparation, as is the case for other neuronal membranes.

have been due to Na-dependent presynaptic action potentials, since they could be blocked by the addition of 1 μM TTX, which then left only small (miniature) spontaneous potentials (Fig. 5D). In many cases, the addition of the $GABA_A$ receptor antagonist, bicuculline, reversibly abolished these potentials (Fig. 5E) identifying them as GABA-mediated synaptic potentials. But occasionally bicuculline was unable to block the mIPSPs, and instead strychnine in nanomolar concentrations abolished the miniature potentials (resultant baseline noise records not shown). In either case, after washing in normal physiological saline, the effects of TTX and bicuculline or strychnine were largely reversed (Fig. 5F). Evidence in favour of a neurotransmitter role of strychnine-sensitive glycine in the rat cerebral cortex has also been presented in intracellular recording studies by Levi et al. (1982).

Whole-cell current recordings in cultured hippocampal neurons revealed spontaneously occurring currents, the amplitudes of which increased at first to a relatively stable level and then showed little or no further increase (Fig. 6A). Measurements of reversal potentials for both IPSCs (Fig. 6B) and mIPSCs (Fig. 6C), which were both close to zero, suggested that the activated channels were Cl^--selective. The channel reversal potential for IPSCs in symmetrical chloride concentrations was +3.5 mV and the chord conductance at –100 mV was 10.8 nS. The current–voltage relationship for mean amplitude of mIPSCs, recorded 1–2 min after the bath application of 1 μM TTX, gave a reversal potential of +3.1 mV with a chord conductance at –100 mV of 4.4 nS. The resulting ratio of the two conductances at this same potential suggested an average quantal content of about 2.5 for a typical evoked IPSC in the low Ca^{2+} bath solution (in the absence of TTX). The comparatively high IPSC conductances for these cells may compensate for their low input resistance.

The neurotransmitters were considered to be $GABA_A$ or glycine, since: (1) the currents were abolished by 10 μM bicuculline and/or 10 nM strychnine (the $GABA_A$ and glycine antagonists respectively), and (2) with equal Cl^- concentration on either side of the membrane their reversal

