GABA-MEDIATED INHIBITORY POSTSYNAPTIC CURRENTS RECORDED IN RAT HIPPOCAMPAL SLICES USING INTRACELLULAR AND PATCH CLAMP RECORDING TECHNIQUES.

by Frances Edwards

A thesis submitted to the Australian National University for the degree of Doctor of Philosophy.

May, 1989.
Statement

The thesis consists of two published papers and one submitted manuscript. The contribution of the candidate to each is outlined below.

Edwards F, Gage P (1988) Seasonal changes in the rat hippocampus. Neuroscience Letters 84:266-270. I planned and performed all experiments. The computer program for catching and fitting currents was written by Rod Malbon. The final manuscript was written by Prof. Peter Gage based on and similar to the original manuscript which I had written before leaving Australia.


When I began to work on this project Tomoyuki Takahashi and Arthur Konnerth had already obtained the first patch clamp recordings on 1-3 day old rat spinal cord slices. The method they were using was however complex and much more difficult than that which is described in the paper. The slicing involved embedding tissue in an agar block and Takahashi had developed an elegant but difficult technique for holding down the slices, involving the coagulation of thrombin and fibrinogen. The slices, which were at maximum 100μm thick, were cleaned using two electrodes, one containing the enzyme collagenase. My contribution was first to simplify the slicing procedure and in collaboration with Arthur Konnerth to apply the technique to rat hippocampal slices and greatly to simplify the whole procedure. I developed an easily made grid for holding down the tissue. This I initially constructed on a silver frame but Arthur Konnerth improved this by suggesting the use of heavier platinum wire. I also developed a reliable method of oxygenating slices in a disposable holding chamber. In addition, we found that use of enzyme was not necessary and
changed to using a single cleaning electrode. It eventually became a very simple technique and patches could be obtained with a success rate similar to patch clamp experiments on cultured cells. For the first seven months I worked fairly closely with Arthur Konnerth, though until the last few weeks of that period I did all the tissue preparation. Bert Sakmann then gave me my own set up and I went on over the following year alone to develop methods for apply the technique to a wide range of other tissues and developmental stages and to other species. In addition, during this period I obtained the data on the passive properties of the membranes of these cells. Throughout the whole period, Bert Sakmann was also involved with discussion and ideas in the role of an enthusiastic supervisor.

In the preparation of the manuscript, Bert Sakmann was more actively involved. As I was the only native English speaker in the group, I largely wrote the basic manuscript which we then all worked on. The experiments described and the figures and photos which were finally used were contributed as follows: the schematic diagrams were largely designed by Arthur Konnerth. Figures 4a,b, 5, 7, 9, 11 and 14 were contributed by Arthur Konnerth (hippocampus) or Tomoyuki Takahashi (spinal cord). I contributed the rest of the figures and all the unillustrated sections concerning other tissues and species. In addition, I did all the practical work of actually arranging to put the manuscript together for submission.

Edwards, F.A., Konnerth, A., Sakmann, B., (with Appendix by Busch, C.) Quantal synaptic transmission in the central nervous system. A patch clamp study of IPSCs in rat hippocampal slices. (Submitted to the Journal of Physiology.) The design and execution of experiments and writing of the main manuscript are my work. Bert Sakmann has however been actively involved throughout the project in a supervisory role, being always ready to discuss a new finding or idea and at the end contributing actively to the manuscript. During the seven month period in which I
collaborated with Arthur Konnerth, most of the experiments on miniature IPSCs were carried out and three of the outside out patch recordings. The computer programs used for the main text were written or adapted by Klaus Bauer (measurement, fitting and selection procedures for IPSCs) and Christopher Busch (Gaussian fitting).

On the basis of mathematical contributions and computer programming in the Appendix, Christopher Busch's name has been added. The Appendix was however controlled and written by Bert Sakmann, though to some extent I have polished the English. I contributed all the statistical data in the Appendix with the exception of the binomial analysis, contributed by Busch. Although I actually performed the tests, the idea and methods for using the autocorrelation functions were the combined work of Busch and Sakmann. The rest of the Appendix was however a collaborative effort. I designed the measurement of background noise in collaboration with Sakmann. Klaus Bauer and Klaus Schröter wrote the computer programs for background noise estimation.

In summary, the first paper is the work of the candidate with Peter Gage playing a supervisory role and contributing in the writing of the final manuscript. The work in Germany was an active collaboration with Arthur Konnerth for seven months of the two and a half years of work on the two manuscripts. In addition, although the candidate never worked directly with him, Tomoyuki Takahashi made a major contribution to the methods, particularly in actually having made the first recordings. Bert Sakmann played an active supervisory role throughout but allowed the candidate a great deal of freedom. None of the computer programs were written by the candidate and Christopher Busch made an active contribution in collaboration with Bert Sakmann on statistics in the Appendix of the last manuscript.

Frances Edwards
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Overview and Summary of Results
A comparison of the results obtained using the two methods and an overview of the thesis as a whole.
SECTION 1
Summary

The thesis consists of three manuscripts, the first two of which are already published and a third which has been submitted for publication.

The first and third papers are studies concerning the features of GABA-mediated postsynaptic currents measured in rat hippocampal slices. The second paper describes a method for making patch clamp recordings from brain slices. This method was then employed for the study described in the third paper. As a result, an increase in resolution of synaptic currents of about ten-fold was obtainable, compared to the first study reported in which intracellular recording techniques were used.

Each paper includes a detailed Summary. A very brief description of the main findings is included here.

Paper 1: *Seasonal changes in the rat hippocampus*. The limits of resolution of intracellular recording techniques resulted in the restriction of this study to the measurement of the decay rate of spontaneously occurring stimulated inhibitory postsynaptic currents (IPSCs). The main finding of the study was that the decay time of IPSCs varies with season being longer in the winter months than in the summer.

Paper 2: *A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system*. A method is described for making patch clamp recordings on brain slices. The method is shown to be applicable to all configurations of the patch clamp technique and to a wide variety of brain areas, developmental stages and species.

Paper 3: *Quantal synaptic transmission in the central nervous system*. A patch clamp study of IPSCs in rat hippocampal slices. The application of the techniques described in the above paper allowed resolution not only of miniature IPSCs but also of amplitude fluctuations of IPSCs stimulated via an extracellular electrode placed on a nearby
cell. It was thus possible to perform detailed quantal analysis on the directly measured currents, without using deconvolution techniques or making complex assumptions about release processes. In addition, single channel currents could be measured under similar conditions to the whole cell synaptic currents.

The main results of the study were:
1. The amplitudes of GABA-mediated IPSCs were seen to be quantally distributed with a quantal current being the result of the opening of not more than 30 GABA-receptor (GABAR) channels.
2. The results were compatible with the theory that release of transmitter from each bouton has an all or nothing effect on the postsynaptic receptors and moreover that the quantal size is determined by the arrangement of receptors on the postsynaptic membrane.
3. Various other features of the whole cell and single channel currents are described.
Acknowledgements

I would like to thank all the many and varied people who have helped directly and indirectly towards this thesis.

In Canberra, I would particularly like to thank Peter Gage for taking me on in the first place when I had no experience in electrophysiology. I will also always be grateful to Alasdair Gibb and Pankaj Sah for all their help and patience as I was learning the basics. In addition, I would like to thank the previous director of the John Curtin School, Prof. Bob Porter for being thoroughly cooperative and helpful about my move to Germany and the school secretary, Sue Scales for her help in organizing the administrative side. I am also very grateful to Steve Redman for various good advice along the way. There are, of course many others who also contributed enormously. Rod Malbon was always ready with help and all the others who provided endless discussion and friendship formed a remarkably stimulating and enjoyable group.

In Germany, there are again many people to thank. Bert Sakmann has been an excellent supervisor, teaching me a great deal and always being enthusiastic to discuss an interesting new result or idea. I am grateful to Arthur Konnerth for teaching me the basics of patch clamping and for our very fruitful collaboration during my seven months in Göttingen. Walter Stühmer was also particularly helpful, teaching me many tricks of the trade and for ever coming up with new and useful devices, (not to mention cakes on Sunday afternoons in the lab). Again there are many others, too numerous for individual mention: Frau Friedlein who provided truly remarkable technical assistance, Andreas, Peter and all the other friends who provide companionship on long nights in the lab, Christopher Busch who proof read my thesis and provided lots of chocolate along the way, Klaus Bauer whose extreme patience in all computer matters, both hardware and software is truly unbelievable.
I would also like to thank various members of the John Curtin School who have been particularly helpful and cooperative in sorting out the difficulties of trying to deal with delays and red tape from the other side of the world. Especially helpful throughout was Peter Gage's secretary, Ann Andrews. In addition, I would like to thank the Director of the John Curtin School, Prof. David Curtis and the Director of Neuroscience, Prof. Steve Redman.

Finally and most importantly, I would like to thank Paul Martin, who not only spent hours of proof reading and provided very valuable critical comments but has lived through all the ups and downs along the way and provided constant love and support.
General Introduction

Synaptic transmission in the central nervous system is dependent on the integration of complex arrays of signals, received via excitatory and inhibitory synapses. Early studies of membrane physiology and synaptic transmission involved in vivo experiments using extra or intracellular recording. While in vivo experiments are in principle the closest to the intact situations, such experiments are slow and difficult due to problems of movement, electrode placement and maintenance of the animal. In addition, in vivo studies must be performed on anaesthetized animals and thus the whole of the central nervous system is by necessity depressed, greatly limiting the study of inhibitory currents.

The introduction of brain slice techniques for electrophysiological recordings (e.g. Yamamoto & Milwain, 1966) greatly accelerated the study of central synaptic transmission. Such preparations provide a physically stable, convenient method for extra and intracellular recordings and, despite isolation from surrounding tissues, seem to maintain most properties seen in vivo (Schwartzkroin, 1975). Over the last 20 years, microelectrode studies using slice preparations, together with cultured and isolated cell preparations have provided a notable increase in the understanding of membrane and synaptic physiology, particularly in the central nervous system. More recently, the resolution of membrane currents has been greatly increased by the development of the patch clamp technique (Neher & Sakmann, 1976; Hamill et al, 1981).

The aim of this study is to describe various properties of post synaptic currents at a central nervous system synapse. In the hippocampus, GABA\textsubscript{A}-mediated inhibitory synapses are thought to be located mostly on or near cell somas (Andersen et al, 1964) and thus are particularly suitable for voltage clamp studies such as those described below.

Initially, I intended to study the effect of pentobarbitone on GABA-mediated inhibitory postsynaptic currents in
the rat hippocampus, using the single electrode voltage clamp technique. I began this section at the Australian National University, in the laboratory of Peter Gage. In the course of studying the effects of pentobarbitone, a seasonal change in the control currents was serendipitously observed. The possibilities of studying the changes underlying the observed phenomena was extremely limited using standard techniques, because the signal to noise ratio was much too small. In fact, in the presence of the sodium channel blocking drug tetrodotoxin, the synaptic currents were too small to be distinguished from the noise level and thus it was not possible to study the properties of the miniature currents at all.

After a year, I moved to the laboratory of Bert Sakmann at the Max-Planck Institut für biophysikalische Chemie in Göttingen, West Germany. At this stage I became involved in the development of a technique for making patch clamp recordings from synaptically connected cells in brain slices. I was thus able to study central synaptic currents at a much higher resolution than was previously possible. Rather than continuing the earlier project directly, it seemed more appropriate first to study the basic properties of central synaptic transmission, which had not been accessible with earlier techniques.

The thesis is comprised of three papers:

Paper 1 (Canberra):
A study of the time course of decay of inhibitory postsynaptic currents is described. The single electrode voltage clamp technique was used in a classical hippocampal slice preparation and the effects of season were observed.

Paper 2 (Göttingen):
A newly developed method is described for making patch clamp recordings in brain slices, without enzymatic disruption of connections and membranes. Various applications are demonstrated and discussed.
Using the technique described in the second paper, GABA-mediated inhibitory postsynaptic currents were recorded in the granule cell layer of the rat hippocampus and are described in detail. The increase in resolution resulting from use of patch clamp techniques and the added advantage of being able to see individual neurones for placement of the stimulus electrode allowed more detailed study of the rise times, decay times and amplitudes of spontaneous and stimulated IPSCs. In addition, the distribution of amplitudes of GABA-mediated single channel currents was observed. Thus it was possible to confirm the quantal nature of the postsynaptic response at this synapse and to estimate the number of channels underlying a quantal current.

