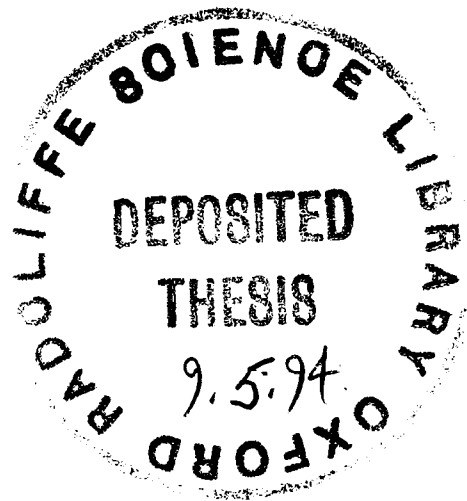


# Quantal Analysis of Synaptic Plasticity in the Rat Hippocampus

Robert Timo Hannay

St. John's College &  
University Laboratory of Physiology



Thesis submitted for the degree of Doctor of Philosophy

at the University of Oxford

Hilary Term, 1994

To AB  
in memory

*"How wonderful, how very wonderful the operations of time, and the changes of the human mind!...If any one faculty of our nature may be called more wonderful than the rest, I do think it is memory. There seems something more speakingly incomprehensible in the powers, the failures, the inequalities of memory, than in any other of our intelligences. The memory is sometimes so retentive, so serviceable, so obedient - at others, so bewildered and so weak - and at others again, so tyrannic, so beyond controul! - We are to be sure a miracle every way - but our powers of recollecting and of forgetting, do seem peculiarly past finding out."*

Jane Austen  
Mansfield Park (1814)

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## **PREFACE**

I have tried to write this thesis in a way that I think a thesis—or, for that matter, anything else—should be written. By this, I mean as clearly and simply as possible. Within the constraints of the subject and bearing in mind my small, specialist readership, I have tried to follow George Orwell's elementary rules of writing:

1. Never use a metaphor, simile or other figure of speech which you are used to seeing in print.
2. Never use a long word where a short one will do.
3. If it is possible to cut out a word, always cut it out.
4. Never use the passive where you can use the active.
5. Never use a foreign phrase, a scientific word or a jargon word if you can think of an everyday English equivalent.
6. Break any of these rules sooner than say anything outright barbarous.

*From Politics and the English Language (1946).*

Scientists tend to flout these rules so, in following them, I have strayed from some of the conventions of scientific writing. My approach might seem misguided because this thesis is part of my training and ought to show that I can adopt scientific orthodoxy. But in modern science, essays and papers by a single author are rare, so it is also an unusual opportunity to indulge myself. I think that scientific writing should follow the same elementary rules as any other type of writing and I do not think that turgid prose is necessarily a sign of scholarship or rigour. The style in which I have written this thesis reflects my opinions. It may not be to everyone's taste but I hope that the result is a better read.

Oxford, January 1994

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Thanks to my friends outside Oxford, especially Andy, Matthew, Nigel, Sam and Scott for reminding me that there is life beyond college walls.

And to Mum, Dad, Buma, Grannie, *Kyoko*, Lara and Max for everything.

## **ABSTRACT**

### *Quantal analysis of synaptic plasticity in the rat hippocampus*

Robert Timo Hannay

St John's College & University Laboratory of Physiology

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Long-term potentiation (LTP) is a long-lasting increase in efficacy shown by some synapses in the brain and elsewhere that might be involved in certain types of learning and memory. It has been most intensively studied in the hippocampus, a brain area known to be important in memory. LTP induction is triggered by a rise in calcium concentration inside the postsynaptic cell. Whether LTP is expressed presynaptically or postsynaptically is less clear, with results from different laboratories appearing to contradict one another.

Quantal analysis is one approach to this question. Neurotransmitter is released from the presynaptic cell in discrete packets of roughly equal size, known as quanta. If the increased response recorded during LTP is composed of a greater number of quanta, it would suggest a presynaptic change. On the other hand, an increase in the postsynaptic effect produced by each quantum would suggest that there had been a change in the postsynaptic cell.

I made intracellular voltage recordings of small excitatory postsynaptic potentials (EPSPs) from pyramidal neurones in area CA1 of rat hippocampal slices. I induced LTP and determined the site of the change using three quantal analysis procedures:  $1/CV^2$  graphs, amplitude frequency histograms and 'constant  $N$  analysis'. The results indicate that EPSPs can show both presynaptic and postsynaptic changes. However, the proportions varied considerably between individual EPSPs and seemed to depend on the initial 'setting' of the synapse. This, in turn, can depend on the experimental conditions, which might explain at least some of the contradictions in earlier results.

I also carried out a similar study of a briefer form of enhancement known as short-term potentiation (STP). I found that this too is caused by both presynaptic and postsynaptic changes and that their proportions seem to obey a similar rule to LTP. This suggests that LTP and STP are not separate phenomena, as some have suggested, but are closely related.

I attempted a similar study of mossy fibre inputs to pyramidal cells in area CA3 of hippocampal slices. However, it was difficult to obtain uncontaminated mossy fibre responses, so the results of this study are preliminary.

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## GLOSSARY

The following is a list of the abbreviations I have used most often. All of these are conventional except for the notation I have used for the binomial parameters,  $N$ ,  $P$  and  $Q$ . There is some inconsistency in the terms used by different researchers. Many use the same letters as I have, but in lower case. However, in certain contexts, these can have alternative meanings so I have chosen to use upper case.

AA	Arachidonic acid.
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate.
AP5	2-amino-5-phosphonovalerate.
CaMK II	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II.
CO	Carbon monoxide.
CV	Coefficient of variation.
DAG	Diacylglycerol.
DG	Dentate gyrus.
EPSC	Excitatory postsynaptic current.
EPSP	Excitatory postsynaptic potential.
IP <sub>3</sub>	Inositol trisphosphate.
LTP	Long-term potentiation
$M$	Mean quantal content. Equal to $NP$ . (Sometimes called $m$ .)
MEND	Maximum entropy noise deconvolution.
MEPP	Miniature end-plate potential.
MLE	Maximum likelihood estimation.
$n$	Number of examples used to produce an average.
$N$	One of the three parameters (along with $P$ and $Q$ ) used in the simple binomial description of quantal synaptic transmission. Usually thought to correspond to the number of release sites. (Sometimes called $n$ but not to be confused with my use of $n$ .) In Chapters Four and Five I use $N$ specifically to refer to the estimate of this binomial parameter from the mean, standard deviation and quantal size of an amplitude frequency histogram.
NMDA	$N$ -methyl-D-aspartate.

NMJ	Neuromuscular junction.
NO	Nitric oxide. Also known as endothelium-derived relaxing factor (EDRF).
NOS	Nitric oxide synthase. Also known as NADPH-diaphorase.
$p$	In the case of a correlation between two variables, $p$ is the probability that the true gradient is zero. In the case of Student's t-test, $p$ is the probability that two groups of data could have come from the same normal (Gaussian) distribution.
$P$	One of the three parameters (along with $N$ and $Q$ ) used in the simple binomial description of quantal synaptic transmission. Usually thought to correspond to the release probability. (Sometimes called $p$ but not to be confused with my use of $p$ .) In Chapters Four and Five, I use $P$ specifically to refer to the estimate of this binomial parameter by 'constant $N$ analysis'.
PKA	Cyclic-adenosine-monophosphate-dependent protein kinase A.
PKC	Calcium/diacylglycerol-dependent protein kinase C.
PPF	Paired-pulse facilitation.
PTP	Post-tetanic potentiation.
$Q$	One of the three parameters (along with $N$ and $P$ ) used in the simple binomial description of quantal synaptic transmission. Usually thought to correspond to the quantal size. (Sometimes called $q$ , $v_1$ , $v_2$ or $a$ but not to be confused with the use of $q$ in classical binomial theory, where $q=1-p$ .) In Chapters Four and Five, I use $Q$ specifically to refer to the estimate of this binomial parameter by 'constant $N$ analysis'. Note that I use the term 'quantal size' to refer to the mean peak spacing of an amplitude frequency histogram, which is an independent estimate of the same parameter and does not necessarily have an identical value.
$r$	Correlation coefficient.
SD	Standard deviation.
STP	Short-term potentiation
TTX	Tetrodotoxin.

# Chapter One

## Introduction

### 1.1 Long-Term Potentiation

#### *1.1.1 Predictions of synaptic plasticity*

If nerve cells, or *neurones*, are the stuff of minds how do they let us remember? At the beginning of this century, the great Spanish neuroanatomist, Santiago Ramón y Cajal, discovered the chemical connections, or *synapses*, through which neurones communicate (Ramón y Cajal, 1911). Ever since, scientists have wondered whether changes in the strengths of these structures might underlie at least some forms of learning and memory. About 45 years ago, Donald Hebb, a Canadian psychologist, produced what has so far been the most influential formalisation of these ideas (Hebb, 1949). He proposed that:

*“When an axon of cell A is near enough to excite cell B and repeatedly or consistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased.”*

These days we might say: *When two neurones are simultaneously active, the strength of any synapses between them will tend to increase.* This has become known as *Hebb’s Rule* and synapses that obey it are said to be *Hebbian*. If it holds true, a group of neurones that are active at the same time become linked by stronger synapses. When a portion of them are subsequently activated, they will tend to excite the others and recreate the original pattern of activity. This, in crude terms, may be the basis of associative memory. More specifically, Hebbian synapses incorporated into neural networks can, in principle, allow these networks to show the salient features of classical Pavlovian conditioning (Brindley, 1967).

*Synaptic plasticity* of this kind has become one of the most intensively studied and discussed ideas in neuroscience. Almost 60 years after the death of Ramón y Cajal, we are just beginning to tease apart the molecular mechanisms involved in these changes and understand the role they might play in learning and memory. The leap from visionary theories to hard empirical facts came barely 20 years ago.