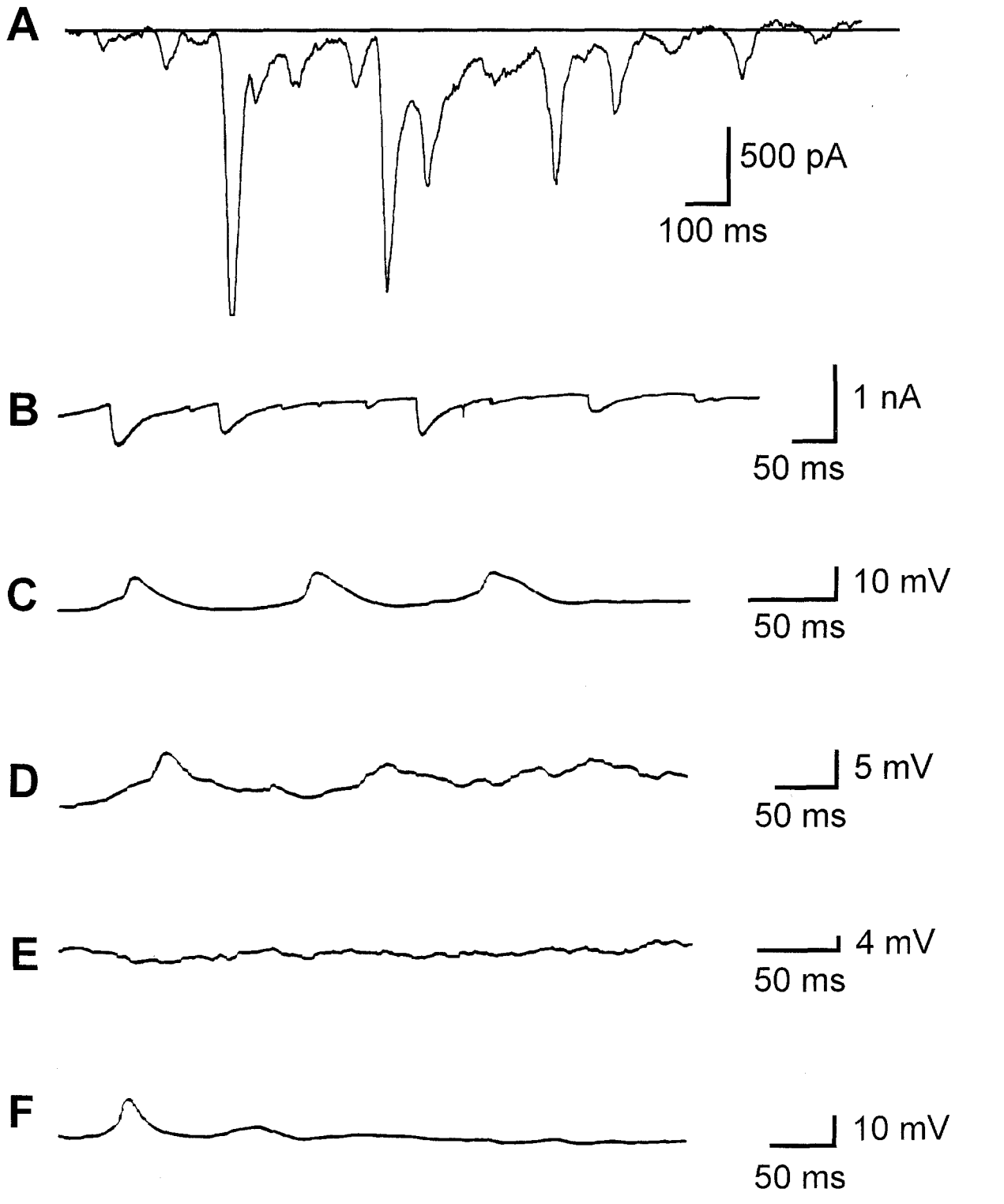

Fig. 5 (A) Inhibitory postsynaptic currents (IPSCs) in symmetrical Cl^- concentrations with K^+ replaced by Cs^+ in the pipette, were seen to occur at the resting potential of about –70 mV in hippocampal neurons in the voltage-clamp mode of the whole-cell configuration, presumably in response to presynaptic action potential activity. (B) After the addition of 1 μM tetradoxin (TTX) in the bath, within 2–3 min the amplitude of the synaptic currents decreased significantly and resultant miniature IPSCs were observed. (C) With symmetrical chloride solutions in the bath and pipette, and low Ca^{2+} to eliminate Ca-dependent action potentials, at negative potentials the openings of Cl^- channels would be expected to result in a significant number of chloride ions flowing out of the cell, thus depolarizing the cell and generating frequently occurring IPSPs, which were observed in the current-clamp mode. (D) Na-dependent presynaptic action potentials should be blocked by the addition of TTX, which resulted in the abolition of all spontaneous IPSPs, leaving only the miniature IPSPs; note the higher gain in (D). (E) Bath application of 10 μM bicuculline often abolished these potentials completely, identifying them as GABA-mediated synaptic potentials. However, occasionally bicuculline was unable to block the mIPSPs and instead strychnine in nanomolar concentration (10 nM) was needed to abolish the miniature potentials (resultant baseline noise records are not shown). (F) In either case, after washing in normal physiological saline the effects of TTX and bicuculline or strychnine were then largely reversed.

potentials were similar to the reversal potentials of directly activated GABA and glycine currents (close to zero millivolts). Assuming the most frequently observed single-channel conductance of about 27 pS for $GABA_A$ channels and 21 pS for glycine-gated channels at –70 mV (Fatima-Shad and Barry, 1991), these results suggest that a single quantum from GABAergic inputs activates about 160, and glycinergic inputs about 200, Cl^- channels, in reasonable agreement with the intracellular recording measurements of Collingridge et al. (1984), who estimated 120–200 channels per quantum for GABA in hippocampal slices.

Kinetics of inhibitory currents

At a resting membrane potential of about –70 mV, the amplitude of 400 averaged mIPSCs (i.e., TTX-resistant IPSCs) was about 490 pA (Fig. 7A) and had a time-to-peak of about 2.3 ms. Figure 7C is a histogram of the time-to-peak for the individual mIPSCs, used to form the average mIPSC in Fig. 7A, fitted by a Gaussian function (mean = 2.3 ms; SD = 0.6 ms; fitted using a least-squares technique). The decay of the mIPSCs could be well fitted by a single exponential with a time constant (Fig. 7B) of about 26 ms obtained by plotting the semilogarithmic plot of current values against the time t (see the equation in the Methods section) in milliseconds. The time course and amplitude of the miniature events should reflect the duration and number of openings of postsynaptic channels following the exocytosis of a single vesicle (Katz and Miledi, 1970).