REFERENCES


SECTION 2
Seasonal changes in inhibitory currents in rat hippocampus

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(Received 25 September 1987; Revised version received 1 October 1987; Accepted 2 October 1987)

Key words: Rat hippocampus; Inhibitory current; Voltage clamp; Season

Spontaneous inhibitory postsynaptic currents were recorded in voltage-clamped neurons (clamp potential -60 to -70 mV) in the pyramidal cell layer of the CA1 region of hippocampal slices from 3- to 4-month-old rats. The average time constant of decay of the currents progressively increased from March (early autumn) to July (mid-winter) and then declined progressively from July to November (late spring). The changes were statistically significant. It is suggested that such seasonal changes in the time course of inhibitory postsynaptic currents may have behavioural consequences and could explain the reported seasonal variations in the occurrence of disorders such as mania.

There have been many clinical reports that the incidence and severity of affective disorders in man vary with season. For example, it has been shown that the first occurrence of mania is more likely in summer than in winter [6]. If such disorders are accompanied by changes in transmission of information from neuron to neuron in the central nervous system, there may be seasonal changes in the underlying processes but no observations of this kind have been reported. We have discovered a seasonal change in the time course of inhibitory postsynaptic currents generated by y-aminobutyric acid (GABA) in CA1 neurones in rat hippocampus; their decay is slower in winter than in summer months. This probably reflects a seasonal alteration in the GABA-activated receptor–channel complex in the membrane of these cells. The overall effect would be a reduction in inhibitory modulation of these cells in summer months and this could conceivably contribute to changes in behaviour.

The animals used in these experiments were 3 to 4-month-old, male Wistar rats, born and kept in our animal house in a room with outside windows that was heated in winter (temperature controlled at 22–23°C). Their diet (food pellets) was constant throughout the year. Rats were killed by cervical dislocation and the brain was immediately removed, hemisected and immersed in ice-cold, oxygenated Krebs solution.

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0304-3940/88/S 03.50 © 1988 Elsevier Scientific Publishers Ireland Ltd.
Slices about 400 \( \mu \text{m} \) thick were cut transversely with a Campden Instruments Vibroslicer from each half of the brain and the hippocampal region dissected out. The hippocampal slices were stored until use in a beaker of oxygenated Krebs solution at room temperature (22–24°C). For recording, one of the slices was placed on a grid in a tissue bath where it was kept barely submerged in flowing, oxygenated Krebs solution at 22-24°C. Details of the methods and solutions used have been described previously [2].

Spontaneous inhibitory postsynaptic currents (IPSCs) were recorded in voltage-clamped cells in the CA1 region of the hippocampus from March (early autumn) to November (late spring) 1986. The membrane potential was clamped at \(-60\) to \(-70\) mV by passing current through a 40- to 65-M\( \Omega \) intracellular microelectrode (filled with 3 M KCl) connected to a single electrode voltage clamp device (Axon 1, Axon Instruments). Results were accepted if the resting membrane potential was more negative than \(-45\) mV, the input resistance was greater than 40 M\( \Omega \) and at least 10 IPSCs were recorded. The decay of IPSCs was exponential with a single time constant \( \tau \); i.e. currents could be well described by \( I(t) = I(0)\exp(-t/\tau) \), where \( I(0) \) was the current shortly after the peak. The lines through the decay of currents in Fig. 1 show best (least squares) fits of this equation to typical records.

When currents recorded from 8 neurons (4 rats) in March and 8 neurons (4 rats) in July, were compared, it was clear the IPSC decay was more rapid on average in March than in July. Representative currents recorded in each of the two months illustrate this observation (Fig. 1); the decay time constants obtained from the fitted exponentials were 10.9 ms (March) and 26.6 ms (July). The average decay time constant of the 152 currents recorded in March was 13.2 ± 0.4 ms (mean ± 1 S.E.M.), whereas, in July, the average decay time constant of 207 currents was 23.2 ± 0.4 ms. When the average decay time constant was calculated for each of the 8 cells from which results were obtained in March and July, the mean of the cell means was 13.0 ± 1.6 ms in March and July, the mean of the cell means was 13.0 ± 1.6 ms in

\begin{figure}[h]
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\includegraphics[width=0.6\textwidth]{fig1.png}
\caption{Typical inhibitory postsynaptic currents recorded from CA1 neurones in March and July. The clamp potential was \(-60\) mV in both cases. The solid lines through the decay phases show the exponentials of best fit from which the time constants were obtained; 10.9 ms in the March current and 26.6 ms in the July current. Horizontal calibration, 10 ms; vertical calibration, 0.15 nA.}
\end{figure}
Fig. 2. Cumulative frequency histograms of decay time constants recorded in 8 cells (4 rats) in March and 8 cells (4 rats) in July. Different patterns represent different cells. Results obtained in each cell are expressed as a percentage of the total number counted in that cell in order to give equal weighting.

March and 23.2 ± 1.4 ms in July, again a highly significant difference. The difference in the distributions of decay time constants in March and July is illustrated in frequency histograms in Fig. 2: in order to weight cells equally, frequencies are expressed as a percentage of a cell's total count.

The change in time course did not occur suddenly but was progressive over a peri-
There is insufficient information available to allow us to predict the influence that these seasonal changes in inhibitory currents would have on behaviour, but it does seem very likely that they would have an effect. Some drugs that modify behaviour, e.g. barbiturates and diazepam, also increase the time constant of decay of IPSCs in rat and guinea pig hippocampus [3, 4]. However, it has not yet been demonstrated that such changes in inhibitory currents underlie the behavioural effects of these drugs.

We do not know what environmental factors changing with season influence the decay of the currents, though preliminary experiments indicate that manipulation of light cycles changes the decay of these currents; nor do we know how these cues are transmitted from sensory input to the inhibitory synapse but it is possible that chemical transmitters such as hormones are involved. More information about these changes may well lead to new insights into the regulation of synaptic transmission in normal and abnormal animals. If similar changes in inhibitory currents occur with season in man, there could be far-reaching clinical implications.

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7 Sakmann, B., Methfessel, C., Mishina, M., Takahashi, T., Takai, T., Kurasaki, M., Fukuda, K. and Numa, S., Role of acetylcholine receptor subunits in gating of the channel, Nature (Lond.), 318 (1985) 538-543.
**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.

**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 it is glued to a platinum frame (F). Such a grid, placed over the slice, fixes it firmly in position on the bottom of the recording chamber by a grid of nylon threads (V) 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 x 9 x 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 x 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, CaCl₂ 1, MgCl₂ 1, NaHCO₃ 26, NaH₂PO₄ 1.25, glucose 25, [pH 7.4 when bubbled with carbogen (95% O₂, 5% CO₂)]. 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): KC1 140, MgCl₂ 1, CaCl₂ 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).

**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 e 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. 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, MgCl₂ 1, CaCl₂ 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 and hyperpolarizing potentials are represented by a downward deflection of the trace.

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 recordings 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.
elicited in response to depolarizing pulses from a holding potential of —80 mV to the test potentials indicated on the traces. 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, MgCl₂ 1, CaCl₂ 2, HEPES 10 (pH 7.3). The records represent traces taken on a chart recorder with a frequency response of 300 Hz (—3 dB). Additions of the GABA receptor antagonist, Bicuculline (Fig. 6c), reversibly abolished all synaptic activity. Thus these potentials represent i.p.s.p.s mediated by GABAₐ 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 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-
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, CaCl₂ 1, MgCl₂ 1, HEPES 10 (pH 7.3) and TTX 1 pM. 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.

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 Ca²⁺ was added. The bath solution was changes to the “intracellular” solution (containing in mM): KCl 140, HEPES 10, EGTA 10, 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.

**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 synthetically 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 around 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 11a, 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 synchronically 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 11c 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).

**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.
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 x 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 x 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 GQ) 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 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 picture 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 fluorescence 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.
one day previously with Evan's Blue (0.3 ml, 1 mg/ml in H2O) in the left hind leg muscle, a, Spinal cord slices were taken from a 4 day old rat which was injected labelled. The other half of the spinal cord showed no labelling, fluorescence A motoneurone from the above slice seen with high magnification under low power magnification (x 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 (x 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 (Äslund 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 Ca²⁺ 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.

Acknowledgements. We would like to thank Drs. O. Creutzfeldt, E. Neher, M. Kuno and A. Berger for critically reading the manuscript. We are also grateful to Dr. E. Neher for assistance in early trials with Fura-2 measurements, Dr. W. Stühmer for help with on line analysis of K⁺ currents and Dr. T. Vidyasagar for supplying cat brains. We would also like to thank F. Friedlein for technical assistance. The work was in part supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 236, A14).

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Received November 7, 1988/Received after revision January 31/Accepted February 14, 1989
QUANTAL SYNAPTIC TRANSMISSION IN THE CENTRAL NERVOUS SYSTEM
A PATCH CLAMP STUDY OF IPSCS IN RAT HIPPOCAMPAL SLICES.

by

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Running title: QUANTAL TRANSMISSION AT A CNS SYNAPSE
Key words Quantal transmission, central nervous system, patch clamp technique

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SUMMARY

1. Synaptically connected neurones were identified in the granule cell layer of slices of 17-21 day old rat hippocampus. Whole-cell current recording using the patch clamp technique revealed synaptic currents ranging from less than 10 to about 100 pA in symmetrical Cl⁻ conditions. These currents were blocked by 2 μM Bicuculline, indicating that they result from the activation of postsynaptic γ-aminobutyric acid receptor (GABAR) channels.

2. Addition of Tetrodotoxin (TTX, 1 μM) resulted in the loss of most currents of more than 40 pA in amplitude. Currents which disappeared after TTX treatment were assumed to be the result of spontaneous presynaptic action potentials. The currents seen in the absence of TTX are referred to as spontaneously occurring inhibitory postsynaptic currents (IPSCs), those remaining in the presence of TTX were defined as miniature IPSCs.

3. Similar currents were observed when recording in the whole-cell configuration while extracellular stimulation was applied to a nearby neurone. These currents were also completely blocked by 2 μM Bicuculline and by 0.5 μM TTX. They were thus defined as stimulus-evoked IPSCs.

4. The half rise time of both miniature and stimulus-evoked IPSCs was fast (<1 ms). The time course of decay of both miniature IPSCs and stimulus-evoked IPSCs could be well fitted with the sum of two exponentials. At a membrane potential of -50 mV, the mean decay time constants of the two components were 2.0±0.38 ms and 54.4±18 ms (mean ± S.D.) for miniature IPSCs (6 cells) and 2.2 ± 1.3 ms and 66 ± 20 ms (3 cells) for stimulus-evoked IPSCs.

5. Stimulus-evoked IPSCs varied in amplitude from less than ten to hundreds of pAs. In eight of eleven cells histograms of IPSC amplitudes showed several clear peaks which, when fitted with the sum of Gaussian curves, were found to be equidistant. This is consistent with the view that stimulus-evoked IPSC amplitudes vary in a quantal
fashion. The quantal size varied between 7 and 20 pA, at a membrane potential of -50 mV.

6. Decreasing the Ca\(^{2+}\) and increasing the Mg\(^{2+}\) concentration in the extracellular solution decreased the number of peaks in the IPSC amplitude histogram but did not affect the size of the quantal event.

7. In one cell where the recording was stable for more than an hour, changing the membrane voltage from -50 mV to -120 mV increased the quantal size by a factor of 2.1, close to that expected if the current-voltage relation of IPSC peak amplitudes were linear.

8. The peak of miniature IPSC amplitude histograms measured in the presence of 1 μM TTX was comparable with the quantal size of stimulus-evoked IPSCs. However, in all cells, a tail of larger amplitude miniature IPSCs was observed. The amplitudes in the tail in six of twelve cases were quantally distributed.

9. Single channel currents activated by GABA, applied locally to outside-out patches isolated from the soma membrane of granule cells, indicated that GABAR channels had two conductance levels of 14 and 23 pS. Thus for IPSCs in hippocampal granule cells, one quantal current represents the simultaneous opening of less than 30 GABAR channels.