### 1.1.2 *The discovery of long-term potentiation*

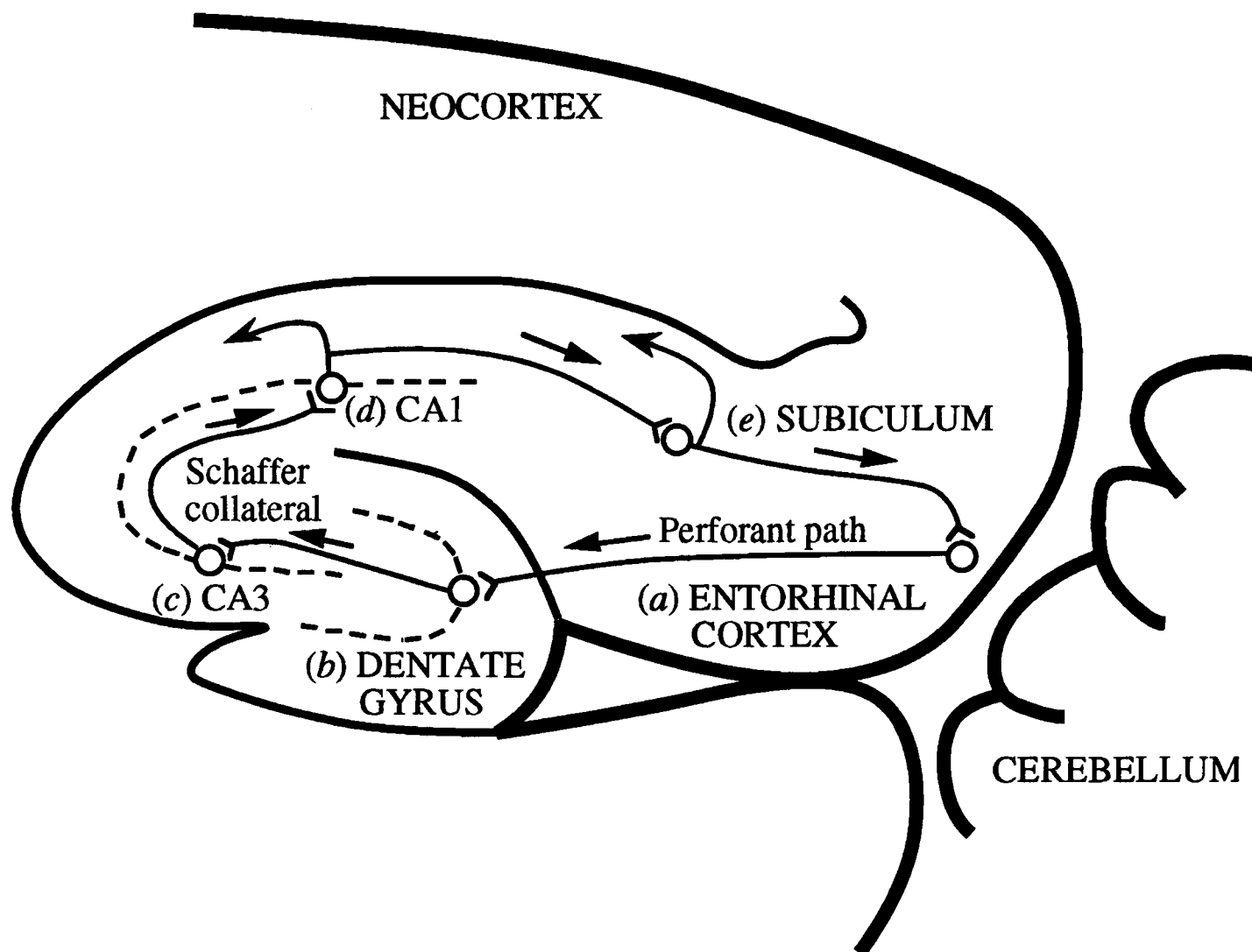
Experiments by Bliss & Lømo in the late 60s and early 70s were the first to demonstrate that durable changes in synaptic strength can occur in the brain<sup>1</sup>. Using anaesthetised rabbits, they electrically stimulated neurones of the *perforant path*, a bundle of nerve fibres that emerge from the *entorhinal cortex* at the back of the brain and make synaptic connections onto *granule cells* in the *dentate gyrus*. The dentate gyrus lies buried underneath the cerebral cortex, next to the *hippocampus* and forms part of the *hippocampal formation* (see Figure 1.1).

Bliss & Lømo made test stimulations of perforant path fibres every 2-3 seconds and recorded the collective response of a large number of granule cells using an extracellular electrode. A high-frequency (10-100Hz) train of stimuli, known as a *tetanus*, was applied for 3-15s and usually resulted in an enhanced response to subsequent test stimulation. The enhancement lasted for 30 minutes to 10 hours without the need for further tetani (Bliss & Lømo, 1970, 1973). Similar results were then obtained from unanaesthetized rabbits (Bliss & Gardner-Medwin, 1973). In this preparation, potentiation lasted up to 3 days. This phenomenon, initially called long-lasting potentiation, later became known as *long-term potentiation* (LTP)<sup>2</sup>.

---

<sup>1</sup>Although Beswick & Conroy (1965) had reported some years earlier that synaptic enhancement in the spinal cord could last for an hour or more.

<sup>2</sup>Except by McNaughton (1993) who calls this form of synaptic plasticity long-term enhancement (LTE). Ironically, McNaughton insists on this nomenclature to avoid confusion in the literature since the term 'potentiation' has already been used to describe other non-associative, very brief forms of synaptic strengthening such as post-tetanic potentiation (see Chapter Three).



**Figure 1.1 Principle structures and basic circuitry of the hippocampal formation** This diagram shows a transverse section through the brain of a rat or similar small mammal. (a) Axons emerging from the *entorhinal cortex* form the *perforant path* and make synapses onto (b) *granule cells* in the *dentate gyrus*. (c) These, in turn, form giant *mossy fibre* synapses with *pyramidal neurones* in area CA3 of the hippocampus. (d) The axons of CA3 cells form *Schaffer collaterals*, which synapse onto other pyramidal cells in area CA1. (e) These send their outputs to the *subiculum*, which completes the loop by connecting to the entorhinal cortex. This is a highly simplified diagram and there are many other important pathways (see Chapter Two). All connections shown are excitatory and glutamatergic. Local inhibitory interneurons are present but are not shown. Areas CA1 to CA3 make up the hippocampus proper. The hippocampal formation is composed of the hippocampus, dentate gyrus, subicular complex and entorhinal cortex. There is one hippocampal formation in each of the brain's two hemispheres.

### *1.1.3 LTP is specific, cooperative and associative*

Andersen *et al.* (1977) used guinea pig hippocampal slices maintained *in vitro* to demonstrate that LTP at synapses in area CA1 of the hippocampus (see Figure 1.1) is confined to the tetanised input and that other synapses are unaffected. At the same time, Lynch *et al.* (1977; Dunwiddie & Lynch, 1978), also studying area CA1, showed that activation of the postsynaptic cell in the absence of presynaptic activity tends to decrease synaptic efficacy, so untetanised inputs are actually slightly depressed. This is known as *heterosynaptic depression*.

A certain minimum number of axons must be activated during the tetanus to trigger LTP induction (McNaughton *et al.*, 1978). This is known as *cooperativity*. A related phenomenon, *associativity*, was demonstrated in rats by Levy & Steward (1979). Dentate granule cells in each of the brain's two hemispheres receive separate inputs from regions of entorhinal cortex in the same (*ipsilateral*) and opposite (*contralateral*) sides of the brain. The contralateral input is sparse and LTP cannot be induced in it unless the ipsilateral input is stimulated at the same time. The ipsilateral input is more dense and LTP can be induced in these synapses by stimulation of this input alone. So it seems that some critical number of synapses must be activated in order to induce LTP. Similar results were obtained in the CA1 region of hippocampal slices (Barrionuevo & Brown, 1983). More recent work on the cellular and molecular details of LTP induction have revealed the reasons behind this.

### *1.1.4 LTP induction involves NMDA-type glutamate receptors and raised postsynaptic calcium concentration*

LTP induction requires a certain critical number of synapses because the *postsynaptic* neurone must be substantially *depolarised* (see Figure 1.2) for LTP to be triggered (Kelso *et al.*, 1986; Malinow & Miller, 1986). LTP therefore fulfils the principle

requirement of Hebb's Rule: that synapses between two neurones will only strengthen if both cells are active at the same time. This close agreement between theory and experiment was an important and exciting breakthrough.

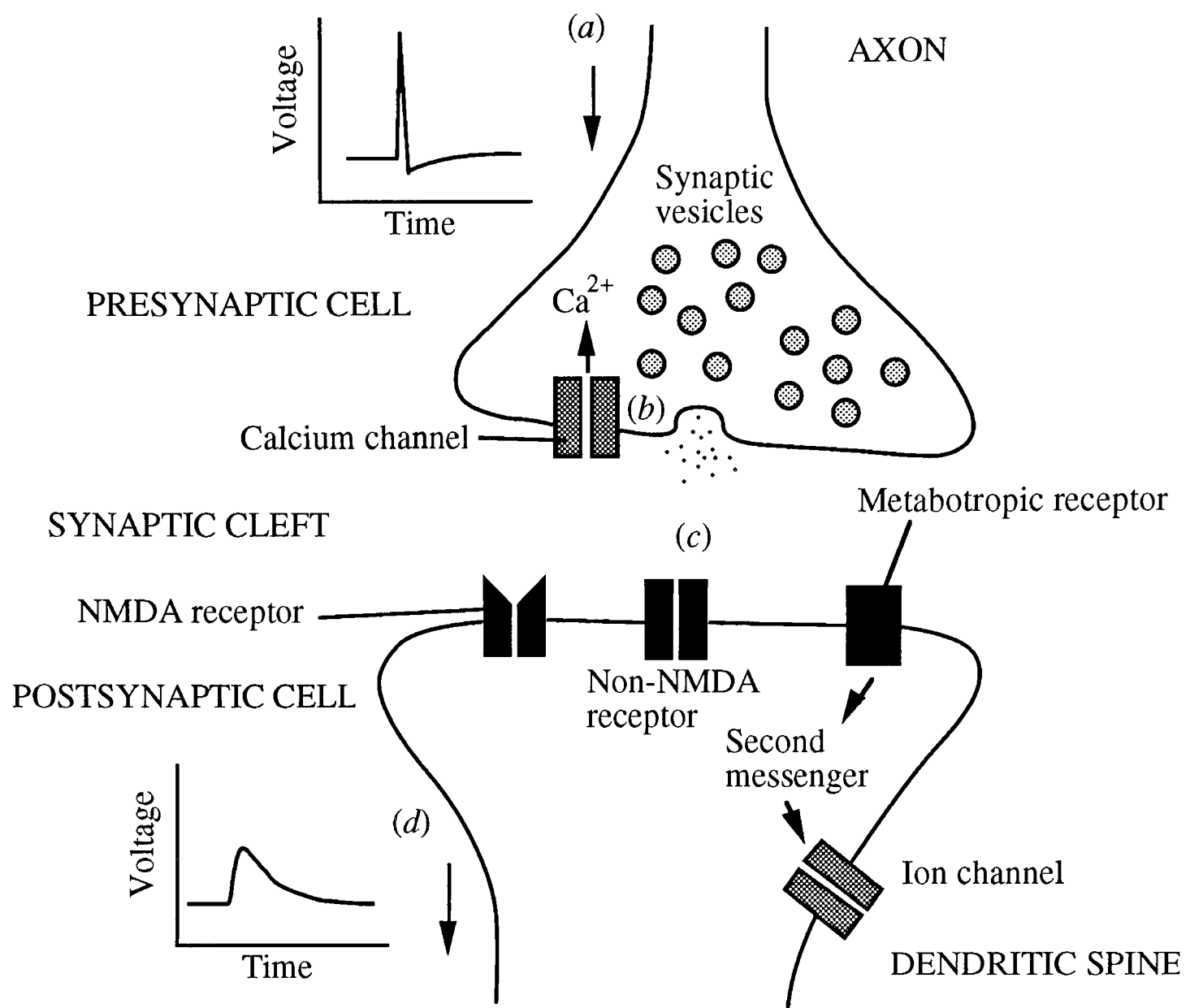
The finding that postsynaptic depolarisation is important in triggering LTP also suggests that induction takes place in the postsynaptic cell, an idea supported by studies into the role of calcium ( $Ca^{2+}$ ) ions.