The time-to-peak of spontaneous inhibitory currents was typically 3–5 ms but sometimes events with a longer time-to-peak were also observed. Since the rise time of a synaptic current depends upon its electrotonic distance from the recording site, this difference in time-to-peak between various IPSCs may be due to variations in the electrotonic distance between synapses at different locations along its dendritic processes and the soma, the further the synaptic release site from the soma the longer the time-to-peak and the duration of the IPSC.

Discussion

Our principal objective was to define a system for culturing hippocampal neurons from rat pups (1-day-old) which would be suitable for electrophysiological studies of inhibitory neurotransmitters. The method that finally evolved differed in several respects from those commonly employed in culturing dissociated neurons from the CNS. The most critical features were the small pH range (between 7.3 and 7.5), osmolality (between 330 and 350 mOsm/kg), mechanical trituration and the replating of neurons on old plates of non-neuronal cells or on a poly-L-lysine-treated glass surface, although in the latter case the growth rate of neurons was reduced. Using this approach, we have obtained cultures which show little or no reaggregation of neurons and with relatively few non-neuronal cells. Within an hour or two of dissociation, about 60% of the viable, dissociated hippocampal cells attached to the culture surface, and of these, about one-third extended neurites in culture.

Hippocampal neurons also seemed to differ from peripheral neurons in the appearance of their growth cones (Bunge, 1973), being typically small (<15 μm) and characterized by a flattened, undulating, membranous expansion at the top of the processes, from which microspikes arise (Fig. 3B). When growing axons reached the cell on which they would finally make synaptic contacts, changes occurred in the shape of their growth cones, and the filopodia extended from the tip of the neurite in an irregular pattern. This change in the appearance of growth cones was associated with a slower rate of elongation. When a growth cone finally contacted a cell with which it formed a synapse, a further change took place in its structure.

One rather specific feature of our culture system was that we never had to use any antiproliferating agents to decrease the rate of proliferation of non-neuronal cells nor did we have to use any exogenous nerve growth factor (NGF). Hippocampal neurons under these cultural conditions in our laboratory seemed to have no requirement for any of the above factors for their normal survival and growth.

Living cells photographed under interference polarizing optics (Zeiss, Jamin-Lebedeff) or under an inverted tissue-culture light microscope (Olympus) showed that immediately after isolation, the cells were well dissociated and round in shape without any neuronal processes. Within a few hours in culture, aggregation of cells and the extrusion of fine neuronal processes were observed. Initially the cells appeared to be unpolarized; they extended several, short neurites, but all of these appeared identical in morphology and in pattern of growth. Polarity was first expressed when one of these neurites began to grow rapidly, becoming significantly longer than the others (see Fig. 3E, anticlockwise). This process was the cell axon, and at this stage it could be distinguished from the remaining processes by its growth properties (see e.g., Dotti et al., 1991).

Previous studies of embryonic hippocampal neurons in isolated cell cultures have demonstrated three major types of neurons including bipolar, basket and pyramidal-like (Banker and Cowan, 1979). These three cell types were also present in cultures established from early postnatal hippocampal cells and the neuronal types in cell culture can be related to their putative counterparts in the intact brain. Pyramidal-like neurons in cell culture showed the characteristics of pyramidal and dentate gyrus granule cells in situ of elaboration of one long axon and several short dendrites.

It is well known that one of the most important morphological characteristics of the neuron is the formation of synapses, and one of the important functions of the synapse is the synthesis of neurotransmitters.

Both chemical and electrical synaptic transmission exist in most nervous systems. Morphologically, a synapse consists of a presynaptic release site containing vesicles,