10. The small number of postsynaptic channels mediating a quantal IPSC, the small variation in quantal size and the fast rise of the IPSCs are consistent with an "all or none" hypothesis of synaptic transmission at granule cell synapses where the size of the quantal event is determined by the number of available postsynaptic GABAR channels opposite a release site.
INTRODUCTION

Inhibitory postsynaptic currents (IPSCs) mediated by $\gamma$-aminobutyric acid (GABA) arise from activation of Cl$^-$ selective ion channels formed by the GABA-receptor (GABAR) subunits (Curtis, Duggan, Felix & Johnson, 1970; Allen, Eccles, Nicoll, Oshima & Rubin, 1977; Schofield et al. 1987). We have investigated the properties of IPSCs in dentate gyrus granule cells of the rat hippocampus. Patch clamp recordings can be made in slices from anatomically and developmentally defined neurones with their synaptic connections functionally intact (Edwards, Konnerth, Sakmann & Takahashi, 1989). We have used this preparation to resolve miniature and evoked IPSCs and the elementary currents underlying them. The more than ten-fold improvement in resolution of the patch clamp technique over intracellular recording allows the direct measurement of amplitudes of miniature and evoked IPSCs with high enough precision for detailed analysis of their amplitude distributions. Previous estimates of quantal size and its variability in mammalian CNS synapses have been forced to rely on indirect methods such as a priori assumptions about release statistics, deconvolution analysis and comparison of derived release parameters to anatomical evidence (for review see Korn & Faber, 1987; Redman, 1990). We describe time courses of IPSCs in granule cells, the quantal nature of their amplitudes and the amplitudes of the underlying single channel currents.
METHODS

Tissue and Preparation

Hippocampal slices were prepared as previously described (Edwards et al. 1989), from the brains of Wistar rats (17-21 days of age). In the early stages of the study, 120 μm thick slices were used but it was found later that thicker slices (300 μm) could be used (Sakmann, Edwards, Konnerth & Takahashi, 1989) and that the cells survived longer. The thickness of the slice did not affect any of the measured parameters.

Solutions and Drugs

The slices were placed in a flow chamber perfused continuously with a solution containing (in mM): NaCl 125; KCl 2.5, CaCl₂ 2, MgCl₂ 1, NaHCO₃ 26, NaH₂PO₄ 1.25, glucose 25, (pH 7.4 when bubbled with carbogen (95% O₂, 5% CO₂)). This solution will be referred to as physiological saline. The pipette solution (intracellular solution) contained (in mM): N-methyl glucamine 140, HCl 125, CaCl₂ 2, EGTA 10, ATP 2, MgCl₂ 1, HEPES 10 (pH 7.3). In experiments using (-)Bicuculline methiodide or Tetrodotoxin (TTX) (both from Sigma, St. Louis, U.S.A), drugs were added to the perfusion medium. For single channel current measurements, GABA (1 μM) was applied locally using an application tool, consisting of a patch pipette (<5 MΩ) attached via silicone tubing to a 0.25 ml glass syringe. The syringe barrel was fitted with a screw which when turned applied positive pressure to the solution in the pipette. It was necessary to place this pipette more than 200 μm from the membrane patch to avoid excessive activation of single channel currents.

Current Recording

All current recordings were made from neurones in the granule cell layer of dentate gyrus of the hippocampus at room temperature (21-23°C). For whole-cell current recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), patch pipettes (<6 MΩ) were pulled from borosilicate glass (2 mm diameter, 0.3 mm wall thickness) and filled
with intracellular solution (see Solutions). Pipettes for outside-out patch recording were also filled with N-methyl glucamine intracellular solution but were pulled from thicker walled borosilicate glass (2 mm diameter, 0.5 mm wall thickness) and had higher resistances (9-12 MΩ). In some single channel current recordings, N-methyl glucamine was replaced by Cs⁺ without any measurable effect on the single channel amplitude. All currents were recorded using an EPC-7 patch clamp amplifier (List, Darmstadt, FRG).

**Stimulation**

After establishing a stable whole-cell recording configuration on a cell in the granule cell layer, a second patch electrode (2-6 MΩ) filled with physiological saline was lowered onto the surface of a nearby cell, also in the granule cell layer (Plate 1A). A voltage pulse (200 μs) was delivered extracellularly between the stimulus pipette and the bath electrode. The voltage was increased (2-8 V) until a response was seen in the cell from which the recording was being made.

**Analysis**

Currents were initially recorded on video tape after filtering at 10 kHz (Bessel filter on EPC-7). Recorded data were later sampled with a VME-bus computer system. Whole-cell currents were filtered at 2 kHz (-3dB; Frequency Devices, Haverhill, USA.; 8-pole Bessel filter) and sampled at 10 kHz. Single channel currents were filtered at 600 Hz and sampled at 2 kHz. For analysis, a program was designed to catch IPSCs and semiautomatically to measure their amplitudes, rise times and decay times. Stimulus-evoked IPSCs, caught with a trigger signal, were accepted for analysis if no spontaneous synaptic activity was detected which could interfere with the measured rise. The latency, rise time to half amplitude and peak amplitude were measured automatically or by placing cursors by eye at the beginning of the stimulus artefact, at the beginning of the IPSC, and at the peak of the rise of the IPSC. The decay time course was measured from the peak of the IPSC by fitting up to two exponential components by eye.
IPSC amplitude distributions. In the majority of experiments, peaks in the IPSC amplitude histograms were clearly detectable by eye. It was thus not necessary to use deconvolution techniques to remove background noise or to make assumptions on the release statistics for fitting of IPSC amplitude histograms with sums of Gaussian curves. Fits were made in two ways: Gaussians were fitted to binned IPSC amplitudes using a least squares criterion and, in addition, the data were fitted without binning using a maximum likelihood method.

The IPSC amplitude histograms were analysed using a program which determines the best fit for a sum of Gaussian distributions to the histogram using a least squares criterion. In each case the number of Gaussians to be fitted was determined by eye and the range of amplitudes restricted as detailed in the figure legends. The bin width was chosen to ensure that at least 5 bins lay between two peaks. In addition, for all distributions least squares fits were made at least twice with different bin widths and in no case were significant differences seen. The program had additional options used in the analysis of simulated IPSC amplitude distributions, where the standard deviation $\sigma_N$ of the baseline noise recording could be fixed such that the standard deviation of any of the fitted Gaussians was at least as large as $\sigma_N$. Finally the standard deviation $\sigma_q$ characterizing variability of the quantal event could be fixed. The error function for the least squares fit minimized the square of the difference between the height of each bin and the height of the fitting function at the centre of each bin using an adaptation of a Simplex routine (Cacelli & Cacheris, 1984).

To check that peaks in IPSC histograms were not the result of binning, the amplitudes from 7 cells were also fitted using the maximum likelihood method which did not require binning of the data (Colquhoun & Sigworth, 1983). The function maximized was

$$\text{LogProb} = \sum_i \log \left( \sum_k A(k) \exp \left( -0.5 \star \left( \frac{(Y(i) - M(k))/\sigma_p(k)}{\sqrt{2 \star \pi} \star \sigma_p(k)} \right)^2 \right) \right).$$
Y(i) are the measured amplitude values, k is the incremental number of the Gaussian and M(k) is the mean, σp (k) is the standard deviation (S.D.) and A(k) the relative area under the Gaussian k.

*Single channel current amplitudes.* Amplitudes of single channel currents were analyzed using a program which measured the distance between a baseline and amplitude level by eye. The resulting amplitude distributions were also fitted by sums of Gaussian distributions using the least squares method.
RESULTS

Despite the extensive dendritic trees of granule cells in hippocampal slices, the cells have a high input resistance (> 1 GΩ) in the whole-cell recording configuration. This may result from the very small cross sectional area of the dendrites even quite close to the soma (Plate 1B). The passive properties of these cells have been previously described (Edwards et al. 1989).

*Spontaneously occurring IPSCs, miniature IPSCs and stimulus-evoked IPSCs*

**General description and effects of applied drugs.** Whole-cell current recordings in the granule cell layer revealed spontaneously occurring currents with fast rise times (< 1 ms) and slower decays. The decay phase was fitted with the sum of two exponentials: one fast (τ₁ < 3 ms) and a second much slower (τ₂ > 30 ms). The amplitude of spontaneously occurring currents varied with the membrane potential, reversing at 0 mV in symmetrical Cl⁻.

At a membrane potential of -50 mV in physiological saline, amplitudes of spontaneously-occurring currents ranged from less than 10 pA to more than 100 pA. When TTX (1 μM) was added to the bathing solution, the larger currents disappeared and almost all of remaining currents had amplitudes of < 40 pA (Fig. 1A). In the presence of Bicuculline (2 μM), no currents were detectable. Strychnine (0.2 μM) blocked whole-cell currents induced by bath application of glycine (60 μM) but did not affect the average amplitude of the synaptic currents. The currents can thus be defined as GABA-mediated Cl⁻ currents and will be referred to as inhibitory postsynaptic currents (IPSCs). The effect of TTX suggested that the larger IPSCs seen in physiological saline were caused by spontaneous action potentials in presynaptic neurones. The IPSCs remaining in the presence of TTX were due to release of transmitter independent of presynaptic TTX sensitive sodium action potentials and will be referred to as miniature IPSCs.
Synaptic currents could also be elicited by application of short extracellular voltage pulses to a nearby cell (Plate 1A, Fig. 1B). Similar to spontaneously-occurring IPSCs, stimulus-evoked IPSCs were completely blocked by Bicuculline (2 μM), unaffected by Strychnine (0.2 μM) and they reversed at a membrane potential of 0 mV in symmetrical Cl\(^-\). When TTX (0.5 μM) was added to the bathing medium, no currents were seen in response to stimulation. These evoked IPSCs were thus due to release of GABA in response to presynaptic action potentials and not due to electrotonic spread to presynaptic terminals. This suggestion was further supported by the all or nothing nature of the response to presynaptic stimulation, i.e. the stimulus voltage was increased until a response was consistently seen. Further increasing the voltage did not change the response. These currents are thus referred to as stimulus-evoked IPSCs.

**Decay of IPSCs.** The decay time course of both miniature and stimulus-evoked IPSCs were fitted with two exponential components (Fig. 2A,B). At -50 mV, the mean decay time constants (± standard deviation (S.D.)) from averaged miniature IPSCs were 2.0±0.38 ms and 54.4±18 ms (6 cells). The decay time constants of stimulus-evoked IPSCs recorded from three cells were 2.2 ± 1.3 ms and 66.4 ± 20.0 ms (n=3). The slow component always contributed at least 50% of the measured peak amplitude.

Somewhat similar spontaneously-occurring IPSCs have previously been observed using the single electrode voltage clamp technique in the hippocampal CA1 region (Collingridge, Gage & Robertson, 1984), but in this study the decay time course of the currents could be fitted with a single exponential.

**Latency of stimulus-evoked IPSCs.** The response to a stimulus began within 3 ms of the start of the stimulus artefact (Fig. 3A). In some cases, the response began to rise before the stimulus artefact had subsided. Latency histograms from cells in which the latency was long enough to be measured revealed a single population. This suggests that only one presynaptic cell was stimulated, consistent with the observation that moving the stimulation pipette a few micrometers away from the cell soma abolished the response.
In one cell of the eleven reported here, two distinct populations were evident from the latency distribution. This was assumed to be the result of stimulating two inputs perhaps via a polysynaptic pathway.

**Rise time of IPSCs.** Fig. 3A shows a record of an IPSC with high time resolution, demonstrating the fast rise time. The step response of the recording system under the standard conditions is superimposed on the current trace, showing that the measured rise time is not limited by the setting of the low pass filter (2 kHz, -3 dB). In histograms of the rise time to half amplitude with bins of 0.1 ms, the distribution always showed a single peak with the mode falling between 0.2 and 0.6 ms. Fig. 3B shows a histogram of rise times to half peak amplitude from a particular cell with its single peak value between 0.2 and 0.3 ms. The single peak indicates that a homogenous population of IPSCs was recorded. In two cells a wider range of rise times was seen but in each case more than 70% of IPSCs had half rise times of less than 1 ms. The rise time of averaged miniature IPSCs was also fast (0.34 ± 0.12 ms, 3 cells).