An increased concentration of extracellular  $Ca^{2+}$  alone can lead to LTP in the absence of high-frequency stimulation (Turner *et al.*, 1982). Intracellular injection of a  $Ca^{2+}$ -chelating agent into the postsynaptic neurone blocks the induction of LTP (Lynch *et al.*, 1983) and photolysis of a photolabile  $Ca^{2+}$ -containing molecule injected into postsynaptic hippocampal CA1 pyramidal neurones results in potentiation with a similar amplitude and duration to that of LTP (Malenka *et al.*, 1988). This strongly suggests that a rise in postsynaptic  $Ca^{2+}$  plays a role in (and may even be sufficient for) inducing LTP but does not identify the source of this  $Ca^{2+}$  in tetanus-induced LTP.

The hippocampal synapses I have described, like most excitatory synapses in the central nervous system (CNS), are *glutamatergic*. In other words, the chemical that carries the synaptic message from one cell to the other (the *neurotransmitter*) is the amino acid, *glutamate* (Storm-Mathisen *et al.*, 1983)<sup>3</sup>. Fast synaptic transmission involves *ionotropic receptors* (see Figure 1.2). Broadly speaking, there are two types of ionotropic receptor at glutamatergic synapses: *NMDA receptors* and *non-NMDA receptors* (for reviews see Barnes & Henley, 1992; Seeburg, 1993). NMDA receptors are named after their preferred artificial agonist, *N-methyl-D-aspartate* (NMDA). Non-NMDA receptors, can be subdivided into two receptor classes according to their preferred artificial agonists,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate

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<sup>3</sup>Another amino acid neurotransmitter, aspartate, is thought to be released together with glutamate (Fleck *et al.*, 1993). Glutamate and aspartate act at the same receptors but show different affinities for the various types.



**Figure 1.2 The basic elements of a glutamatergic synapse** (a) An action potential arriving at the *presynaptic* terminal, or *bouton*, causes the terminal to depolarise and voltage-dependent calcium ( $Ca^{2+}$ ) channels to open.  $Ca^{2+}$  flows down its electrochemical gradient, into the cell. The intracellular concentration briefly rises from a low nanomolar level to several hundred micromolar. (b) This stimulates the release of glutamate-containing synaptic vesicles by a mechanism that is not yet well understood but involves a variety of proteins (for reviews see Südhof & Jahn, 1991; Linstedt & Kelly, 1991; Thomas & Almers, 1992). (c) The released glutamate diffuses across the *synaptic cleft* (which is typically about 200nm wide) in a few microseconds. It binds to receptor proteins on the membrane of the *postsynaptic* cell. Some of these are *ionotropic* receptors, which have their own ion channels that open directly in response to the binding of neurotransmitter and act over time scales of milliseconds. Ionotropic glutamate receptors can be divided into *NMDA-type* and *non-NMDA-type* receptors. The significance of this distinction is discussed in the text. Other receptors are *metabotropic*. These do not have integral ion channels but affect the opening of separate ion channel proteins via second messengers such as inositol trisphosphate ( $IP_3$ ) and cyclic adenosine monophosphate ( $cAMP$ ). They act over time scales of seconds. Generally speaking, these different types of receptors are thought to exist together at individual synapses. (d) Glutamatergic synapses are excitatory so the opening and closing of these various ion channels in the postsynaptic cell usually causes the electrical potential across its membrane to *depolarise* from the negative resting level. In this way the chemical synaptic message is converted back into an electrical signal. The postsynaptic side of an excitatory synapses usually takes the form of a *dendritic spine*, which is connected to the dendrite of the postsynaptic cell by a narrow neck. Biochemically (but not electrically) spines appear to act as semi-isolated compartments (Müller & Connor, 1991; Guthrie *et al.*, 1991; for reviews see Wickens, 1988; Koch & Zador, 1993).

(AMPA) and *kainate*. Most, but not all, glutamatergic synapses seem to contain a mixture of both NMDA and non-NMDA receptors (Bekkers & Stevens, 1989; Silver *et al.*, 1992).

Collingridge *et al.* (1983) found that 2-amino-5-phosphonovalerate (AP5), a specific antagonist of the NMDA receptor, has no significant effect on normal synaptic transmission, which is mediated mainly by non-NMDA receptors, but blocks the induction of LTP. This is because NMDA receptors play a significant role during the tetani used to induce LTP (Herron *et al.*, 1986). Their action is normally blocked by physiological concentrations of magnesium ions ( $Mg^{2+}$ ) but this effect is relieved by the strong postsynaptic depolarisation that occurs when successive small depolarisations summate during high-frequency stimulation (Nowak *et al.*, 1984). The ion channels associated with NMDA receptors are highly permeable to  $Ca^{2+}$ . When they are activated,  $Ca^{2+}$  ions pass down their electrochemical gradient, into the neuronal cytoplasm, where they trigger biochemical events that lead to LTP *expression*. Thus it is the ability of NMDA receptors to respond to both presynaptic neurotransmitter release and postsynaptic depolarisation that accounts for the Hebbian behaviour of these synapses.

$Ca^{2+}$  does not necessarily have to enter the cell through NMDA-receptor ion channels in order to induce LTP.  $Ca^{2+}$  passing through dihydropyridine-sensitive voltage-dependent  $Ca^{2+}$  channels can also produce synapse-specific potentiation (Grover & Teyler, 1990; 1992). However, saturated  $Ca^{2+}$ -channel-induced enhancement does not completely occlude NMDA receptor-dependent LTP and *vice versa* (Huang & Malenka, 1993), so there might be important differences between the two.

Under some conditions,  $Ca^{2+}$  entering the postsynaptic cell through voltage-dependent channels results not in LTP, which typically lasts for an hour or more, but in short-term potentiation (STP) that lasts about 30 minutes (Kullmann *et al.*, 1992)<sup>4</sup>.

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<sup>4</sup>LTP and STP are usually considered to be two distinct processes rather than the extremes of a continuous range of durations (see Chapter Five).

Similar  $\text{Ca}^{2+}$  entry combined with presynaptic activity, however, does produce robust LTP. This enhancing effect of presynaptic activity cannot be explained by activation of NMDA receptors, which were blocked with AP5. Applying NMDA to hippocampal slices has also been reported to produce STP instead of LTP (Collingridge *et al.*, 1983; Kauer *et al.* 1988a, but see Thibault, *et al.*, 1989). These findings suggest that LTP induction requires not only postsynaptic NMDA receptor activation and  $\text{Ca}^{2+}$  influx but also some other signal that accompanies presynaptic activity. However, there may be a simpler explanation. Whether induction results in STP or LTP seems to depend on the amount of  $\text{Ca}^{2+}$  that enters the postsynaptic cell, with LTP requiring more  $\text{Ca}^{2+}$  than STP does (see Chapter Five). During presynaptic stimulation, *metabotropic* glutamate receptors (see Figure 1.2) might have been activated and these could have increased the amount of  $\text{Ca}^{2+}$  in the cytoplasm either by their modulatory effects on various postsynaptic ion channels or by stimulating  $\text{Ca}^{2+}$  release from intracellular stores (see section 1.1.8).

### *1.1.5 The role of protein kinases in LTP*

The cellular processes that lead from  $\text{Ca}^{2+}$  influx to LTP expression are not yet well understood. Protein kinase enzymes control almost every aspect of cellular metabolism so they are likely to play a central role. Two prime candidates, whose activities are both influenced by  $\text{Ca}^{2+}$  levels, are the serine-threonine kinases,  $\text{Ca}^{2+}$ /diacylglycerol-dependent protein kinase C (*PKC*) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (*CaMK II*).

After LTP induction, PKC seems to move from soluble to membrane fractions of cells (Akers *et al.*, 1986) and remain persistently active (Klann *et al.*, 1991). Activation of PKC by phorbol esters (Malenka *et al.*, 1986) or injection of active PKC into the postsynaptic neurone (Hu *et al.*, 1987) results in enhanced synaptic transmission. Bath application of PKC inhibitors is reported to prevent induction of robust LTP (synaptic responses decaying to baseline within one hour) and also to

reverse LTP during the first hour after induction (Lovinger *et al.*, 1987; Reymann *et al.*, 1988a,b, 1990). By bath-applying drugs with different modes of inhibition, Malinow *et al.* (1988) provided evidence that LTP beyond about 30 minutes is maintained by a persistently active kinase. Later experiments involving application of inhibitors through the intracellular recording electrode showed that postsynaptic PKC activity (and, incidentally, CaMK II activity too) is needed for induction of robust LTP lasting longer than about 30 minutes (Malenka *et al.*, 1989; Malinow *et al.*, 1989; Huang *et al.*, 1992b). The same studies also showed that expression of previously-induced LTP is dependent on presynaptic PKC but does not require postsynaptic kinase activity (but see Wang & Feng, 1992).

Thus presynaptic but not postsynaptic PKC activity might maintain LTP expression. This idea is consistent with reports that PKC can phosphorylate neuromodulin (also called F1, GAP-43 and B-50) (Akers & Routtenberg, 1985), which seems to be involved in neurotransmitter release and LTP (Routtenberg *et al.*, 1985; Gianotti *et al.*, 1992) and with the finding that PKC can enhance transmitter release by affecting presynaptic ion channel activity (Barrie *et al.*, 1991; Parfitt & Madison, 1993). It also agrees with the fact that, although the sequences of non-NMDA receptor subunits contain consensus sites for phosphorylation by PKC (reviewed by Barnard & Henley, 1990), there is not yet any direct evidence that PKC can phosphorylate them.