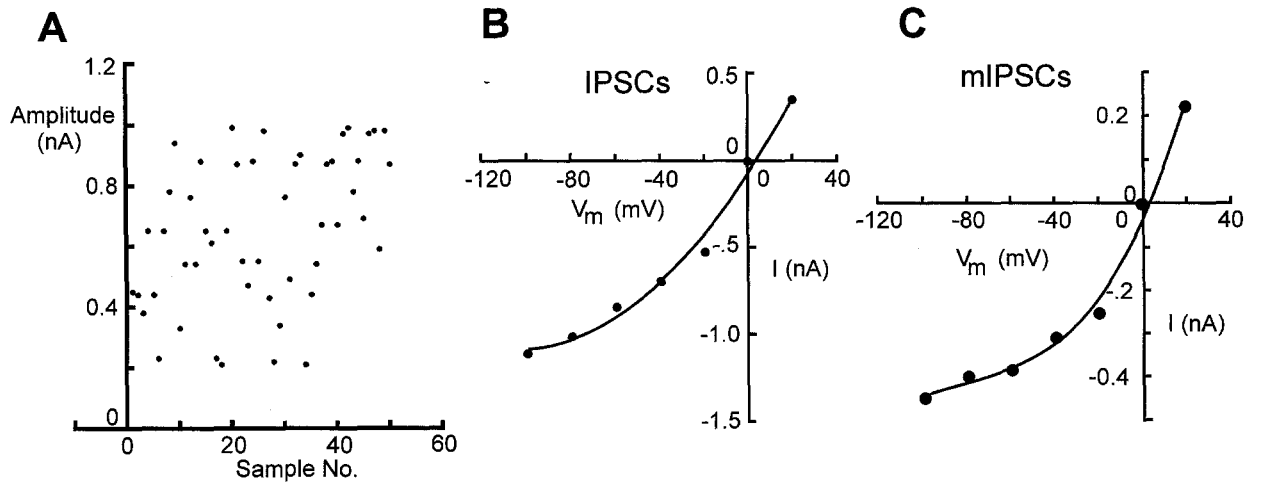

Fig. 6 Following the establishment of the whole-cell configuration, the amplitude of currents increased at first to a relatively stable level at higher sample numbers and then showed little or no further increase. (A) The amplitudes of IPSCs during a recording period of 20 min. (B) The current–voltage relationship for the mean amplitude of IPSCs. These IPSCs were recorded with mammalian Ringer (with TEA and low Ca^{2+}) in the bath, and a pipette solution in which K^+ had been replaced by Cs^+. The channel reversal potential was predicted to be close to 0 mV (as expected for Cl^--selective channels), with symmetrical chloride solutions on both sides of the membrane. The chord conductance at –100 mV was 10.8 nS. (C) The current–voltage relationship for the mean amplitude of mIPSCs, which were recorded 1–2 min after the bath application of 1 μM TTX. The channel reversal potential was +3.1 mV and the chord conductance at –100 mV was 4.4 nS. The ratio of the two conductances suggests an average quantal content of about 2.5 for a typical evoked IPSC in the low Ca^{2+} bath solution. In both (B) and (C), the error bars for the SEM are smaller than the symbol size (n = 16 in each case).