**Amplitude of stimulus-evoked IPSCs**

**Quantal nature of stimulus-evoked IPSC amplitudes**

Successive stimulus-evoked IPSCs fluctuated in peak amplitude (Fig. 4). In most cells, at -50 mV, the range of amplitudes was between a measurable minimum of about 6-8 pA and a maximum of 100-200 pA. In two cells, IPSCs with peak amplitudes of up to 300 pA were observed. Some IPSCs of less than 8 pA also occurred but were not regularly shaped and were close to the noise band. They were thus considered to be ambiguous and were not included in the analysis. Amplitude histograms were constructed for 11 cells held at a membrane potential of -50 mV. In 8 of these cells regularly spaced peaks were clearly visible. Fitting the histogram with a sum of Gaussian curves resulted in peaks with means that were nearly equally spaced (Figs. 5A-C, 8A, 9A and 11C). The curves also conformed to additional criteria for equi-spaced peaks described in the
Appendix. The amplitude distributions of the remaining 3 cells could also be fitted with equal spaced Gaussians curves, but the distributions were less clear and the additional criteria were not met. Table 1 lists the means and standard deviations obtained from the eight neurones from which clearly quantal IPSC amplitude distributions were seen. Due to the difficulties of resolving the smallest IPSCs, the first peak was not always fitted and so is not listed. At -50 mV, in 5 of these 8 cells, separation between the peaks was 7-12 pA and in the other 3 cells the separation was 17-20 pA (Table 1). In each case the standard deviation of the fitted Gaussians remained fairly constant throughout successive peaks. The averaged standard deviations in different experiments varied between 2.4 pA and 6.4 pA (S.D.s of Gaussians fitted to peaks 2-5 were averaged). They were thus larger than the range of standard deviations $\sigma_N$ measured for background noise recordings in these cells which varied between 1.9 pA and 3.2 pA (Appendix).

It should also be noted that in all cells some stimuli did not result in a postsynaptic response (Fig. 4, upper panel). The proportion of blank responses varied between cells and increased if the stimulation frequency was higher than 2 s$^{-1}$. As the stimulation was extracellular, it was not possible to assess whether failures were due to failure of the presynaptic neurone to generate an action potential or a failure of the presynaptic terminal to release transmitter in response to the incoming action potential. Presumably the number of failures reflected the sum of these two possibilities. In addition it was not always possible to distinguish between small current responses and failures. The meaning of failures was thus ambiguous and they were not included in the histograms except for one experiment with the largest quantal size. In this experiment, illustrated in Fig. 7, the measured number of failures conformed to that expected assuming binomial release statistics (Appendix).

The time course of stimulus-evoked IPSCs was almost independent of the peak amplitude. Averages of the IPSCs constituting the first, second, third and fourth peak of the histogram in Fig.5A are shown in Fig.6. Both the time constants of the two decay components as well as their relative amplitudes were comparable for IPSCs of each peak.
Effects of changing the extracellular Ca\(^{2+}\) and Mg\(^{2+}\) concentration on stimulus-evoked IPSCs

In the two cells tested, when the extracellular solution was changed from physiological saline (2 mM Ca\(^{2+}\); 1 mM Mg\(^{2+}\)) to a solution containing a lower Ca\(^{2+}\) (0.5 mM) and higher Mg\(^{2+}\) (2.5 mM) concentration, only one clear peak remained in the IPSC amplitude distribution. Fig. 7A shows the fit of a sum of Gaussians to the amplitude distribution measured in one cell during bath perfusion of solution containing the normal concentration of 2 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\). The mean peak separation for peaks 2-5 is 20.5 pA. In low Ca\(^{2+}\), high Mg\(^{2+}\), one peak remained which was best fitted by a single Gaussian with a mean of 19.7 pA (Fig. 7B). This is consistent with previous observations in the neuromuscular junction that extracellular Ca\(^{2+}\) and Mg\(^{2+}\) influence the release probability of quanta but not the quantal size (del Castillo & Katz, 1954).

Comparison of stimulus-evoked IPSC amplitude distributions of one cell at different voltages

Fig. 8 shows amplitude distributions from a cell that was held for long enough to obtain extended recordings at -50 mV (Fig. 8A), -100 mV and -120 mV (Fig. 8B). At -100 mV (not shown) and at -120 mV (Fig. 8B), the shapes of the distributions were very similar to the distribution of stimulus-evoked IPSCs recorded at -50 mV but with a greater separation between the peaks. At -50 mV the peak separation was 7.1±0.03 pA (n=851) while at -120 mV the peak separation was 14.9±0.64 pA (n=527). At -100 mV, the distribution obtained was somewhat less clear but did comply with the requirements for a quantal distribution (see Appendix). The peak separation at -100 mV was 12.5±0.63 pA (n = 610). This demonstrates an almost linear dependence of the peak IPSC amplitude on membrane potential, as would be expected if the IPSCs were mediated by Cl\(^-\) channels with no rectification in their instantaneous current-voltage relation (Bormann, Hamill & Sakmann, 1987). Note that the much larger sample sizes for this cell allowed a very small quantal size to be distinguished.
Comparison of spontaneously-occurring and stimulus-evoked IPSCs amplitude distributions in the same cell

Amplitudes of the spontaneously-occurring IPSCs which occurred in the time between stimulations were also measured in the same cells described in the previous two sections. In the cell shown in Fig. 7, a range of spontaneously-occurring IPSCs amplitudes was seen, but only the first peak contained enough data to be fitted with a Gaussian distribution. The mean of this fit was 18.1 pA. The peak separation measured for stimulus-evoked IPSCs in physiological saline was similar, falling at 20.5 pA (Fig. 7A). In the other cell, larger spontaneously-occurring IPSCs were more frequent and were also compared to stimulus-evoked IPSCs. Similar amplitude distributions were seen for both groups (Fig. 9). The peak separations in the distributions of stimulus-evoked and spontaneously-occurring IPSCs were 7.1 pA and 7.0 pA, respectively.

Taken together, these results show that, although the peak separation of quantal distributions varies between different neurones, it is consistent within any one cell when different populations of IPSCs are compared at the same membrane voltage.

Tests to confirm the biological origin of the quantal nature of stimulus-evoked IPSC amplitude distributions

Due to the limitations involved in holding cells in the whole-cell recording configuration for long periods without change in recording conditions, in most cases the sample sizes were fairly small (< 500 IPSCs per amplitude histogram). To confirm that the quantal distributions observed were not due to random occurrence as a result of small sample sizes, various checks were made. The tests listed in this section were all made using the experimental data. In addition, the Appendix details a further test using random selection of events from a simulated IPSC distribution not containing equidistant peaks.

Effect of selecting IPSCs for rise time or latency. In the two cells where the rise time distribution was broad, IPSCs were only accepted if the half rise time was less than 1
ms. This selection procedure excluded less than one third of the IPSCs and resulted in
peaks in the amplitude distribution being more clearly separated. In the one cell where
two populations were evident in the latency histogram, selection for latency was
necessary before a quantal distribution was seen (Fig. 10).

*Contribution of histogram bin size.* Fig. 11 shows the effect of displaying the data
previously shown in Fig. 7A with different bin sizes. Whereas the distribution was
somewhat clarified by use of a suitable bin size, the overall distribution and position of
peaks was very little affected. The possibility of such an artefact is anyway avoided by
fitting with the maximum likelihood method, which does not depend on binning.

*Consistency of distributions through a data set.* Fig. 12 shows the effect of analysing
subsets of the same total data set shown in Fig. 7A. Fig. 12A again shows the total data
set. Figs. 12B-C show the distributions of the first 175 IPSCs sampled and of the last
197 IPSCs respectively. Though the smaller numbers resulted in less clear distributions,
the peaks fall in the same places. If the quantal features of the distributions were an
artefact due to small sample size, different subsets of the data would not be expected to
show distributions with comparable peaks and increasing the sample size should
gradually smooth the data. These results in which the opposite trend is seen, are also
inconsistent with a statistical artefact and rule out shifts in the recording parameters with
time.

*Amplitude of miniature IPSCs*

IPSCs recorded in the presence of 1 μM TTX and in some cases 2 mM Mn$^{2+}$ to
block sodium and calcium action potentials usually occurred at a rate of only about 2-10
per minute. It was thus not possible to collect long enough stretches to obtain enough
miniature IPSCs for detailed quantal analysis in the same cells in which stimulus-evoked
IPSCs were recorded. However, one case is shown in which 43 miniature IPSCs were
recorded (Fig. 6B, filled columns) in a neurone from which an amplitude histogram of
stimulus-evoked IPSCs was also constructed. The least squares or maximum likelihood fits of the amplitude of miniature IPSCs with amplitudes less than 23 pA resulted in a mean of 13.8 pA. This may reflect a combination of miniature IPSCs from the stimulated synapse, in which the quantal size was measured to be 17.4 pA, and from other synapses on the same cell where the quantal size may be in the range of 7-12 pA. The stimulus-evoked IPSCs were completely blocked during recording of miniature IPSCs.

From 12 other cells, more than 100 miniature IPSCs per cell were collected in the presence of TTX. The distributions of miniature IPSC amplitudes shown in Fig. 13 are typical. In all cases a main peak was seen that was consistent with the small quantal sizes estimated by analysis of stimulus-evoked IPSCs. There was, however, always a "tail" of larger amplitude IPSCs (Fig. 13A). In 6 of the 12 miniature IPSC amplitude distributions, peaks were detected in the tail. Fig. 13B shows the most clearly quantal of these distributions in which the histogram could be well fitted with the sum of three equally spaced Gaussian curves.

No significant correlation was seen between rise time and amplitude of miniature IPSCs or between rise time and decay time constants of miniature IPSCs arguing against the possibility that miniature IPSCs were sampled from different, e.g. somatic or dendritic locations, respectively.

**GABA-activated single channel currents**

To estimate the number of postsynaptic channels that underlie a quantal synaptic response, the amplitudes of GABA-activated single channel currents in outside-out patches were measured under the same conditions as those used for the measurement of IPSCs in the whole-cell recording configuration. Fig. 14 shows examples of GABA-activated currents from an outside-out patch at -50 mV. The GABA-activated single channel currents in different patches fall into two main amplitude classes. The proportion of single channel currents with the two amplitudes varied between different patches.
ranging from almost pure populations of either subtype to mixtures of both subtypes (Fig. 15A-C).

The variation in the proportion of the occurrence of the two types of channels in different patches (Fig. 15D) suggests that they reflect the expression of two different GABAR channel subtypes in the soma membrane of granule cells, rather than activation of two sublevels of one GABAR channel type. The amplitude distribution of GABA-activated single channel currents (n = 1217) pooled from all patches collected at -50 mV (9 cells from 5 animals) was well fitted with the sum of two Gaussian distributions with means (± S.D.) of 0.70 ± 0.12 pA and 1.16 ± 0.18 pA. It is thus possible to estimate that the size of a quantal contribution of evoked IPSCs is the result of the simultaneous opening of between 6 and 30 single GABAR channels.
DISCUSSION

The application of patch clamp techniques to the recording of synaptic currents in hippocampal granule cells has increased the resolution sufficiently to allow the direct measurement of quantal IPSCs resulting from stimulation of a single presynaptic neurone. The experiments suggest that transmission at these synapses is quantal and that transmitter release is Ca\(^{2+}\) dependent as in other peripheral and central synapses (for reviews see Martin, 1966; Katz, 1969; Atwood & Wojtowicz, 1986; Korn & Faber, 1987; Augustine, Charlton & Smith, 1988; Redman, 1990; for quantal inhibitory transmission in hippocampus see Miles and Wong, 1984). Other features of the IPSCs, such as the small conductance change underlying quantal components of stimulus-evoked IPSCs, the small number of postsynaptic channels mediating a quantal event, the small variance of Gaussian curves fitted to IPSC amplitude histograms and the multiquantantal amplitudes of miniature IPSCs suggest that transmission at this synapse is characterized by properties that could be specific for this and possibly other mammalian CNS synapses.