These findings suggest a role for PKC in LTP expression but other results contradict the idea. The potentiation seen after injecting PKC into the postsynaptic cell does not seem to be identical to LTP (Gustafsson *et al.*, 1988; Muller *et al.*, 1988b, 1990). Inhibition of PKC activity does not always inhibit expression of previously induced LTP (Denny *et al.*, 1990) and sufficiently vigorous induction can produce robust LTP even in the presence of a PKC inhibitor (Muller *et al.*, 1988b; Muller *et al.*, 1992). Ben-Ari *et al.* (1992) suggest that PKC may merely modulate Ca<sup>2+</sup> entry during LTP induction and play no role in mediating expression. This is supported by findings that

PKC can enhance NMDA receptor action (Aniksztejn *et al.*, 1992; Kelso *et al.*, 1992; Chen & Huang, 1992).

So, although some reports suggest that presynaptic PKC activity may be necessary to maintain LTP expression, most are consistent with a scheme in which postsynaptic PKC plays a modulatory role by influencing NMDA receptor activity (and hence the amount of  $\text{Ca}^{2+}$  that enters the cell) during LTP induction.

CaMK II might be a more attractive alternative. It is the major protein in the *postsynaptic density* (Kennedy *et al.*, 1983; Kelly *et al.*, 1984), a thickening of the postsynaptic membrane that can be seen with an electron microscope. The sequences of non-NMDA receptor subunits contain consensus sites for phosphorylation by CaMK II (reviewed by Barnard & Henley, 1990) and CaMK II action has been shown to enhance the activity of these receptors (McGlade-McCulloh *et al.*, 1993). Silva *et al.* (1992a) used gene targeting to produce mutant mice lacking the  $\alpha$ -isoform of CaMK II and found that LTP was inhibited in these animals. It was not abolished all together but this could be explained by the fact that other isoforms of the enzyme were still active. CaMK II activity can be sustained even after brief NMDA receptor stimulation because the enzyme, once active, can switch to a  $\text{Ca}^{2+}$ -independent state by phosphorylating itself (*autophosphorylation*) (Miller & Kennedy, 1986; Fukunaga *et al.*, 1992). This could, in principle, explain how a brief induction can lead to the sustained synaptic enhancement seen in LTP (Lisman, 1985).

Tyrosine kinases have also been implicated in LTP. O'Dell *et al.* (1991b) found that tyrosine kinase inhibitors applied postsynaptically prevent induction of LTP but not STP. There was no effect on established LTP. Grant *et al.* (1992) showed that mice with mutations in a specific tyrosine kinase gene show impaired LTP, though, as with the gene-knockout study of CaMK II, LTP was inhibited rather than abolished all together.

Protein kinase A (*PKA*) is another possible mediator of LTP expression. At first sight it seems like a less suitable candidate because its activity is controlled not by  $\text{Ca}^{2+}$  levels but by the second messenger, cyclic adenosine 3'-5'-monophosphate (*cAMP*). However, this does not rule out a direct role in linking LTP induction to expression because NMDA receptor activity in the hippocampus increases *cAMP* levels (Chetkovich *et al.*, 1991) by stimulating a  $\text{Ca}^{2+}$ -dependent adenylyl cyclase (Chetkovich & Sweatt, 1993). *PKA* can upregulate non-NMDA receptors (Greengard *et al.*, 1991; Wang *et al.*, 1991, 1993; Raymond *et al.*, 1993) and its activation by *cAMP* increases synaptic strength in hippocampal slices (Chavez-Noriega & Stevens, 1992).

Studies of protein kinase activity are difficult to interpret with any degree of certainty. One reason is that most protein kinase inhibitors are not particularly specific. Another is that even when highly specific blockers or gene targeting methods are available and can be used reliably, mediatory roles for the enzymes are very difficult to tease apart from modulatory ones. If, for example, no potentiation is seen, it is impossible to be sure that LTP has been truly abolished: the threshold for induction may have merely been raised. On the other hand, if LTP still occurs in the presence of a blocker, it does not rule out a mediatory role: it is possible that there are several different enzyme types and isoforms involved, with parallel pathways that are capable of interacting or standing alone. However, most of the current evidence suggests that protein kinases are involved in linking LTP induction to expression. The evidence is perhaps strongest for *CaMK II*, but *PKC*, *PKA* and tyrosine kinases might be involved too.

With this said, it is striking that even inhibition experiments that used the most non-specific blockers at the highest concentrations did not show any effect on the size of the initial enhancement. There is typically no difference from control experiments for at least 5-10 minutes after induction and response amplitudes do not usually decay to baseline for at least 30 minutes. This means that none of these kinases seem to be involved in the changes that are responsible for the first few minutes of LTP or STP

expression. This early phase is often considered to be an entirely separate phenomenon from LTP and is sometimes assumed to be presynaptic (e.g. Stevens, 1989).

### *1.1.6 LTP expression: the 'pre' versus 'post' debate*

Which neurone is responsible for the increase in synaptic strength seen in LTP? Does the presynaptic cell release more neurotransmitter or does the postsynaptic cell become more sensitive to it?

Some of the earliest evidence pointed to a postsynaptic site for LTP expression. Baudry *et al.* (1981) suggested that a rise in postsynaptic  $Ca^{2+}$  could activate proteases that cleave membrane proteins to expose a new population of glutamate receptors, hence increasing postsynaptic sensitivity to neurotransmitter. Synaptic membranes prepared after LTP induction do indeed appear to show a greater number of glutamate binding sites than control tissue (Baudry *et al.*, 1980; Lynch *et al.*, 1982; but see Lynch *et al.*, 1985; Kessler *et al.*, 1991). The subsequent discovery that LTP induction takes place postsynaptically and that it involves a  $Ca^{2+}$  signal (see section 1.1.4) made it seem even more likely that this idea was right, particularly since synapses were thought of as biological diodes, with no backward information transfer from postsynaptic to presynaptic neurone.

But this view was challenged by Skrede & Malthe-Sørensen (1981), who worked on area CA1, and shortly afterwards by Bliss and his co-workers (Dolphin *et al.*, 1982; Bliss *et al.*, 1986), who studied the dentate gyrus. They showed that LTP is accompanied by an increase in glutamate or aspartate found in the extracellular fluid. However, Aniksztejn *et al.* (1989) were unable to replicate these findings and, in any case, this effect might be due to reduced glutamate uptake by non-neuronal cells (*glial cells*) rather than increased release by neurones (see section 1.1.7).

Postsynaptic changes seemed to be ruled out by the finding that LTP is not associated with an increase in postsynaptic responses to iontophoretically applied glutamate (Turner *et al.*, 1982). However, these results too were called into question. Davies *et al.* (1989) showed that the sensitivity of CA1 neurones to iontophoretically applied AMPA receptor agonists increases during LTP. This effect takes tens of minutes or hours to develop, which may explain why it was not seen by other groups. It also supports the view that the early phase of LTP involves another, perhaps presynaptic, mechanism (Stevens, 1989). But this study is open to the criticism that it might have measured mainly the sensitivity of kainate receptors rather than AMPA receptors, which mediate most transmission at these synapses. This is because the experiments involved slow application of agonist and AMPA receptors (but not kainate receptors) desensitise very rapidly under these conditions (Trussell *et al.*, 1988; Trussell & Fischbach, 1989).

Kauer *et al.* (1988b; see also Perkel & Nicoll, 1993) and Muller *et al.* (1988a; Muller & Lynch, 1988) took advantage of the fact that pharmacological manipulation of glutamate receptors can separate the effects of NMDA receptors from those of non-NMDA receptors. They showed that, following LTP induction, the component mediated by non-NMDA receptors is enhanced while that mediated by NMDA receptors is not significantly changed. Assuming that both receptor types are saturated during normal transmission, an increase in presynaptic neurotransmitter release would be expected to affect both components equally so this suggests a predominantly postsynaptic change. However, these results have been contradicted by several other groups (Bashir *et al.*, 1991; Berretta *et al.*, 1991; Xie *et al.*, 1992) who have reported being able to induce LTP in EPSPs mediated by NMDA receptors alone. Asztely *et al.* (1992) report that LTP is accompanied by enhancements in components mediated by both NMDA and non-NMDA receptors but the NMDA-receptor-mediated part shows only about a third of the potentiation of the non-NMDA-receptor-mediated one, making a purely presynaptic change unlikely.

There have been several reports that paired-pulse facilitation (*PPF*, generally agreed to be a presynaptic phenomenon, see Chapter Three) is not affected by LTP (McNaughton, 1982; Gustafsson *et al.*, 1988; Muller & Lynch, 1989; Manabe *et al.*, 1993). This indicates that these two forms of potentiation do not share common mechanisms and therefore suggests that LTP expression might be postsynaptic. Yet these experiments have also been contradicted by reports that PPF is significantly reduced during LTP (Voronin & Kuhnt, 1990).

A selective review of this literature could provide a convincing case for either a presynaptic or a postsynaptic locus of change but the overall picture seems full of contradictions. Of course, presynaptic and postsynaptic changes are not mutually exclusive and both could happen at the same time. If a conclusion can be drawn from these studies, it is that both presynaptic and postsynaptic changes can occur but which one predominates seems to depend on the laboratory in which the experiments are carried out!

### *1.1.7 Retrograde messengers*

If LTP induction takes place postsynaptically and its expression is at least partly presynaptic then a *retrograde messenger* must cross the synaptic cleft. Proposed candidates include arachidonic acid (AA) and its metabolites as well as nitric oxide (*NO*)<sup>5</sup> and carbon monoxide (*CO*). In order to act as a retrograde messenger, a substance must be produced during LTP induction, pass through the cell membrane of the postsynaptic neurone, diffuse across the synaptic cleft and then cause some effect on the presynaptic side that enhances transmitter release.

AA can be released from membrane phospholipids by phospholipase A<sub>2</sub> or by the combined action of a phospholipase C and a diacylglycerol-lipase. Activation of NMDA receptors on cultured neurones results in the appearance of AA in the

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<sup>5</sup>Also known as endothelium-derived relaxing factor (EDRF) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987).

extracellular space, probably as a result of Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> activity (Lazarewicz *et al.*, 1988; Dumuis *et al.*, 1988). Induction of LTP in the dentate gyrus of anaesthetised rats is accompanied by AA production (Lynch *et al.*, 1989).