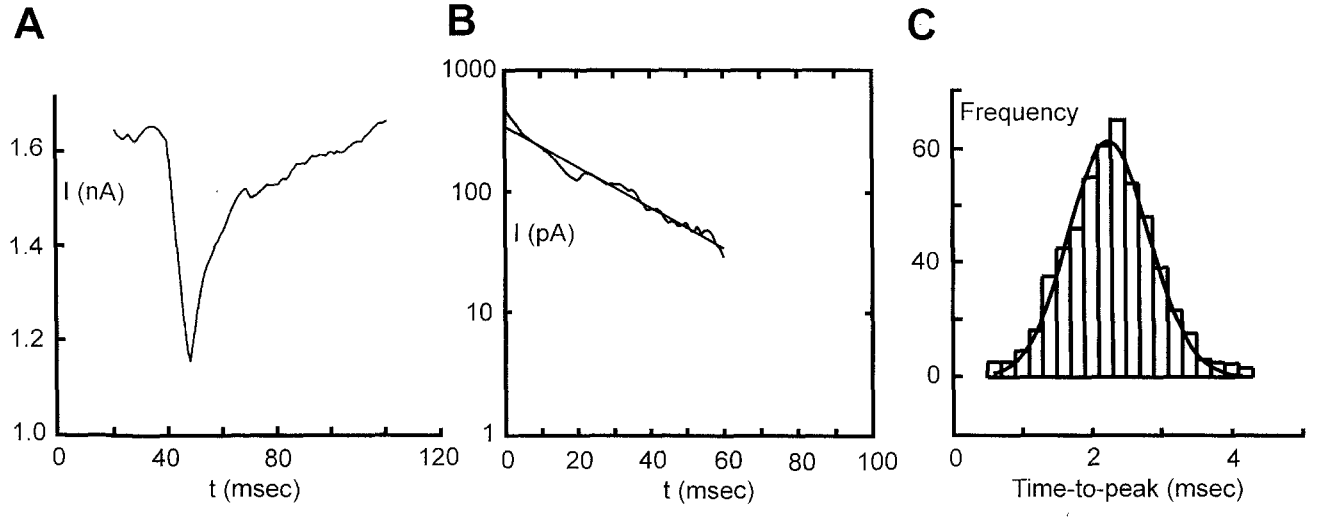

Fig. 7 (A) A typical computer-averaged mIPSC (i.e. TTX-resistant IPSCs) obtained by averaging about 400 mIPSCs. (B) A semilogarithmic plot of averaged mIPSC amplitude vs. time in milliseconds and a calculated linear regression line yielding a decay time constant of 25.8 ms. (C) A histogram of the time-to-peak for the individual mIPSCs used to form the average mIPSC in (A) and fitted by a Gaussian function (mean = 2.3 ms; SD = 0.6 ms; using least-squares techniques).

a synaptic cleft and a postsynaptic site with specialized regions on the membrane. The number of presynaptic release sites is directly proportional to the extent of synaptic transmission between neurons. Although excitatory interactions between cortical neurons have been examined (Miles, 1990), the number of presynaptic release sites remained unknown until recently when Gulyas et al. (1994) discovered that a single pyramidal cell is responsible for evoking excitatory postsynaptic potentials in inhibitory neurons. Their data showed that pyramidal cells form single axonal contacts with postsynaptic inhibitory neurons. In the hippocampus, morphological studies have suggested that inhibitory cells form 5–30 synaptic contacts with a single pyramidal cell (Somogyi et al., 1983; Freund et al., 1985), indicating that there may be different strategies for inhibitory and excitatory transmission.

In this study we were interested in chemical synapses. According to the morphological identification of the regions of contact, there are three main types of chemical synapses, and as most presynaptic endings are axon terminals, postsynaptic elements of central nervous system synapses are dendrites, somas and axons of other cells. In spinal motor neurons, chemical synapses were studied by Eccles (1964), and he found that in most central neurons, inhibitory transmitter activities were mediated by Cl^- channels. Inhibition mediated by second messengers can involve the opening of K^+ channels as well (e.g., $GABA_B$). Both Cl^- and K^+ ion channels are similar in that their reversal potentials (the Nernst potentials for E_K and E_{Cl}) are generally more negative than the resting membrane potential, E_m. In a typical nerve cell, E_{Cl} and E_K are more negative than the resting membrane potential. E_{Cl} may be about –70 mV at 37°C, and E_K about –80 mV, and the membrane potential –65 mV. The concentration of Cl^- is high on the outside of the cell (150 mM) and low inside (10 mM), so that the opening of Cl^- channels leads to the movement of Cl^- down its electrochemical gradient and into the cell. This influx of Cl^- adds to the negative charge inside the cell, while the efflux of K^+ removes the positive charge. Thus, provided E_{Cl} is less than E_m, opening either Cl^- or K^+ channels leads to a positive (outward) current and a net hyperpolarization.

The flow of current due to an inhibitory postsynaptic potential (I_{IPSP}) can be analysed by changing the membrane potential (V_m) systematically and determining the reversal potential for I_{IPS}. When the membrane potential is depolarized, the I_{IPS} become larger with increasing depolarization because the electrochemical force on Cl^- ($V_m - E_{Cl}$) becomes larger. In the above example, when the membrane potential is hyperpolarized from its resting level of –65 mV to –70 mV, which is the reversal potential for the IPSP (E_{IPSP}) and also the Nernst equilibrium potential for Cl^- (E_{Cl}), the IPSP decreases to zero. Thus, at such a potential, any increase in g_{Cl}, due to the opening of Cl^- channels, results in no net current through these channels. When the membrane potential is decreased further to –80 mV, Cl^- will now move out of the cell and the membrane will depolarize.

The resting potential of a central neuron is usually close to E_{Cl}. Indeed, if it equalled E_{Cl}, an inhibitory transmitter, which opens Cl^- channels, still helps to prevent the cells from firing, because the open Cl^- channels tend to clamp the membrane potential at E_{Cl} and to increase the level of excitatory input needed to drive V_m towards threshold.