Size of quantal conductance change

The first obvious difference between the present and previous studies of synaptic transmission at peripheral and central synapses is the small size of the postsynaptic current constituting a quantal contribution to a stimulus-evoked IPSC. The conductance change mediating a quantal event in granule cells is \(2-4 \times 10^{-10} \, \text{S}\). This value is about 1-2 orders of magnitude smaller than the values reported for the neuromuscular junction (\(10^{-7} \, \text{S}\); del Castillo & Katz, 1954; Takeuchi & Takeuchi, 1960), for inhibitory CNS synapses in the Mauthner cell of gold fish (\(3.5 \times 10^{-8} \, \text{S}\); Faber and Korn, 1982) and Müller cell in lamprey spinal cord (\(10^{-7} \, \text{S}\); Gold & Martin, 1983) as well as for inhibitory (\(5-8 \times 10^{-9} \, \text{S}\); Kuno & Weakly, 1972) and excitatory synapses (\(5 \times 10^{-9} \, \text{S}\); Finkel and Redman, 1983) in the motoneurone of cat spinal cord. It should, however, be noted that the high input resistance of hippocampal granule cells (> 1 G\(\Omega\)) will greatly increase the efficacy of a
small current flux at this synapse compared, for example, to the neuromuscular junction where the input resistance is less than one MΩ (Katz, 1966). Thus the relative efficacy of the conductance change would be greater at the synapse of hippocampal granule cells described here. Interestingly, the input resistances of Müller and Mauthner cells are low (like muscle in the low MΩ range). Thus this difference in the size of the conductance change underlying a quantal event in different synapses may reflect a matching of the size of quantal current to the input resistance of the postsynaptic cell.

**Number of channels activated during a quantal event.** The small conductance change resulting from a quantal event in granule cell synapses is due to the opening of a small number (< 30) of postsynaptic GABAR channels. This is about two orders of magnitude fewer than at other neuronal synapses of vertebrates. Gold and Martin (1983) estimated that the opening of about 1500 glycine receptor (GlyR) channels underlies the quantal IPSCs in Müller cells of lamprey spinal cord. Similarly, Korn, Burnod & Faber (1987) estimated 1400 GlyR channels underlying a quantal IPSC in the goldfish Mauthner cell. At the excitatory synapse activated by Ia afferents in motoneurones in cat spinal cord, release of a quantal EPSC at somatic synapses is generated by the opening of about 500-1000 channels activated by excitatory amino acids (Redman, 1990). Barker & McBurney (1979) estimated activation of 300 GABAR channels per quantal current, measured in cultured mouse spinal cord neurones. As TTX was not included in the bathing solution, these much larger 'unitary' currents may have been due to presynaptic action potentials and represent superpositions of several quantal events. Only one study previously suggested similarly small quantal sizes at neuronal synapses. Rang (1981) estimated that the opening of fewer than 100 AChR channels underlies the miniature excitatory synaptic currents in rat submandibular ganglion cells.

**Small variation in quantal size**

The variance of Gaussians fitted to IPSC amplitude histograms is larger than the variance of the background noise recording and thus indicates quantal variability as observed in other synapses. A difference between the amplitude distributions of IPSCs
reported here and, for example, end-plate potential amplitude distributions (Katz, 1969),
IPSC amplitude distributions in cat motoneurones (Kuno & Weakly, 1972) or goldfish
Müller cells (Gold & Martin, 1983) is, however, that the variance of Gaussian curves
does not appear to increase over successive peaks as expected for independent
superposition of quantal events. This apparent constancy of variance in incremental
amplitude peaks has been observed previously for amplitudes of evoked IPSCs in the
Mauthner cell (Korn et al. 1987) and was inferred for distributions of evoked EPSCs
measured in cat motoneurones (Jack, Redman & Wong, 1981).

The inability to detect an increase in the standard deviation of Gaussian curves
fitted to successive peaks in evoked IPSC amplitude histograms could result from the
relatively small sample size. In simulated IPSC amplitude histograms, taking into account
the experimentally determined background noise, peaks were detected if the standard
deviation $\sigma_q$ of the quantal event was not larger than 15% of the quantal event size $q$. An
increase in the variance of incremental peaks however was not seen consistently with
small sample sizes (see Appendix). In fact, when a curve representing a sum of
Gaussian curves, assuming that $\sigma_q/q$ is 15%, is superimposed on a stimulus-evoked
IPSC amplitude histogram, the fit appears satisfactory over 4-5 peaks (Fig. A4). This
estimate for $\sigma_q/q$ is within the range found at most other synapses but it is very small
when the number of channels generating a quantal event is considered. In the maximum
measured case of a granule cell, synapses with quantal sizes of around 20 pA (at a
membrane potential of -50 mV), a $\sigma_q/q$ of 15% amounts to a variation of only three
channel openings per quantal event.

The apparent lack of increase in variance could, however, also indicate that the
assumption that the larger multiquantal IPSCs result from the superposition of statistically
independent quantal events might not hold for granule cell synapses. Instead presynaptic
processes such as vesicle filling could be statistically coupled or the GABARs in the
postsynaptic densities could be arranged in multiples of a unit aggregate as discussed
below.
Possible sources of error

The small quantal conductance change, the small quantal variability and the constancy of variance lead to the question of whether the peaks in the IPSC amplitude distributions are genuine or whether they could reflect a statistical error, for example due to the relatively small number of observations. This problem has been addressed previously by Magleby and Miller (1981) in reference to the data of Kriebel and his coworkers (for review see Kriebel & Erxleben, 1984).

Several methods have been used to check for the possibility of statistical errors in the present data. When the data is divided into two groups according to time of recording rather than biological criteria, the resulting subdistributions show the same peaks as the distribution of the whole data set, albeit less distinctly. This demonstrates that a consistent distribution of amplitudes is seen throughout the recording, and the distribution is not dependent on the number of IPSCs sampled. This point was further checked by simulations. Although taking subsets of 400 random samples from a simulated smooth parent IPSC amplitude distribution occasionally results in an apparent quantal distribution (2/50 cases), subsets of these distributions did not show consistent peaks.

Strong evidence for the biological source of the quantal IPSC amplitude distributions comes also from the difference in peak separation between different cells but consistency within each cell, when different populations of IPSCs are observed. Although the peak separation varies between 7 pA and 20 pA in different cells, when stimulus-evoked and spontaneously occurring IPSCs are measured in the same cell, amplitude distributions with similar peak separation result. Furthermore, in low Ca\(^{2+}\) solution, the majority of stimulus-evoked IPSC amplitudes fall in a single peak corresponding to the first peak in the amplitude distribution of stimulus-evoked IPSCs from the same cell in physiological saline. In contrast, increasing the membrane potential results in a similarly shaped IPSC amplitude distribution but with greater peak separation. Additional experimental evidence comes from the improved resolution of peaks which results from selecting stimulus-evoked IPSCs for fast rise time or a narrow range of latency. Any instrumental artefact would not be expected to be correlated to these biological variables.

These observations are inconsistent with an instrumental variation or a random statistical effect. They show that the peaks in IPSC amplitude histograms, despite the small quantal size and the apparent constancy of the peaks, are not due to statistical error. They reflect the quantal transmission at this inhibitory CNS synapse.

Miniature IPSCs

Another feature of synaptic transmission at the synapse studied here that is different from what is observed at neuromuscular junctions, is the consistent observation of skewed amplitude distributions of miniature IPSCs. Skewed amplitude distributions have been seen only occasionally at mammalian neuromuscular synapses (Liley, 1957) but regularly at various neuronal synapses (e.g. Blackman, Ginsborg & Ray, 1963;
Martin & Pilar, 1964; Dennis, Harris & Kuffler, 1971; Bornstein, 1974; McLachlan, 1975, but see Kojima & Takahashi, 1985 for a Gaussian distribution of IPSPs). This presents an apparent inconsistency in that amplitude histograms of stimulus-evoked IPSCs could be fitted with sums of equally spaced Gaussians while the amplitude distributions of miniature IPSCs were skewed. Several explanations are possible for this.

If the synaptic inputs were spread out along the dendritic tree, such that some synapses were electrotonically distant from the recording pipette, skewed distributions could result. This is however unlikely, as factors causing such a distortion of IPSC amplitudes would be expected to affect miniature IPSC rise and decay times and no correlations were found between these variables.

An explanation which would be more consistent with the observations is that miniature IPSC amplitudes are also quantally distributed for example due to TTX and Mn$^{2+}$ insensitive action potentials or other mechanisms as discussed in Martin and Pilar (1964) and Martin (1966). Clearly quantal IPSC distributions were only seen in half of the experiments, presumably due to small sample sizes. Detection of mono and multiquantal peaks in the miniature IPSC amplitude histograms could also be obscured by the fact that the recorded miniature IPSCs may originate from several synapses of different presynaptic neurones which could vary in their quantal sizes. In one cell (Fig. 7) at low extracellular Ca$^{2+}$ stimulus-evoked IPSC amplitudes were described well by a single Gaussian, suggesting that quantal currents may show a single Gaussian amplitude distribution.

Models for synaptic transmission

It has been suggested that in CNS synapses presynaptic terminals operate in an "all or none" fashion, implying that an action potential in a single synaptic bouton contributes a single quantal event (Edwards, Redman & Walmsley, 1976; Faber & Korn, 1982; for review see Korn & Faber, 1987; Redman, 1990). Different mechanisms have been invoked to explain this all or none behaviour of a bouton. At the inhibitory synapse of Mauthner cells it has been attributed to a particular release mechanism where
at one bouton only a single vesicle is released upon a presynaptic action potential. This view is based on the observation that the number of boutons is almost identical to the number of release sites calculated from IPSC amplitude distributions (Korn et al. 1982; Korn & Faber, 1987). A corollary of this assumption was that the size of the quantal event is determined by the amount of transmitter released similar to what has been found at the neuromuscular junction (Hartzell, Kuffler & Yoshikami, 1975; Land, Salpeter & Salpeter, 1980). At excitatory synapses of cat motoneurones on the other hand the "all or none" behaviour of boutons has been attributed to a postsynaptic mechanism by assuming that following the release of a single vesicle, all postsynaptic receptors of the bouton are saturated (Jack et al. 1981; Redman, 1990). Therefore the size of a quantal event is determined by the number of available postsynaptic receptors. Differences in quantal size between different boutons of the same synapse were invoked and were attributed to different numbers of receptors in the postsynaptic densities.

It is difficult to propose a mechanism for the granule cell synapses studied here which assumes that during a quantal event not all postsynaptic receptors opposite a release site are saturated. In order to achieve the small variability in the size of the quantal event (± 1-3 channel openings), it would be necessary to propose that each vesicle contained almost the same small number of transmitter molecules (e.g. < 40) and that the synapse was structured so that every molecule released would diffuse across the synaptic cleft and bind to the postsynaptic receptors, with virtually no loss of transmitter. An additional problem with such a model is that the fast rise time of the observed IPSCs (half rise times < 1 ms) is inconsistent with release of a small number of transmitter molecules using conventional transmitter-receptor association and receptor isomerization rates (B.S., unpublished).

An alternative model more consistent with the present observations, would be that the subsynaptic membrane opposite a release site contains a regular number of GABAR channels, possibly in form of an aggregate, which cover a small area compared to the postsynaptic spread of transmitter. Release of a large number (thousands) of transmitter molecules from a vesicle would invariably saturate all available receptor binding sites and
cause an almost synchronous opening of all GABAR channels. The size of a quantal event would therefore be determined by the number of available GABAR channels opposite the release site. Assuming that the synaptic contact between pre- and postsynaptic neurone consisted of many independent release sites with usually one GABAR channel aggregate under each site, a regular repetitive IPSC amplitude distribution could be generated. To account for multiquantal miniature IPSCs one possibility would be that spontaneous release from several sites is synchronized. Alternatively some boutons could have several GABAR aggregates opposite a release site which would all be saturated by the content of a single vesicle. This hypothesis could account for both the occurrence of multiquantal miniature IPSCs and for the constant variance of incremental IPSC amplitude peaks. In any case, the very similar time course of miniature IPSCs and of stimulus-evoked IPSCs of different sizes implies that different receptor aggregates respond to transmitter release in a highly similar fashion.