Lipoxygenases and cyclo-oxygenases act on AA to produce various biologically active oxidation products known collectively as eicosanoids (reviewed by Needleman *et al.*, 1986). These can modulate ion channel activity by direct or indirect action (e.g. Buttner *et al.*, 1989; Kurachi *et al.*, 1989; Kim *et al.*, 1989; Schwartz & Yu, 1992) and can increase transmitter release from presynaptic terminals (Lynch & Voss, 1990). The presence of phospholipase and lipoxygenase (but not cyclo-oxygenase) inhibitors results in STP after procedures that otherwise produce LTP (Williams & Bliss, 1988; Lynch *et al.*, 1989; Okada *et al.*, 1989; Williams & Bliss, 1989). When paired with weak activation of the perforant path, AA induces synaptic enhancement that develops over one or two hours and occludes tetanus-induced LTP (Williams *et al.*, 1989). Interestingly, there is also evidence that an AA metabolite can act as a retrograde signal at the neuromuscular junction to upregulate release of acetylcholine (Harish & Poo, 1992).

However, nordihydroguaiaretic acid, the only drug used in all but one of the inhibitor studies, also interferes with the NO system (O'Dell *et al.*, 1991a). And the potentiation seen after AA application is dependent on NMDA receptor activation (O'Dell *et al.*, 1991a), which is not consistent with a role as a retrograde messenger. Potentiation may instead be caused by the ability of AA to inhibit glutamate uptake from the extracellular space (Barbour *et al.*, 1989). Applying AA might produce a chronic rise in extracellular glutamate levels in the region of active synapses. This could cause tonic NMDA receptor activity and result in potentiation.

NO is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS)<sup>6</sup>. At first sight this noxious free radical gas seems an unlikely cellular messenger but there are a number of reasons for thinking that NO might play a role in LTP.

Firstly, NOS activity is Ca<sup>2+</sup>/calmodulin-dependent (Knowles *et al.*, 1989; Bredt & Snyder, 1990; Bredt *et al.*, 1991b). Secondly, NO is assumed to be sufficiently lipophilic to pass through cell membranes and small enough to diffuse rapidly across the synaptic cleft (although neither its phospholipid membrane permeability nor its diffusion coefficient in water have yet been reported (Stamler *et al.*, 1992)). Thirdly, NO stimulates soluble guanylyl cyclase, an enzyme that produces cyclic guanosine 3',5'-monophosphate (cGMP) (Miki *et al.*, 1977; Arnold, *et al.*, 1977), which can, in turn, regulate a range of cellular proteins (e.g. Schlichter *et al.*, 1980; Sonnenburg *et al.*, 1991; Brüne & Lapetina, 1989). Cyclic GMP phosphodiesterase, which breaks down cGMP, is activated by Ca<sup>2+</sup>/calmodulin at the same concentration that activates NOS (Mayer *et al.*, 1992). This presumably prevents cGMP accumulation in the vicinity of NO production and suggests that NO has its effects at distant sites, possibly in other cells or subcellular compartments.

In more direct support of a role for NO as a retrograde messenger in LTP, NMDA receptor activation in the hippocampus and other brain areas can result in the production of cGMP and this effect seems to be mediated by NO (Garthwaite *et al.*, 1988; East & Garthwaite, 1991). Application of NO to neurones in dissociated culture enhances spontaneous neurotransmitter release (O'Dell *et al.*, 1991a) and in the CA1 region of hippocampal slices, NO causes synaptic enhancement that occludes tetanus-induced LTP (Böhme *et al.*, 1991; Bon *et al.*, 1992). This is specific to presynaptically active connections and does not require the activity of NMDA receptors or voltage-dependent Ca<sup>2+</sup> channels (Zhuo *et al.*, 1993). Haemoglobin, which scavenges NO from the extracellular space, and NOS inhibitors are reported to

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<sup>6</sup>Also known as NADPH-diaphorase (Hope *et al.*, 1991).

inhibit LTP in area CA1 (Böhme *et al.*, 1991; O'Dell *et al.*, 1991a; Schuman & Madison, 1991; Bon *et al.*, 1992; Haley *et al.*, 1992).

Perhaps unsurprisingly in this field, there is also evidence that NO is not a retrograde messenger. The effect of NOS inhibitors on LTP depends on the induction procedure (Gribkoff & Lum-Ragan, 1992; Haley *et al.*, 1993; Chetkovich *et al.*, 1993), which suggests that it plays a modulatory rather than a mediatory role. Also, Bliss and his colleagues report that photolytic release of NO in area CA1 does not produce LTP (Williams *et al.*, 1993a) and that NOS inhibitors block LTP only in young animals at room temperature (Williams *et al.*, 1993b).

Another difficulty with the idea that NO is a retrograde messenger is that only about 2% of neurones seem to contain NOS and hippocampal CA1 pyramidal cells in particular show very low levels of NOS immunoreactivity (Bredt *et al.*, 1990), NOS mRNA (Bredt *et al.*, 1991a) or NADPH-diaphorase activity (Vincent and Kimura, 1992; Valtschanoff *et al.*, 1993; but see Divac *et al.*, 1993). One idea is that other isoforms of NOS are present and that these are not detected by current methods, but this remains to be confirmed.

The distribution of NOS in the brain does not precisely match that of guanylyl cyclase (Matsuoka *et al.*, 1992), suggesting that NO has other targets of action. NMDA receptors, which NO is known to inhibit (Manzoni *et al.*, 1992; Lei *et al.*, 1992; Hoyt *et al.*, 1992), are likely targets. This could act as a negative feedback loop to prevent excessive NO production during continued stimulation. It might also explain why prior activation of NMDA receptors raises the threshold of tetanus-induced LTP (Huang *et al.*, 1992a) in an NO-dependent manner (Izumi *et al.*, 1992a) and why some researchers find that NOS inhibitors enhance LTP in area CA1 (Kato & Zorumski, 1993).

Carbon monoxide can also activate guanylyl cyclase (Brüne & Ullrich, 1987; Brüne *et al.*, 1990). It is formed by *haem oxygenase*, an enzyme expressed throughout the

brain in a pattern closely (but not absolutely) matched by guanylyl cyclase mRNA (Verma *et al.*, 1993). Haem oxygenase is expressed in hippocampal pyramidal cells and in the dentate gyrus. Interestingly, CO binds tightly to haemoglobin so the inhibition of LTP by this protein (see above) is as good evidence for CO playing a role as it is for NO.

CO can produce LTP specifically at synapses that are presynaptically active even when NMDA receptors and voltage-dependent  $\text{Ca}^{2+}$  channels are blocked (Zhuo *et al.*, 1993). Other evidence for a role in LTP induction comes from findings that zinc protoporphyrin (*ZnPP*), an inhibitor of haem oxygenase, prevents induction of hippocampal LTP (Stevens & Wang, 1993; Zhuo *et al.*, 1993). However, *ZnPP* is not specific: it inhibits guanylyl cyclase too (Ignarro *et al.*, 1984). *ZnPP* also reverses established LTP (Stevens & Wang, 1993), which suggests that it might affect expression rather than induction.

Many of the problems encountered in investigating the roles of protein kinases also apply to retrograde messengers: it is hard to tell modulatory and mediatory roles apart, and there may be several interacting retrograde messengers. Once again, a consistent finding is that the potentiation seen immediately after induction is not affected by any of the inhibitory drugs used, so these putative retrograde messengers do not appear to be involved in the early minutes of LTP or STP. This is consistent with the view that the initial stage of potentiation is a separate process from LTP but is difficult to reconcile with the idea that it is presynaptic (Stevens, 1989). I will discuss this point in light of my own results in Chapter Seven.

### *1.1.8 The role of metabotropic glutamate receptors in LTP*

$\text{Ca}^{2+}$  is not the only intracellular signal that accompanies LTP. During induction, levels of the second messengers, inositol 1, 4, 5 trisphosphate (*IP<sub>3</sub>*) and diacylglycerol (*DAG*) also rise (Bär *et al.*, 1984; Lynch *et al.*, 1988). This probably comes about by

activation of metabotropic glutamate receptors (Sugiyama *et al.*, 1987; Masu *et al.*, 1991; Houamed *et al.*, 1991).

Blocking these receptors with the antagonist, (*RS*)- $\alpha$ -methyl-4-carboxyphenylglycine (*MCPG*), during a tetanus that would normally result in LTP causes potentiation to decline back to baseline in about 30 minutes and activating them with the agonist aminocyclopentane dicarboxylate (*ACPD*) produces a slow-forming potentiation (Bashir *et al.*, 1993). These findings are similar to some of the results obtained for PKC. This is not surprising since activation of metabotropic glutamate receptors stimulates PKC activity (Manzoni, *et al.*, 1990) via a well-established chain of biochemical events that involves IP<sub>3</sub> and DAG<sup>7</sup>. PKC, in turn, can phosphorylate NMDA receptors and enhance their action (see section 1.1.5). Metabotropic receptors can therefore have modulatory effects on NMDA receptor activity and hence on LTP induction (Aniksztejn *et al.*, 1992).

However, McGuinness *et al.* (1991a) report that the enhancement of LTP seen when they apply metabotropic receptor agonists during induction does not depend on protein kinase activity. There are other routes by which metabotropic receptors could be modulating this process. The release of Ca<sup>2+</sup> from intracellular stores by IP<sub>3</sub> could enhance the effects produced by Ca<sup>2+</sup> entry through NMDA-receptor channels. This agrees with the finding that blocking intracellular Ca<sup>2+</sup> release inhibits LTP induction (Obenaus *et al.*, 1989; Harvey & Collingridge, 1992). IP<sub>3</sub> can also block potassium channels in hippocampal neurones (Charpak *et al.*, 1990; Gerber *et al.*, 1992), allowing the cell to depolarise more deeply during tetanic stimulation. This effect is independent of PKC or PKA activity and could be mediated either by the direct action of a G-protein or by other diffusible second messengers, such as cAMP. Several members of the metabotropic glutamate receptor family are linked to cAMP

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<sup>7</sup>Metabotropic glutamate receptors stimulate phospholipase C (PLC) via a membrane-associated GTP-binding protein (G-protein). PLC cleaves certain cell membrane phospholipids to produce inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> triggers release of Ca<sup>2+</sup> from intracellular stores. Ca<sup>2+</sup> and DAG act together to activate PKC. (described in Stryer, 1988)

production (reviewed by Schoepp & Conn, 1993) and these have also been implicated in LTP induction (Slack & Pockett, 1991; Musgrave *et al.*, 1993).