In addition, the opening of Cl^- channels increases the overall conductance of the membrane of the postsynaptic cell (g_m). Since the amplitude of an excitatory synaptic potential is dependent on g_m, any increase in g_m during the opening of inhibitory channels will reduce the amplitude of any excitatory input that occurs whilst these inhibitory channels are open.

Thus the opening of Cl^- channels should inhibit the postsynaptic cell in three ways. First, an IPSP can hyperpolarize the membrane and move the membrane potential further away from threshold. Second, by increasing the permeability of the cell to Cl^-, the inhibitory transmitter acts to stabilize or clamp the membrane potential near E_{Cl}, preventing it from reaching threshold. Finally, an IPSP increases the membrane conductance, thereby reducing the amplitude of any concurrent EPSP.

The opening of Cl^- channels has yet another important feature that requires comment. As with most forms of synaptic excitation, the opening of the inhibitory channels is not influenced by membrane voltage, i.e., a change in the membrane potential does not alter the *number* of channels opened by the transmitter. This again demonstrates the important difference between most transmitter-gated channels (apart from the NMDA-activated channel) and voltage-gated channels. Spontaneous IPSCs are responsible for tonic neuronal inhibition, which can explain the pronounced excitatory effects produced by GABA antagonists at the intact hippocampus as they block this inhibition. GABAergic transmission is thought to play a very important role in hippocampal inhibitory mechanisms. In the hippocampus, GABAergic terminals and fibres form a dense plexus around the somata of pyramidal cells in the CA1–CA3 regions and around granule cells in the fascia dentate, and a particularly high density of GABA-positive punctate cells surrounds CA3 pyramidal cell somata (Ribak et al., 1978; Woodson et al, 1989). GABAergic boutons are not confined to the somata of pyramidal and granule cells, but cover their axon hillock and initial segment (Somogyi et al., 1983; Soriano and Frotscher, 1989). Woodson et al. (1989) have also observed that in dendritic fields the density of GABA-positive grains is fairly homogeneous in the CA1–CA3 areas, and they also found that 11% of the total population of hippocampal neurons are GABAergic cells.

This patch-clamp study of inhibitory synapses between neurons of early postnatal rat hippocampus in dissociated culture indicates the presence of TTX-sensitive and TTX-resistant IPSPs in current-clamp mode and IPSCs in voltage-clamp mode. The coincidence of the reversal potentials for the IPSCs, for the $GABA_A$- and glycine-evoked currents and the Cl^- equilibrium potential, together with the sensitivity of the IPSCs to bicuculline and strychnine, indicate that the IPSCs described here are Cl^--dependent and mediated by either $GABA_A$ or glycine receptors. Inhibitory postsynaptic currents, recorded under voltage clamp conditions, decayed with kinetics that could be fitted by a single exponential. This observation is consistent with previous recordings of IPSCs made with intracellular microelectrode techniques (Collingridge et al., 1984; Segal and Barker, 1984). Exponential kinetics of synaptic current decay recorded at these and other types of chemical synapses have been interpreted as reflecting the probabilistic closure of ion channels opened as a consequence of postsynaptic receptor occupancy by the synaptic transmitter (Magleby and Stevens, 1972; Gage and McBurney, 1975). The amplitudes of spontaneous IPSCs observed in our preparation varied substantially over the total population of cells, probably mainly due to a large

variability in the number of presynaptic release sites per cell. Their amplitudes were of a similar order of magnitude to those reported by Collingridge et al. (1984) for spontaneous IPSCs in their hippocampal slices.

The recordings of spontaneous synaptic currents from the postnatal rat hippocampus support the hypothesis that they are generated by a transient release of GABA, acting at $GABA_A$ receptors, and glycine acting at glycine receptors to produce an increase in Cl^- conductance. The synaptic current decays exponentially in a manner that is compatible with the probabilistic closure of individual ion channels. The ability to record these spontaneous and miniature inhibitory synaptic currents should extend our understanding of GABA, glycine and other types of chemical synapses between central neurons grown in tissue culture. Our study suggests that these tissue-cultured cells with their interneuronal synaptic connections are behaving in a manner which is reasonably similar to normal cells under in vivo conditions. The preparation may also prove to be particularly useful for further investigation of the properties and mechanisms underlying inhibitory neurotransmission in the CNS. This study also confirmed our previous finding (Fatima-Shad and Barry, 1992): glycine receptor channels are present in the rat hippocampus during an early postnatal age.

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