Inhibitory circuitry

Another surprising observation is that nearly all cells in the granule cell layer appear to elicit IPSCs in nearly all other cells. This is inconsistent with the accepted model for hippocampal circuitry which suggests that most of the cells in the cell layer of the dentate gyrus are granule cells which send excitatory projections to the CA3 pyramidal cell layer (for review see Dingledine, 1984). The stimulation is certainly local as moving the stimulus pipette away from the cell soma as little as two micrometer generally abolishes the response. Further, the response is apparently dependent on a presynaptic action potential since it is prevented by the sodium channel blocker TTX. However it is possible that each cell is densely covered with axon terminals from a branched interneurone and that the extracellular stimulation results in antidromic action potentials in the axon which, via another branch, reaches the recorded neurone. It will be necessary to stimulate presynaptic neurones intracellularly or in whole-cell configuration to be sure of the effective stimulus arrangement.
Elementary events

With respect to the postsynaptic mechanism, it should also be noted that the GABA-activated currents, measured in outside-out patches taken from rat brain slices, showed only two different conductance states in contrast with the great variety previously reported in various other preparations of rodent cultured neurones (Bormann, Sakmann & Seifert, 1983; Bormann et al. 1987; MacDonald, Rogers & Twyman, 1989; Smith, Zoreq & McBurney, 1989). In hippocampal granule cells, single channel analysis revealed only two common conductance levels (14 and 23 pS), probably representing two different subtypes of GABAR channels. *In situ* hybridization experiments on rat dentate gyrus showed expression of mRNAs encoding four different GABAR subunits (Séquier et al. 1988; Shivers et al. 1989). It has been reported that GABAR channel subtypes with different conductances are assembled from different GABAR subunit combinations (Verdoorn, Draguhn, Sakmann, Pritchett & Seeburg, 1989). The two functionally different GABAR channels described here may represent two channel subtypes assembled from different subunit combinations.

From the present experiments it is not possible to determine whether one or both of the GABAR channel subtypes measured from somal membrane patches mediate the IPSCs. It is interesting to speculate that both channel types could mediate synaptic events but at different synapses. Thus at -50 mV, the synapses which show the smaller quantal size (7 - 12 pA) may comprise only openings of the lower conductance channel (0.7 pA) and openings of the larger conductance channels (1.16 pA) may mediate IPSCs at synapses where a larger quantal size is observed (17.4 - 20.5 pA). If this were the case, the number of channels underlying a quantal event would be 10 - 17 in the case of the smaller IPSCs and 15 - 18 in the case of the larger IPSCs.

Conclusions

The quantal nature of transmitter-activated postsynaptic conductances at a CNS synapse is directly demonstrated and the quantal size is shown to be small in comparison to other synapses. This reflects the fact that only a small number of postsynaptic GABAR
channels is activated by a quantum of transmitter. The small number of postsynaptic channels mediating a quantal IPSC, the small variation in quantal size and the fast rise of the IPSCs are consistent with an all or none hypothesis of synaptic transmission at a single synaptic bouton where the number of postsynaptic channels determines the size of a quantal event.
ACKNOWLEDGEMENTS

We would like to acknowledge the contribution of K. Bauer for the programs used for measuring the IPSCs. We would also like to thank Professor D. Colquhoun for valuable advice on statistics and Professor A.R. Martin for his suggestion about the matching of cell input resistance and quantal size. We thank Drs. P. Martin, T. Verdoorn, M. Jackson and R. Rahamimoff for reading the manuscript.
APPENDIX

The purpose of the Appendix is to use simulations of IPSC amplitude histograms to assess the random occurrence of peaks in essentially non quantal distributions and to derive an upper estimate of the quantal variability that, given the experimental background noise, would still allow the detection of several consecutive peaks in IPSC histograms. Finally it is shown, for one cell, that the IPSC amplitude distribution is consistent with a release mechanism following binominal statistics as inferred for other CNS synapses.

Assessment of peaks in amplitude histograms

Histograms of experimental and simulated amplitude distributions were first assessed by eye. If apparently equidistant peaks were detectable, the data were fitted with a sum of Gaussians distributions where only the number of Gaussians and the fitting range were restricted. A mean peak separation $q$ (in pA) was calculated from the means of the fitted distributions. For the data shown in Table 1, the mean $M(k)$ of each of the successive peaks, numbered $k=2-5$, was divided by its peak number $(k)$. Mean peak separation $(q)$ was calculated according to:

$$q = \frac{1}{4} \sum M(k)/k; \text{ for } k=2-5 \text{ in experimental histograms.}$$

The peaks were considered to be equidistant if the standard deviation of the weighted means $M(k)/k$ was less than 5% of $q$. This was the case for all experiments listed in Table 1.

A further test was then applied to obtain an additional criterion for the occurrence of peaks. The autocorrelation function (ACF) of the amplitude histograms was calculated according to Aseltine (1958) using the expression:

$$ACF(j) = \frac{\sum H(i) \ast H(i+j)}{\sum H(i) \ast H(i)}$$
where $H(i)$ is the height of the $i$-th bin which is centred at the amplitude value given by $i \times$ bin width. The shift along the x-axis is given by $j \times$ bin width (interval in pA).

The ACF was compared with a smoothed autocorrelation function in which each point was replaced by a five point rolling average. The two functions were superimposed and if the ACF showed at least two consecutive and equally spaced peaks and dips (as illustrated in Fig. A1A) the amplitudes were judged to be quantally distributed.

**Random occurrence of quantal distributions**

To examine the random occurrence of quantal distributions in subsets of an essentially non-quantal parental amplitude distribution subsets consisting of 400 events were chosen randomly from a much larger parental distribution and analyzed in the same way as the experimental IPSC histograms. A smooth parental distribution of 50,000 events was created from the following equation:

$$H(a)=900*\exp\left(-\frac{a}{100}1.5\right)\times(1-\exp\left(-5\times\frac{a}{100}2\right)).$$

This results in a smooth distribution (i.e. without peaks), the overall shape of which was similar to the envelope of the histogram shown in Fig. 7A. Here $a$ is defined as amplitude in bins of 1 pA and $H(a)$ is the proportional height of bin $a$. Fifty subsets of 400 events were selected from the parent population using the random number generator described by Wichman and Hill (1985), kindly supplied by D. Colquhoun, University College London. If, upon visual inspection, histograms of these sub-distributions showed any tendency towards containing equidistant peaks, they were further tested using the criteria described above. From the fifty subsets only two fitted the criterion for equal peak separation but they did not show repetitive peaks in their ACFs. In addition, when these distributions were divided into subsets of 200 events, consistent peaks were not seen. These simulations thus demonstrate that random occurrence is an unlikely explanation for the peaks in experimental IPSC amplitude histograms.
Variation of successive peaks in amplitude distributions

On inspection of the experimental IPSC amplitude distributions no clear trend is seen in the standard deviation of the Gaussians fitted to successive peaks (Table 1). Since such an increase is expected if it is assumed that multiquantal peaks in IPSC amplitude histograms result from the independent superposition of quantal events, two questions were investigated by simulations. Firstly, what would be an upper estimate of variation in quantal size which would still allow detection of 4-5 consecutive peaks as seen in IPSC amplitude histograms, given the measured background noise variance? Secondly, is the apparent constancy of the variance of Gaussians fitted to successive peaks real and therefore indicating that either quantal variance is small with respect to background variance or that quantal events do not superimpose independently or could the constancy be an apparent one as a result of relatively small sample sizes?

Sources of variation. The standard deviation of Gaussians fitted to peaks of IPSC amplitude histograms can be attributed to two independent sources: 1. Standard deviation due to instrumental background noise (σ_N) which would be expected to remain constant throughout peaks. 2. Standard deviation arising from biological sources (σ_Q) such as vesicle filling, loss of transmitter during diffusion across the synapse, variability in channel conductance, receptor density and fluctuations in the number of successful transmitter-receptor binding events. If quantal events superimpose independently, as shown for the neuromuscular junction (del Castillo and Katz, 1954; Boyd and Martin, 1956), then the variance of Gaussians fitted to incremental peaks is expected to increase with successive peaks.

Thus, if these same assumptions hold for quantal transmission at the granule cell synapse studied here, the standard deviation S.D.(k) of the Gaussian fitted to peak k in the IPSC amplitude histogram would be:

\[ S.D.(k) = \sqrt{\sigma_N^2 + k \cdot \sigma_Q^2} \]
Contribution of background noise. To estimate the contribution of the background noise (including sampling error) to the shape of Gaussians fitted to IPSC amplitude histograms, IPSCs of 20, 40, 60, 80 and 100 pA size were simulated and added to a baseline noise recording (using the whole-cell recording configuration). These simulated currents (rise time 1 ms, decay time 3 ms) were filtered at 2 kHz (-3 dB), sampled at 10 kHz and measured using exactly the same methods as for the experimental data. Fig. A2 shows one such distribution. From two independent runs using baselines from two of the experimental cells, the mean of the standard deviations of the ten resulting peaks (five from each trial) was 2.5 pA with a range of 1.8 to 3.0 pA. This was very close to \( \sigma_N \) assessed by calculating amplitude distributions of 100 stretches of 5 ms of recorded baseline noise. This estimate \( \sigma_N \) was made for all cells listed in Table 1 and gave an average of 2.5 pA with a range of 1.9 to 3.2 pA. For subsequent simulations a value of 2.5 pA was assumed for \( \sigma_N \).

Contribution of quantal variation. To estimate the quantal variability that would still allow detection of peaks in IPSC amplitude histograms, a parental distribution of 50,000 events was created with the events distributed as the sum of 5 Gaussians. The Gaussians had means of 20, 40, 60, 80 and 100 pA and relative peak amplitudes similar to the data shown in Fig.7A. The value of \( \sigma_Q \) was varied between 2 pA and 5 pA (10% to 25% of quantal size) and \( \sigma_N \) was assumed to be 2.5 pA. In 45 random subsets consisting of 400 events, 7/9 distributions showed 4-5 clear peaks if \( \sigma_Q \) was 15% of the quantal size. Increasing \( \sigma_Q/q \) to 18% resulted in subsets with no more than the first three peaks detectable. Thus the upper limit of \( \sigma_Q \) which still allows detection of clear peaks in simulated amplitude histograms which are comparable to those obtained experimentally, is about 15% of the quantal size.

To assess whether the expected increase in standard deviation of Gaussians fitted to incremental peaks in IPSC amplitude histograms would be detectable with small sample sizes, the curves fitted to the subsets were examined. Fig. A3 shows results from
one simulation of nine subsets. Despite the fact that the parental distributions had
variances that increased linearly over successive peaks (\(\sigma_q/q\) was 15 %) such an increase
was not consistently detectable in the smaller subsets.

The simulations suggest that despite the apparently constant variance of the
Gaussians fitted to successive peaks in experimental IPSC amplitude histograms, the
results are not inconsistent with the view that these IPSC amplitude histograms could
represent subsets of a parental distribution where the quantal size is indeed variable but
where \(\sigma_q\) is not much larger than 15% of the quantal size and where the larger peaks
represent independent superpositions of quantal events. To illustrate this point further,
Fig. A4 shows a calculated curve of the sum of 6 Gaussian distributions superimposed
on an experimental IPSC amplitude histogram. The assumed value of \(\sigma_q/q\) was 15% and
this calculated curve appears to give a good visual fit of the data over the first 4-5 peaks.

**Release statistics**

A further argument supporting the view that IPSC amplitude distributions reflect the
quantal nature of synaptic transmission in granule cells derives from the statistical
analysis of the amplitude distributions. In one cell several clear peaks were visible in the
amplitude histogram of IPSCs measured in physiological saline (Fig.7A). In low
extracellular Ca\(^{2+}\) the IPSC amplitudes fell into a single peak (Fig.7B) with a mean
amplitude similar to the peak separation of the multiquantal histogram. This suggested
that the larger peaks are in fact superpositions of a quantal event with normally distributed
amplitude.

The probabilities of occurrence of different IPSC amplitudes and of the number of
failures were fitted, assuming that the release of quanta follows binomial statistics
(Martin, 1966). The number of measured failures was seen as predicted by binomial
statistics. The series of areas can be fitted well with a binomial model with \(n = 40, p = 0.1\). These estimates are within the range of estimates at other CNS inhibitory synapses
(Korn, Mallet, Triller and Faber, 1982; Korn and Faber, 1987).
REFERENCES


FIGURES

Fig. 1. Examples of miniature and stimulus-evoked IPSCs. Recording at -50 mV membrane potential from a hippocampal granule cell of a 19 day old rat. A, Three selected traces of miniature IPSCs recorded in the presence of TTX (1 μM). The traces were aligned by the peak of first miniature IPSC. B, Traces of consecutive IPSCs evoked by extracellular stimulation of a nearby neurone. The stimulus (5 V, 200 μs) was applied at a rate of 1 s⁻¹. All traces were sampled at 10 kHz and filtered at 2 kHz (-3 dB). The scale bars refer to all traces. Thin lines represent baseline, inward currents are downwards.
Fig. 1, Edwards et al.