Metabotropic receptors exist on the presynaptic as well as the postsynaptic side. They can inhibit vesicle discharge and therefore provide a negative feedback mechanism for glutamate release at the synapse (Forsythe & Clements, 1990; Baskys & Malenka, 1991). This may come about by inhibition of presynaptic voltage-dependent  $\text{Ca}^{2+}$  channels (Lester & Jahr, 1990; Swartz & Bean, 1992; Swartz *et al.*, 1993). However, under some conditions, the action of presynaptic metabotropic receptors can enhance neurotransmitter release. *Synaptosomes* (isolated presynaptic terminals together with attached postsynaptic membranes) prepared from adult rats respond to metabotropic glutamate receptor stimulation by producing  $\text{IP}_3$  and DAG, and releasing  $\text{Ca}^{2+}$  from intracellular stores (Brammer *et al.*, 1991). This stimulates PKC, which in turn increases the amount of glutamate released in response to depolarisation of the presynaptic terminal (Herrero *et al.*, 1992). PKC might do this either by modulating ion channels and thus increasing presynaptic depolarisation (discussed by Nicholls, 1992) or by affecting the machinery that links depolarisation to vesicle release.

One possibility for which there is direct evidence is that PKC activation enhances the activity of voltage-dependent  $\text{Ca}^{2+}$  channels on the presynaptic terminal (Swartz *et al.*, 1993). Although the normal action of presynaptic metabotropic receptors is to inhibit these channels by a mechanism that does not involve protein kinases (see above), PKC is activated by these receptors in the presence of the candidate retrograde messenger, AA (Herrero *et al.*, 1992). The action of PKC on the  $\text{Ca}^{2+}$  channels reverses the normal inhibitory effects that these receptors have on them. If AA is a retrograde messenger, this could provide a neat mechanism by which only active presynaptic terminals connected to recently active postsynaptic terminals would show enhanced release. It also agrees with the idea that presynaptic PKC activity is needed for LTP expression (see section 1.1.5). Further support for the involvement of a presynaptic metabotropic receptor comes from the finding that

pertussis toxin (a blocker of certain *G-proteins*, through which metabotropic receptors mediate many of their effects) inhibits LTP, but saturating postsynaptic G-protein activity does not (Goh & Pennefather, 1989).

### *1.1.9 Distribution of synapses showing LTP*

Although LTP was discovered in the dentate gyrus, most research is now carried out in area CA1, largely because this region is more convenient to study using *in vitro* slice preparations. These two areas show superficially similar LTP but there are some reports of substantial differences. LTP in the lateral perforant path does not seem to require activation of NMDA receptors (Bramham *et al.*, 1991a). This is in contrast to the medial perforant path, which behaves much more like area CA1 and does require NMDA receptor activity. Also, Errington *et al.* (1991) report that a NOS inhibitor reduces LTP in the dentate gyrus but has no effect in area CA1.

LTP at mossy fibre synapses in area CA3 certainly has very different properties from LTP in area CA1. It does not depend on the activity of NMDA receptors and might be non-Hebbian. This form of LTP is discussed in more detail in Chapter Six.

Certain glutamatergic synapses from other regions of the CNS also express LTP. Particular interest has been taken in the discovery of LTP in neocortex (Artola & Singer, 1987; Iriki *et al.*, 1989; reviewed by Bindman *et al.*, 1991) since this is thought to be the site of permanent storage for certain types of memory. LTP has also been seen in the spinal cord (Iriki *et al.*, 1990; Pockett & Figurov, 1993). Long-lasting synaptic enhancement is not limited to glutamatergic synapses: LTP-like potentiation is seen, for example, in cholinergic synapses of the superior cervical ganglion (Brown & McAfee, 1982).

### *1.1.10 The role of LTP in learning and memory*

Learning is the process by which experience shapes our behaviour. Memory is the ability to store and retrieve the information gathered through experience and can be broadly divided into two categories, *declarative memory* (for example, recall of past events and general facts, spatial memory) and *procedural memory* (for example, manual skills) (reviewed by Squire, 1986). Learning and memory are usually considered to be two aspects of the same underlying physiological processes and have become almost synonymous.

LTP was the first example of brief synaptic activity in the brain leading to a durable, specific change in synaptic strength. So, although it is an undeniably artificial phenomenon, LTP is considered to be a useful model for cellular and molecular changes that could underlie learning and memory in normal, living animals.

The fact that LTP conforms so well to Hebb's predictions is itself a strong indication of a role in memory. Furthermore, the hippocampus is known to play an important role in spatial memory in rats (Morris *et al.*, 1982) and declarative memory in humans (briefly reviewed by Milner, 1968). It may act as a temporary buffer for memories (on time scales of days or weeks) before they are passed on to the cortex for longer-term storage (Zola-Morgan & Squire, 1990).

But does LTP, or something similar, happen naturally in living animals? LTP is usually induced by stimulation frequencies of 100Hz or more so it is prudent to question whether natural patterns of neuronal activity can lead to this kind of potentiation. Researchers addressing this point have concentrated on two features of hippocampal neuronal activity—CA3 cell bursting and theta rhythm—and have shown that LTP can be induced by methods that mimic patterns of activity that are thought to occur naturally (Buzsáki *et al.*, 1987; Diamond *et al.*, 1988; Capocchi *et al.*, 1992).

Blocking LTP seems to impair learning. Morris *et al.* (1986) used AP5, to inhibit LTP in living rats. After training, AP5-treated rats took longer than control animals to find a hidden platform in a Morris water maze. Importantly, the two groups also seemed to employ different strategies in searching for the platform. Control animals spent most of the time in the immediate vicinity of the platform while AP5-treated animals swam in circles, a constant distance from the perimeter and spent a similar amount of time in all quadrants of the pool. This is good evidence for *anterograde spatial amnesia*—an inability to store new spatial memories. The inhibition by AP5 of both LTP and learning have very similar dose-response relations and, crucially, no concentration of AP5 blocked LTP without affecting learning (Davis *et al.*, 1992). However, AP5 clearly has effects other than those on memory alone. Morris *et al.* showed that even before training, AP5-treated rats took longer than control animals to find the hidden platform. The difference in escape time before training is about the same as that after training and, using this measure, the rate at which the two groups learned was virtually indistinguishable. Keith and Rudy (1990) have argued that the current evidence is, at best, inconclusive and, at worst, directly contradicts the hypothesis that LTP has a role in learning and memory.

Another method of blocking LTP in intact animals is to saturate the process by artificial stimulation with chronically implanted electrodes. Initial investigations (McNaughton *et al.*, 1986; Castro *et al.*, 1989) indicated that this causes anterograde spatial amnesia but more recent studies have been unable to replicate these findings (Robinson, 1992; Cain *et al.*, 1993; Jeffery & Morris, 1993; Korol *et al.*, 1993; McNamara *et al.*, 1993, Sutherland *et al.*, 1993). This need not be a devastating blow to theories that give LTP or similar processes a central place in mechanisms of learning and memory. In this type of experiment, it is impossible both to know what proportion of synapses have been potentiated and to be sure that LTP at these synapses has been truly saturated (discussed by Bliss & Richter-Levin, 1993).

But many other studies point to a link between LTP and learning. Amnesic drugs inhibit the formation of robust LTP (Krug *et al.*, 1991) and substances that improve learning enhance LTP (Krug *et al.*, 1989; Ashton & Werbrouck, 1991; Thompson *et al.*, 1992). Both old age (Deupree *et al.*, 1991) and hyperthyroidism (Pavrides *et al.*, 1991) disrupt LTP and spatial learning together. Genetically engineered mice with a mutation in the  $\alpha$ -isoform of CaMK II (Silva *et al.*, 1992a,b) or a specific tyrosine kinase (Grant *et al.*, 1992) show impaired LTP and spatial learning, although these studies are open to some of the same criticisms levelled at the AP5 experiments described above (Deutsch, 1993).

All of these studies suffer from the possibility that the effects on learning could come about by mechanisms unrelated to LTP, so any individual report does not constitute conclusive evidence. But the fact that such a wide range of manipulations should have comparable effects on both LTP and learning is strong evidence for a link. A constant finding throughout these data is that spatial memory is affected while certain other types of memory (such as visual discrimination learning) are not, so other mechanisms must also be involved.

LTP is a phenomenon seen at glutamatergic synapses but many other neurotransmitters, including acetylcholine, the opioids, noradrenaline and  $\gamma$ -amino butyric acid (*GABA*) have been implicated in learning and memory (see references in Izquierdo, 1991). One function they might have is to modulate LTP at glutamatergic synapses (e.g. Blitzer *et al.*, 1990; Davies *et al.*, 1991; Mott & Lewis, 1991; Bramham *et al.*, 1991b; Markram & Segal, 1992; Izumi *et al.*, 1992b; Wagner *et al.*, 1993). However, it is also possible that synapses using some of these transmitters can show activity-dependent changes in strength (Brown & McAfee, 1982).

It is important to remember that LTP is long-term only in a neurophysiologist's sense of the word (i.e. hours or days). LTP as we currently know it seems unlikely to account for memories that psychologists describe as long-term, which last for years or decades. In this regard, it will be important to understand more about the changes in

gene expression (Mackler *et al.*, 1992; Patterson *et al.*, 1992) and neuronal structure (Lee *et al.*, 1980) that seem to accompany LTP. Equally important will be a proper understanding of the way in which memories appear to be able to move from one site in the brain (e.g. the hippocampus) to another (e.g. neocortex) (Zola-Morgan & Squire, 1990).

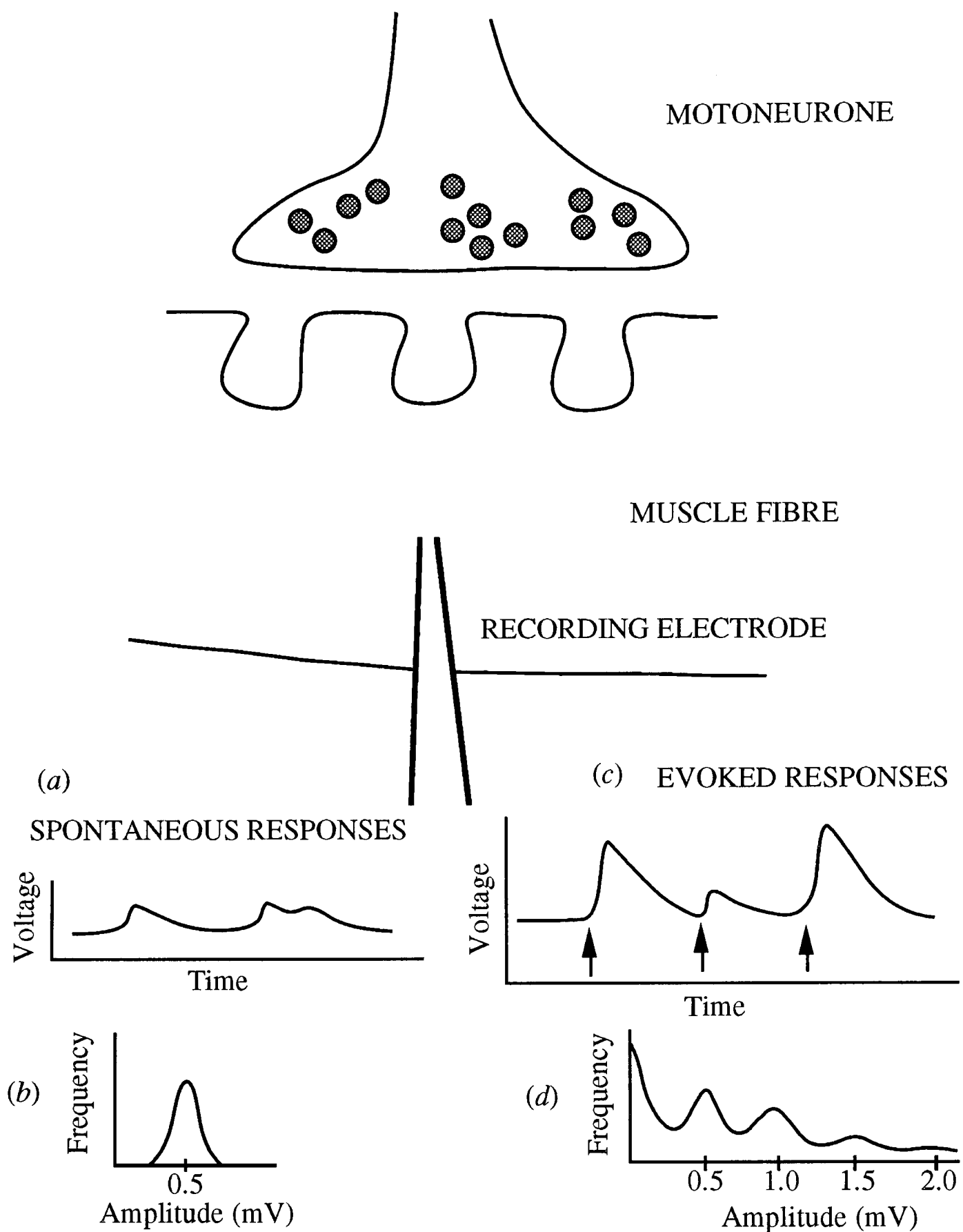
Thus there is strong evidence that LTP-like processes can happen in living animals and that they are involved in memory. But it is also clear that LTP is not the whole story as far as memory is concerned.

## 1.2 Quantal Analysis of Synaptic Transmission

### 1.2.1 *Quantal analysis of the neuromuscular junction*

*Quantal analysis* of synaptic transmission was pioneered at the frog neuromuscular junction (*NMJ*) in the 1950's. It arose from the observation (Fatt & Katz, 1952) that unstimulated motor end-plates could show spontaneous activity in the form of a random succession of miniature end-plate potentials (*MEPPs*). Their amplitudes showed a bell-shaped *Gaussian* distribution about a preferred value, typically about 0.5 mV (see Figure 1.2). This is about one hundredth of the amplitude of the usual response to motor nerve stimulation. The frequency of MEPPs is affected by temperature, osmotic pressure and extracellular  $\text{Ca}^{2+}$  concentration and their amplitudes are affected by curarine, prostigmine and extracellular sodium concentration. This led Fatt & Katz to conclude that each MEPP is due to the spontaneous release of many molecules of acetylcholine, which then act on postjunctional acetylcholine receptors to produce a small depolarisation. The reason for their relatively uniform size, though, was not clear.

Fatt and Katz (1952) also stimulated motoneurone axons and studied the resulting *evoked* end-plate potentials (*EPPs*) in the muscle fibre. Lowering the extracellular  $\text{Ca}^{2+}$  concentration causes the mean amplitude of EPPs to fall dramatically and, under



**Figure 1.3 Quantal analysis at the neuromuscular junction** A fine glass recording electrode can be used to detect voltage fluctuations across the membrane of a muscle fibre that occur in response to neurotransmitter release from a nearby motoneurone. **(a)** Even when the motoneurone is not being stimulated, spontaneous miniature end-plate potentials (*MEPPs*) can be seen. **(b)** The amplitude of these varies but has a mean of about 0.5 mV. **(c)** Stimulation of the motoneurone normally evokes a response amplitude of about 100 times this and causes the muscle fibre to contract. However, if the neurone is stimulated under conditions that artificially reduce the amount of neurotransmitter released, the muscle fibre shows relatively small evoked responses that do not reach the threshold for contraction. These vary in amplitude from trial to trial (arrows). **(d)** The amplitudes of evoked responses show certain preferred levels that correspond to integer multiples of the mean MEPP amplitude (in this case 0.5 mV).

these conditions, EPPs show large random fluctuations in amplitude from trial to trial. Amplitude frequency histograms (which I will refer to from now on simply as *histograms*) show clear peaks, corresponding to certain preferred amplitudes (see Figure 1.3). These are integer multiples of the mean MEPP amplitude, which led to the suggestion that EPPs are composed of discrete, irreducible units or *quanta* and that each MEPP corresponds to a single quantum (del Castillo & Katz, 1954a). Because their results involved random EPP amplitude fluctuations, del Castillo and Katz (1954a) developed a statistical model to describe them (see Box 1.1). Their data were well described by *Poisson statistics*.

### **Box 1.1 A statistical model of neurotransmission**

The presynaptic terminal is composed of  $N$  sites, each of which releases one quantum in an all-or-nothing (*binomial*) manner when stimulated by an action potential. The probability that a given site will release a quantum in response to an action potential is  $P$ . For the *simple binomial* case, where  $P$  is the same at all sites, the mean number of quanta released per trial,  $M = NP$  and the probability,  $P_x$  that any given trial will result in the release of  $x$  quanta is given by:

$$P_x = \frac{N!}{(N-x)!x!} P^x (1-P)^{N-x}$$

When  $P$  is not identical at all release sites, *compound binomial* statistics will apply and  $M = N\bar{P}$ , where  $\bar{P}$  is the mean value of  $P$ . Provided that the variations in  $P$  are not extreme, simple binomial statistics may provide a good approximation. If  $P$  is very small in relation to  $N$ , (as is the case at the neuromuscular junction when release is artificially depressed) release will approximate to *Poisson* statistics. In this case:  $P_x = \frac{M^x e^{-M}}{x!}$

In all cases the mean postsynaptic response,  $E = MQ$  where  $Q$  is the mean postsynaptic response to a single quantum of transmitter.

These results were soon confirmed in cat (Boyd & Martin, 1956) and rat (Liley, 1956) NMJ preparations. Once again, the results conformed well to Poisson statistics.

At this time, the first electron micrographs of the neuromuscular junction were published (Robertson, 1956; de Robertis, 1958). These showed that the presynaptic terminal contains small membranous *vesicles* of roughly uniform size. This immediately suggested a possible anatomical correlate for the electrophysiological quantum—the discharge of neurotransmitter from individual vesicles could give rise to a quantal postjunctional response (del Castillo & Katz, 1956). More than a decade later Heuser & Miledi (1971) provided the first direct evidence that synaptic vesicles are responsible for transmitter release.

The number of release sites at a single neuromuscular junction has been estimated at 300-1,000 and one quantum of transmitter is thought to open 1,000-1,700 postsynaptic channels (Katz and Miledi, 1972; Anderson & Stevens, 1973).

### *1.2.2 Quantal analysis in the mammalian spinal cord (and goldfish)*

Neurons in the CNS contain synaptic vesicles too, suggesting that transmission here might also be quantal. Unfortunately, recording excitatory postsynaptic potentials (*EPSPs*) in the CNS is technically more difficult than similar experiments at the neuromuscular junction. Spontaneous miniature EPSPs (*miniatures*) recorded at the soma of a CNS neurone can arise from any one of thousands of synaptic contacts, not just the ones of interest, so it might be difficult or impossible to obtain a measure of quantal size from the amplitude of miniatures. Also, a single afferent axon typically has several *boutons* (see Figure 1.2) that each make a synaptic connection with individual spines on the postsynaptic cell (Jack *et al.*, 1971). These might be widely dispersed and each could show different transmission properties. Any variation in *P* between individual boutons causes the statistics to deviate from a simple binomial distribution and makes a reliable analysis more difficult (Brown *et al.*, 1976; Barton & Cohen, 1977). A substantial variation in *Q* would preclude a quantal analysis all

together. Finally, recordings made in the CNS usually have lower signal-to-noise ratios than experiments at the NMJ because finer (and thus higher-resistance) recording electrodes must be used.

Intracellular recordings of spontaneous activity in frog spinal motor neurones showed responses that seemed to be analogous to MEPPs at the NMJ but these had a greater variation in amplitude and time course (Katz & Miledi, 1963). A study of evoked EPSPs in spinal motoneurones showed that when one or a few afferent fibres were stimulated the postsynaptic response fluctuated in amplitude from trial to trial and included apparent failures of response (Kuno, 1964). This was taken to be evidence of a quantal transmission mechanism similar to that which operates at the neuromuscular junction, although histograms of evoked responses did not show clear peaks. The overall distribution of amplitudes could be adequately described by assuming an underlying quantal process with a Poisson distribution. Estimated quantal sizes were in the range 0.12-0.24 mV and values of  $M$  were low, ranging from less than 1 to about 15 (Kuno, 1964).

Histograms with peaks too indistinct to be reliably located by eye can be analysed using a *deconvolution* method to extract underlying components (Edwards *et al.*, 1976). This assumes that:

1. EPSP amplitudes fluctuate between a small number of discrete values.
2. Fluctuations in the noise and the noise-free EPSP are independent and add linearly.

If there is no quantal variance, each underlying component will have the same SD, which, to a first approximation, is often taken to be the same as the baseline noise. The positions and heights of these components are estimated and used to plot theoretical histograms, which are then compared to the empirical data. The estimates are modified until the best fit is obtained according to the sum of the squared differences or the  $\chi^2$  statistic.