A

B

30pA

30ms
Fig. 2. Decay of miniature and stimulus-evoked IPSCs. A, Average time course of 40 miniature IPSCs recorded at -50 mV membrane potential in the presence of TTX (1 μM). The average trace was obtained by aligning individual miniature IPSCs at their peaks. B, Record of a single stimulus-evoked IPSC at -50 mV. The currents were digitized at 0.5 ms per point and low pass filtered at 2 kHz (-3 dB). The continuous lines superimposed on the decay time course of both traces represent the sum of two exponentials. For miniature IPSCs the exponentials had time constants of $\tau_1$: 2.7 ms and $\tau_2$: 53.6 ms. The amplitudes were $A_1$: 10 pA; and $A_2$: 15 pA. The stimulus-evoked IPSCs were also fitted with two exponentials with the average time constants $\tau_1$: 2.3 ms and $\tau_2$: 57.5 ms. The amplitudes were $A_1$: 16 pA and $A_2$: 73 pA.
Fig. 3. Rise time of stimulus-evoked IPSCs. A, Digitized record of a stimulated IPSC shown at high time resolution. The initial deflections represent the stimulus artefact which is followed by the rising phase of the IPSC. The half rise time was measured as the time for a current to reach half its maximum amplitude starting from the last sample point before the rise (0.4 ms for this current). The dashed line represents a digitized record of the step response of the recording system under similar conditions. It is aligned with the IPSC record to facilitate comparison. The record was filtered at 2 kHz (-3 dB) and sampled at 100 μs per point. B, Histogram of half rise times of stimulus-evoked IPSCs measured in a neurone of the granule cell layer of a 21 day old rat. n = 171.
Fig. 3, Edwards et al.

A

Half Rise Time (ms)

50pA

2ms

B

Number of Events

Half Rise Time (ms)
Fig. 4. Fluctuation in peak amplitude of consecutive stimulus-evoked IPSCs. The three panels represent recordings of 9 responses to consecutive stimulations applied to a nearby neurone. In each panel three consecutive traces were aligned by the trigger pulse given 10 ms before the stimulus was applied. The initial biphasic deflections represent the stimulus artefact. Stimulus frequency was 1 s\(^{-1}\), stimulus intensity 5 V and duration 200 \(\mu\)s. Stimulus was applied via a patch pipette placed on the surface of a nearby neurone. The currents were filtered at 2 kHz (-3dB) and sampled at 10 kHz.
Fig. 5. Quantal distribution of stimulus-evoked IPSC amplitudes. All experiments at -50 mV membrane potential. A-C. The three histograms shown represent the distribution of IPSC amplitudes collected from three different neurones, in physiological saline (containing 2 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$). The thick smooth lines superimposed on the histogram represent the sum of Gaussian distributions which best fit the data. The thin smooth lines represent the individual Gaussians of this fit. The fit was restricted only by the number of Gaussian distributions fitted and the amplitude range to be fitted (A, 25-95 pA; B, 25-115 pA; C, 0-82 pA). For ease of comparison the bin width shown is 3 pA for all panels. Details of the fitted Gaussians are listed in Table 1. The standard deviations of background noise, $\sigma_N$, were for A: 5 pA, B: 2.8 pA, C: 2.1 pA. The filled columns in B represent the amplitudes of 43 miniature IPSCs recorded in the same cell in the presence of 0.5 μM TTX. The peak of the miniature IPSC amplitude distribution is 13.8 pA.
Fig. 6. Average decay time course of stimulus-evoked IPSCs. 40 IPSCs falling into the amplitude range of different peaks of the amplitude histogram shown in Fig. 5B were averaged. -50 mV membrane potential. The decay time course in the averaged IPSCs is fitted by the sum of two exponential components. The continuous line represents the baseline. The average decay time constants $\tau_1$ and $\tau_2$ of the fast and slow decay components, their amplitudes $A_1$ and $A_2$ and the ratio of $A_1$ to the peak amplitude $A_p$ were, for IPSCs falling into the range of A (20-40 pA), $\tau_1$: 1.88 ms, $\tau_2$: 78 ms, $A_1$: 14 pA, $A_2$: 19 pA and $A_1/A_p$: 42%. B (45-60 pA), $\tau_1$: 1.85 ms, $\tau_2$: 71 ms, $A_1$: 23 pA, $A_2$: 29 pA and $A_1/A_p$: 44%. C (65-80 pA), $\tau_1$: 2.8 ms, $\tau_2$: 62 ms, $A_1$: 29 pA, $A_2$: 41 pA and $A_1/A_p$: 41%. D (IPSCs with amplitudes larger than 90 pA), $\tau_1$: 3.06 ms and $\tau_2$: 66 ms, $A_1$: 47 pA, $A_2$: 60 pA and $A_1/A_p$: 44%. The sum of the two components is represented by the thin line, fits were made by eye.
Fig. 7. Effect of changing extracellular Ca\(^{2+}\) and Mg\(^{2+}\) concentration on amplitudes of stimulus-evoked IPSCs. Both experiments at -50 mV holding potential. A, Amplitude distribution of stimulus-evoked IPSCs in physiological saline (containing 2 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\)). Mean ±S.D of the Gaussians represented by the thin lines are listed in Table 1. Mean peak separation is 20.7 pA, \(\sigma_N\) was 2.4 pA. The thick line represents the sum of five Gaussians. The number of stimuli applied was 369, the number of failures was 7. B, The amplitude distribution of stimulus-evoked IPSCs recorded in the same cell after changing the external solution to saline containing 0.5 mM Ca\(^{2+}\) and 2.5 mM Mg\(^{2+}\). The mean ±S.D. of the only clear peak is 19.7 ± 8.0 pA (fitted from 0-40 pA).
Fig. 8. Voltage dependence of quantal size of stimulus-evoked IPSCs. The membrane potential of the cell was held at -50 mV for 10 min during stimulation, then was changed to -120 mV for ten minutes of stimulation and then returned to -50 mV for a further 3 min. Later various other voltages were tested also and periods at -50 mV membrane potential were always interposed for control. The reversal potential of stimulus-evoked IPSCs was between 0 and -5 mV. A, Amplitude distribution of stimulus-evoked IPSCs recorded at a holding potential of -50 mV (n=851). Thick line represents sum of five Gaussian distributions. Peak separation (as defined in the Appendix) is 7.1 pA. Fitted range 11-46 pA. B, Amplitude distribution of stimulus-evoked IPSCs from the same cell as shown in A but measured at -120 mV (n=527). Peak separation is 14.9 pA. Fitted range 0-95 pA. Note the difference in scales of the x-axes of the two histograms.
Fig. 8, Edwards et al.

Number of Events

Amplitude (pA)

-50 mV

-120 mV

Number of Events
Fig. 9. Comparison between amplitude distributions of stimulus-evoked and spontaneously-occurring IPSCs. Both measurements at -50 mV in the same neurone. A, Amplitude distribution of stimulus-evoked IPSCs (same cell as shown in Fig. 8A). B, Amplitude distribution of the spontaneously-occurring IPSCs recorded between the stimulus-evoked IPSCs (n=406). Fitted range 18-44 pA. Peak separation of spontaneously-occurring IPSCs was 7.0 pA as compared to stimulus-evoked IPSCs which were characterized by a peak separation of 7.1 pA, $\sigma_N$ was 3.2 pA. Spontaneously-occurring IPSCs presumably consist of a mixture of miniature IPSCs and IPSCs evoked by spontaneously-occurring action potentials recorded from all synapses on or near the cell soma.
Fig. 9, Edwards et al.

Number of Events vs. Amplitude (pA)
Fig. 10. Effect of selecting for latency on amplitude distributions of stimulus-evoked IPSCs. A, Distributions of latencies between the beginning of the stimulus artefact and the beginning of the IPSC in the one neurone where the distribution of latencies showed two populations. B, Amplitude distribution of all stimulus-evoked IPSCs recorded in the same neurone. C, Amplitude distribution of stimulus-evoked IPSCs in the same neurone, including only IPSCs with latencies of 3.8 - 6.5 ms (see Table 1, $\sigma_N$ was 1.9 pA). Fitted range 12-56 pA. Even the shorter latencies measured in this neurone were somewhat longer than measured in other neurones (< 3 ms) suggesting stimulation of a polysynaptic pathway resulting in activation of two different presynaptic neurones.
Fig. 10. Edwards et al.

A

Number of Events vs. Latency (ms)

B

Number of Events vs. Amplitude (pA)

C

Number of Events vs. Amplitude (pA)
Fig. 11. Effect of different bin sizes on the shape of stimulus-evoked IPSC amplitude distributions. Consecutive panels show the same data as shown in Fig. 7A sorted into bins ranging from 2-5 pA as indicated by the number in the top right section of each histogram. Least square fits (fit range 0-110 pA) were run on each histogram and the peak separation for peaks 1-5 were: 2 pA bins, 20.7 pA; 3 pA bins, 20.7 pA; 4 pA bins, 20.1 pA; 5 pA bins, 20.8 pA. Note that use of a maximum likelihood fit avoids the problem of binning as unbinned amplitudes are used. The peak separation for the maximum likelihood fit of the same data was 20.4 pA.
Fig. 11, Edwards et al.

Number of Events

Amplitude (pA)

2 pA

3 pA

4 pA

5 pA
Fig. 12. Effect of sampling subsets of data on the shape of stimulus-evoked IPSC amplitude distributions. A, Amplitude histogram of stimulus-evoked IPSCs (the same total data set as shown in Fig. 7A). Peak separation (as defined in Appendix) 20.7 pA. B,C, Subsets of the data shown in panel A. B, Amplitude histogram of the first 175 IPSCs sampled. Peak separation 20.3 pA. C, Amplitude histogram of the remaining 197 IPSCs sampled. Peak separation 20.7 pA. Fitted range 0-110 pA.
Fig. 13. Distributions of miniature IPSC amplitudes. Both experiments at -50 mV membrane potential. A, Histogram of miniature IPSC amplitudes collected over 10 min recording time (n = 198). Bin width is 3 pA. The bath solution was as physiological saline and contained TTX (1 μM) to block sodium dependent action potentials and Mn^{2+} (2 mM) with no added Ca^{2+} to block calcium dependent action potentials. B, Amplitude distribution of miniature IPSCs collected over 15 min from a different cell in physiological saline and TTX (1 μM). The thick line represents the sum of three Gaussian distributions having means ±S.D. of 10.7 ± 2.5 pA, 19.2 ± 2.4 pA and 31.0 ± 4 pA respectively (n = 189). The inclusion of Mn^{2+} did not influence the appearance of several peaks in the distribution.
Fig. 13, Edwards et al.

A

Amplitude (pA)

Number of Events

B

Amplitude (pA)

Number of Events
Fig. 14. Examples of GABA-activated single channel currents. Single channel currents at -50 mV in an outside-out patch isolated from a granule cell of a 20 day old rat when 1 μM GABA was applied to the patch from a nearby patch electrode. The recording pipette contained the standard intracellular solution with N-methyl-glucamine as the main cation. In this patch, channels were activated with conductances of both 14 pS (upper trace) and 23 pS (lower trace). The current was sampled at 0.5 ms per point.
Fig. 15. Relative frequency of openings of the 14 and 23 pS conductance states of GABAR channels. Different patches at -50 mV. A, Distribution of single channel current amplitudes recorded in a patch in which 14 and 23 pS conductance channels opened with similar frequency. B, Distribution of single channel amplitudes in a patch in which only 14 pS conductance channels opened. C, Distribution of single channel openings from a patch in which openings of 23 pS conductance channel predominated. D, Percentages of current amplitudes in different patches which constitute separate Gaussians in the distribution of GABA-activated single channel current amplitudes. The positions of the horizontal lines on the x-axis represent the means ±S.D. of the current amplitudes of each fitted Gaussian distribution. In 6 of the 9 patches both 14 pS and 23 pS conductance channels were found while in the other three only 14 pS channel openings occurred. The percentage of lower and higher conductance channel openings occurring in each patch is represented on the y-axis. The numbers on the graph represent the total number of events recorded in each patch. Six of these numbers appear twice. They represent the percentage of channel openings of each conductance seen in the same patch.
Fig. 15. Edwards et al.
Fig. A1. Criterion for equidistant peaks. A, An example of a simulated distribution with equidistant peaks. Thick line represents the sum of the underlying Gaussians (thin lines). B, Symbols connected by a line represent the autocorrelation function (ACF) of the simulated data shown in panel A. The curve without symbols represents the 'smoothed' ACF, i.e. the curve created by averaging each point of the original ACF and the two points on either side of it. Note that the original ACF crosses the smoothed ACF at regular intervals resulting in three repeats of equally spaced peaks and dips.
Fig. A1. Edwards et al.

A

Number of Events

Amplitude (pA)

0 20 40 60 80 100 120

B

ACF

Interval (pA)

0 20 40 60 80 120

peak
dip

ACF

smoothed ACF
Fig. A2. Amplitude distribution of 400 discretely distributed simulated IPSCs with amplitudes of 20, 40, 60, 80 and 100 pA measured after addition of experimental baseline noise filtered at 2 kHz (-3dB) and sampled at 10 kHz. The baseline recording is that of cell 4 listed in Table 1. The numbers above each peak represent mean±S.D. of the fitted Gaussians. The mean value of the S.D.s of Gaussians fitted to the peaks is 2.5 pA. All conditions were the same as for measurement of experimental data.
Fig. A2, Edwards et al.

Amplitude (pA)

Number of Events

21 ± 2.5
40.6 ± 2.6
60.7 ± 2.0
80.8 ± 2.5
100.7 ± 2.2
Fig. A3. Apparent constancy of standard deviations of Gaussians fitted to successive peaks of simulated IPSC amplitude histograms. A, Subset consisting of 400 events chosen randomly from a parent population consisting of the sum of 8 Gaussian distributions with a peak separation $q$ of 20 pA. The background noise $\sigma_N$ was 2.5 pA and $\sigma_q$ was assumed to be 3 pA (i.e. 15% of the peak separation). Thick line represents the sum of the six Gaussian distributions (thin lines) as fitted by the least squares method. B, S.D.(k) of Gaussians fitted to successive peaks (k=1-6) in amplitude histograms of seven subsets (of 400 events each) chosen randomly from the parent population described in the text. The Gaussians fitted to the histograms did not show consistently increasing S.D.s with incremental peaks. The graph includes only the S.D.s of subsets, represented by different symbols in which at least 3 clear peaks were evident in the histogram. The symbols connected by the broken line represent the S.D.s of Gaussians fitted to one subset of 10,000 events. When the sample size of the subset was \( \geq 1000 \) an increase in the S.D.s of incremental peaks was seen regularly.
Fig. A4. Comparison of an experimental IPSC amplitude distribution and a calculated curve assuming independent superimposition of quantal events. The histogram represents the same experimentally obtained amplitude distribution as shown in Fig. 7A. The thick line represents the sum of 6 Gaussian distributions (thin lines). The $\sigma_N$ was set at 2.5 pA and the $\sigma_Q$ was assumed to be 3 pA (15% of peak separation, Table 1). The number of failures was 7 and the number $N(k)$ of IPSCs constituting the various peaks, as derived from the free fit shown in Fig. 7A were, for the first five peaks $N(1): 30; N(2): 60; N(3): 79; N(4): 75; N(5): 56; N(6):$ The expected values from a binomial fit (with binomial $n$ and $p$ of 40 and 0.1 respectively) were: 7 failures and $N(1): 30; N(2): 64; N(3): 75; N(4): 66; N(5): 59.$
Explanation of Table 1

For each cell the results from fitting Gaussian distributions to the stimulus-evoked IPSC amplitudes are listed. The first column lists consecutive cells and the figure in which the amplitude histogram is shown. n is the number of IPSC amplitudes used for the fit. PEAK NO refers to consecutive peaks in the IPSC amplitude histogram identified by eye. LEAST SQUARES: The means ± S.D. (in pA) of Gaussians fitted to peaks 2-5 of amplitude histograms. Note that in some cases in the figures more peaks have been included in the fit where data was available, resulting in slightly different peak separations (see figure legends). PEAK SEPARATION: Estimate of the quantal size q (in pA), obtained from least squares fits of IPSC amplitude histograms or maximum likelihood fits to all IPSC amplitudes. The value of q was calculated as the mean amplitude interval between peaks as described in the Appendix. In all experiments listed the standard deviation of the mean peak separation was less than 5% of the mean peak separation. EQUAL PEAKS: For ease of comparison, the estimated peak separation was multiplied by the peak number resulting in expected values for equidistant peaks. MAXIMUM LIKELIHOOD: The means ± S.D. of Gaussians fitted to peaks 2-5 of all amplitudes in the same experiment. The background noise standard deviations are given in the respective legends. For cells 7 and 8 the values were 2.8 pA and 2.6 pA.
TABLE 1
Means and standard deviations of Gaussian curves fitted to amplitude distributions of stimulus-evoked IPSCs (all mean values in pA)

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PLATES

Explanation of Plate.

Structure of granule cells in the 3 week old rat hippocampus. A, Photograph of granule cells in the dentate gyrus as seen during the experiment, with patch pipettes in the recording and stimulus configuration. Cells in the granule cell layer can be clearly seen. Whole-cell recording configuration is first established on one neurone (left hand pipette). A second pipette (right hand pipette, containing physiological saline) is then placed as close as possible to the surface of another cell and an electrical pulse is applied via this pipette. B, A 3-dimensional surface reconstruction of a typical granule cell filled with Lucifer Yellow (1.5 mg/ml) included in the filling solution of the whole-cell recording pipette. The slice was then mounted in Moviol solution (Osborn, 1981), without fixation and 35 optical sections were scanned (63x oil immersion objective, 0.5 μm intervals between sections with a resolution of 0.2 μm in the horizontal plane), using a ‘Phoibos’ Laser scanning microscope (Sarastro, Stockholm, Sweden). The 3-D image reconstruction of the neurone reveals the extensive, intact, but very fine dendritic tree. The width of the granule cell soma is about 10 μm. Scale bar refers to A and B.
Overview and Summary of Results

A comparison of the results obtained using the two methods and an overview of the thesis as a whole.

The purpose of this study was to investigate transmission at an identified synapse in the central nervous system. The study was started using the single electrode voltage clamp technique and a standard slice preparation. The development of a technique for using patch clamp techniques in brain slices allowed the study of hippocampal IPSCs to be continued with a much higher resolution. Various differences were seen in the currents recorded using the two different techniques. The results are not directly comparable as the intracellular recordings were made from hippocampal CA1 cells of adult (more than three month old) rats, while the patch clamp recordings were made from hippocampal granule cells of three week old rats. A few patch clamp recordings have been made on adult CA1 cells however and though no detailed analysis was done on these data, the overall features of currents measured in the two cell types were very similar. In both, a fast rise was followed by a slower double exponential decay and the currents remaining in the presence of tetrodotoxin were of a similar size (mostly < 40 pA). Thus the use of a different synapse and the developmental stage is not sufficient to explain the differences in the currents recorded with the two techniques.

For the intracellular recordings, the inhibitory input to hippocampal CA1 cells was chosen because these cells are relatively easily impaled with a microelectrode and the synapses are thought to be located on, or near the cell soma and thus can be well voltage clamped. The resolution obtainable with the single electrode voltage clamp technique allowed only limited analysis. Thus only decay times of currents were measured. The higher resolution of currents obtained by using patch clamp techniques makes it clear
that decay times were also not well resolved with the single electrode voltage clamp (as discussed below). However, while the absolute values measured may not be accurate, the conditions were the same throughout the first study and thus comparisons within this paper remain valid.

The development of a technique for making patch clamp recordings from synaptically connected cells in brain slices presents various new possibilities as discussed in second paper. In the context of the present study the great increase in resolution of synaptic currents allowed a much more accurate analysis, and thus a basic description of IPSCs which was not previously possible. In order to allow direct comparison with the currents described in first paper, this patch clamp study was started using spontaneous IPSCs measured in cells in the CA1 region. It was found, however that the small round cells in the granule cell region had notably higher input resistances and thus resolution of currents in these cells was even higher than in CA1 cells. The study was thus continued using cells of the hippocampal granule cell layer.

In the future it will be of interest further to study the seasonal phenomenon described in the first paper using patch clamp techniques. However it seemed most appropriate to start using this new technique for a more basic analysis of these currents, especially in the light of the differences seen between the currents measured using the two different methods. The consistent appearance of a fast component in the decay phase, not previously reported and the longer time for the total decay are presumably both due to the improved resolution when using the patch clamp technique. Under the recording and filtering conditions in the seasonal study, the rise time of currents appeared to be approximately 2 ms, in which time the first component of the decay would be nearly finished. Thus not only would the fast component be largely lost but the peak of the current would depend on the combination of rise and decay. In addition,
the background noise of > 30 pA, superimposed throughout the current, would tend to cause an underestimate of the tail of the current where it disappears into the baseline. Thus the fitted exponential would be a result of the residue of the fast component and the first section of the slow component of the underlying current. The resulting time constant would fall between the time constants of the underlying exponentials. Another factor contributing to the masking of the fast component could be the fact that only very large currents could be recorded with the intracellular single electrode voltage clamp technique. These currents may be the result of simultaneous firing of several presynaptic cells and thus release of GABA at many synapses. Any variation in delay between the synapses involved would cause smearing of the initial fast phase of the currents, resulting in a slower rise and initial decay.

In addition to the obvious difference in decay times between the currents measured with the single electrode voltage clamp and the patch clamp techniques, the range of amplitudes was also very different. In the first paper, only currents of > 100 pA were accepted for analysis. The range of amplitudes recorded was approximately 100 pA - 1 nA with most currents being 100 - 500 pA. These currents occurred at irregular intervals at a frequency of about 4 - 10 per minute. While these amplitude measurements are not accurate (due to the limitations of the technique), they would tend to be underestimates rather than overestimates. In the case of currents recorded with the patch clamp technique, the range of amplitudes was usually less than 10 - 100 pA with rare occurrences of larger currents (up to 200 pA) in some cells. Although again this could be a developmental difference, it is perhaps more likely that it is a result of the different recording conditions. Cells impaled with a microelectrode generally lie deep within a slice, thus a three dimensional array of short range synaptic inputs would remain intact. In contrast, a cell to which a patch electrode is applied tends to lie relatively close to the
surface of a somewhat thinner slice and any remaining synaptic connections on its upper surface would presumably be removed by the cleaning procedure. This factor alone could explain the difference in amplitudes. In addition, in the former case, the presence of the penetrating electrode in the slice may disturb the surrounding axons causing an increased basal level of firing and thus result in more frequent IPSCs of all sizes.

The increase in resolution introduced by the patch clamp technique also allowed the measurement of postsynaptic currents to a sufficient level of precision that direct quantal analysis was possible. Further, it was possible to measure the amplitudes of currents resulting from the opening of single GABA-mediated Cl⁻ channels in outside out patches. Thus the number of single channel openings which contribute to a synaptic quantum could be estimated. This would not have been possible without the application of patch clamp techniques.

While this thesis brings to light certain new information regarding the nature of transmission at a mammalian central nervous system synapse, it only touches the surface of several interesting questions. Thus various projects present themselves for further investigation. For example, it would be interesting to repeat the observations made in first paper using the more precise techniques described in the second. This approach could clarify exactly which feature of the IPSC alters with season. In conjunction, possible changes in the underlying GABA-mediated channels could be observed. Such an analysis of GABA-mediated single channel kinetics may explain the details of the decay time course of IPSCs and would contribute to an investigation of the molecular basis of the observed seasonal change. In addition many questions arise from the study of amplitudes reported in the last paper. Further statistical and pharmacological study is now required to test the ideas presented in this thesis.
Thus in summary, the thesis reports on features of inhibitory synaptic currents in the rat hippocampus. The overall findings suggest that the transmission at these synapses is quantal in nature with the very small quantal size probably being determined by the arrangement of postsynaptic receptors. In addition it was shown that the kinetics of such synaptic currents can be altered by external environmental cues which change with season. Collection of the quantal data was made possible by the development of a method for making patch clamp recordings in tissue slices.