Edwards *et al.* concluded that underlying components are not equally spaced but a later study, which applied a refined deconvolution procedure to histograms of motoneurone EPSPs, concluded that they are (Jack *et al.*, 1981). This is good evidence for quantal transmission. Amplitude distributions were not well described by Poisson or simple binomial statistics. Individual amplitude components had different time courses, suggesting that they arose from widely-spaced sites on the dendritic tree, and the calculated number of release sites was similar to the number of boutons per afferent fibre determined in earlier anatomical studies. This led Jack *et al.* (1981) to propose that each bouton contains one release site and responds to an action potential in an all-or-nothing fashion. The average quantal size was about 90 $\mu$ V.

The coefficient of variation (*CV*) of the quantal size for any given EPSP was very low (less than 5%, compared to about 30% at the NMJ). There was also little apparent variation in quantal size between EPSPs evoked at different sites on the neurones' branched dendrites (*dendritic trees*). EPSPs are recorded at the cell body, or *soma*, so distal synapses must pass relatively more charge than proximal ones in order to have the same peak amplitude at the recording site. This suggests that the quantal synaptic currents at individual spines are larger at distal connections than at proximal ones. These facts led Jack *et al.* to propose that, in the CNS, the quantal size is set postsynaptically by the number and sensitivity of receptors and that, at any given bouton, sufficient neurotransmitter is released to saturate these receptors. This is unlike the NMJ, where the quantal size is set presynaptically by the amount of transmitter in each vesicle, there being a vast excess of postsynaptic receptors.

Korn and his co-workers provided further evidence for the proposal that each bouton contains one release site that acts in an all-or-nothing fashion. Working with inhibitory glycinergic synapses in the goldfish, they obtained histograms with remarkably clear peaks and found transmission here to be well-described by simple binomial statistics. They showed an almost perfect correlation between the binomial parameter *N* and the number of boutons seen under the microscope (Korn *et al.*, 1981,

1982). Most boutons appear to contain only one *active zone*, an area of synaptic connection that may correspond to a single release site (Triller & Korn, 1982).

Further work on the spinal cord was carried out by Walmsley *et al.* (1987, 1988), this time on dorsal spinocerebellar tract (DSCT) neurones. Quite clear peaks could sometimes be seen in histograms, which were deconvolved by a maximum likelihood estimation (*MLE*) method. This had been developed by Ling and Tolhurst (1983; see also Kullmann, 1989) and is more reliable than the earlier methods described above. Release statistics here, like those in motoneurones, were not adequately described by a simple binomial model in which the release probability at each release site is assumed to be identical. The data were better described by a compound binomial model with different release probabilities at each site.

One of the problems associated with deconvolution is that finite sampling error can lead to spurious inflections in amplitude frequency distributions and this can lead to detection of false underlying components. Kullmann (1992) has recently developed maximum entropy noise deconvolution (*MEND*), a method that reduces this problem by supplying the smoothest distribution that is compatible with data, taking noise and sample size into account.

### *1.2.3 Quantal analysis in the hippocampus*

Spontaneous miniature EPSPs can be seen in CA3 hippocampal neurones in the presence of tetrodotoxin (TTX), which blocks the propagation of action potentials (Brown *et al.*, 1979). This led Yamamoto (1982) to undertake a quantal analysis of synaptic transmission at mossy fibre terminals in the CA3 region of hippocampal slices. He saw no quantal peaks so he calculated values for  $M$  and  $Q$  from the mean and variance of the response by assuming a Poisson distribution. The mean values were  $M=8.3$  and  $Q=280\mu\text{V}$ .

Sayer *et al.* (1989) applied quantal analysis techniques to CA1 hippocampal pyramidal neurones. They made intracellular recordings and stimulated excitatory synapses either by intracellular impalement of connected CA3 cells or by extracellular stimulation of Schaffer collateral fibres. They studied 5 EPSPs using the dual impalement arrangement. These showed an amplitude range of 85-275 $\mu$ V and three of them showed trial-to-trial fluctuations in amplitude too great to be attributed to baseline noise alone, indicating intermittent (and possibly quantal) synaptic transmission. They did not see distinct histogram peaks but two EPSPs induced by extracellular stimulation had sufficiently low background noise levels to use MLE deconvolution. These gave quantal sizes of 224 and 193 $\mu$ V respectively. A subsequent study by the same group (Sayer *et al.*, 1990) gave a quantal size of 278 $\mu$ V for an EPSP recorded by dual impalement.

### 1.3 Quantal Analysis of LTP

Assuming you can overcome the technical difficulties, quantal analysis seems to be an ideal way of resolving the question of whether LTP is expressed presynaptically or postsynaptically. An increase in  $P$  would indicate a presynaptic change in vesicular release probability. An increase in  $N$  would indicate a change in the number of active boutons. This could come about either by a presynaptic increase in the number of responsive release sites or by postsynaptic activation of previously inactive arrays of receptors, but the presynaptic mechanism is generally thought to be more plausible. An increase in  $Q$  would indicate a change in quantal size which, in view of the vast excess of neurotransmitter molecules thought to be contained in each vesicle, is more likely to be a postsynaptic than a presynaptic change.

The first quantal analyses of hippocampal LTP were carried out by Voronin in region CA3. These indicated a presynaptic locus of expression (reviewed by Voronin, 1983). His more recent work in CA1 reached similar conclusions, although small increases in quantal size were often seen (Voronin *et al.*, 1991a,b, 1992a,b,c).

Two studies that applied whole-cell patch clamp recording to quantal analysis of LTP confirmed these conclusions (Malinow & Tsien, 1990; Bekkers & Stevens, 1990). Despite the favourable signal-to-noise ratio, EPSC histograms did not usually show clear peaks. Malinow and Tsien showed that in some cases the 'failures' peak (which corresponds to the release of no quanta) could be resolved and that the amplitude of this decreased following LTP induction. This indicates a presynaptic component to the potentiation. The CV of the fluctuating EPSC amplitudes was used as an indirect measure of changes in binomial parameters (see Chapter 2). Potentiation was accompanied by a substantial increase in  $1/CV^2$  which, according to most models of neurotransmitter release, indicates a presynaptic change. These data were interpreted by Malinow and Tsien as evidence that the changes underlying LTP are mainly presynaptic.

Bekkers and Stevens (1990) made use of neurones in culture as well as hippocampal slices. Assuming simple binomial transmitter release statistics, they showed that the change in overall distribution of histograms following LTP induction in cultured cell pairs was best described by a change in  $P$  rather than in  $N$  or  $Q$ . In hippocampal slices,  $1/CV^2$  increased in direct proportion to mean EPSC amplitude. This was interpreted as indicating Poisson release statistics and a presynaptic change which, in light of their *in vitro* results, was assumed to be in  $P$  rather than  $N$ .

Malinow (1991) used dual impalements to study four neurone pairs in hippocampal slices. By analysing changes in CV and the number of failures, he showed potentiation to be largely presynaptic. Individual peaks in the histograms could be resolved by eye and these indicated an increase of about 50% in the quantal size, which accounted for a small proportion of the 10- to 20-fold potentiation.

Foster and McNaughton (1991) used intracellular recording together with deconvolution methods that assume simple binomial or Poisson release statistics. They showed that quantal size, but not quantal content, increases during LTP, thus contradicting the conclusions of these other quantal analysis experiments.

Two studies have investigated the size of miniature EPSCs (which are thought to be single quanta) before and after LTP induction, but these failed to produce consistent results. One, using hippocampal slices, demonstrated that miniatures get bigger but their frequency does not change (Manabe *et al.*, 1992) and the other, using neurones in culture, showed exactly the reverse (Malgaroli & Tsien, 1992).

So quantal analysis also seemed unable to resolve the controversy surrounding the synaptic locus of LTP expression. Once again, there is good evidence for changes on both sides of the synapse but individual laboratories consistently produced data supporting the predominance of only one of these.

## 1.4 Aims of This Project

My project aimed to investigate long-term potentiation and other forms of synaptic plasticity in rat hippocampal slices using intracellular voltage recordings. I used quantal analysis to determine the relative contributions of presynaptic and postsynaptic modifications to changes in synaptic efficacy.

# Chapter Two

## Methods

### 2.1 Hippocampal slices

#### 2.1.1 *The hippocampal slice as an in vitro preparation*

The main excitatory pathways of the hippocampal formation pass from the entorhinal cortex to the subiculum by running across the width of the hippocampus proper via the dentate gyrus, area CA3 and area CA1 (see Chapter One). These transverse projections are often thought to show little or no significant spread along the length of the hippocampus and other pathways that do run along the length of the hippocampus are usually thought to be less important. In 1971, Andersen *et al.* concluded from their electrophysiological and anatomical studies that:

*"The hippocampal cortex seems to be organized in parallel lamellae, both with regard to the neuronal and the vascular system. By means of this lamellar organization, small strips of the hippocampal cortex may operate as independent functional units...a point source of entorhinal activity projects its impulses through the four membered pathway along a slice or lamella, of the hippocampal tissue oriented normally to the alvear surface."*

If this is true, a transverse hippocampal slice could be a particularly convenient *in vitro* preparation for investigating hippocampal function: their neuronal connections would be largely intact but, at the same time, exposed to electrophysiological and pharmacological manipulation. As a result, this preparation has been extremely popular among electrophysiologists since the early 70s (Yamamoto, 1972). Another advantage is that *in vitro* slices are more stable subjects than live, often awake animals. This is particularly important when making intracellular recordings, which are extremely sensitive to vibrations or instabilities of any kind.

However, it is now clear that hippocampal circuitry is neither as simple nor as lamellar as this picture suggests. Andersen *et al.* (1971) acknowledged that longitudinal connections might exist, but underestimated their importance. A review of more recent anatomical data (Amaral & Witter, 1989) concludes that most of the major hippocampal pathways show considerable spread along the length of the hippocampal formation. The only exceptions are the mossy fibre connections between dentate gyrus granule cells and CA3 pyramidal cells. Important information processing takes place in both the transverse and long axes. It is also true that a slice contains a lot of damaged tissue and might have pathologically high or low levels of important biochemicals. This does not necessarily make the *in vitro* hippocampal slice preparation a less useful tool for investigating connections between individual neurones, but care must be taken in using *in vitro* results alone to conclude too much about the function of the hippocampus *in vivo*.

Since long-term potentiation (LTP) is seen in several regions of the hippocampus, transverse slices are an effective way of investigating the induction and early expression of this phenomenon (Schwartzkroin & Wester, 1975). Since the life span of neurones in a slice is limited to a matter of hours, this preparation is less useful for studying the later stages and ultimate duration of the longest-lasting forms of LTP.

The most widely studied region of the hippocampal slice is area CA1. The major excitatory inputs here are the Schaffer collaterals from area CA3 (see Chapter One). One of the advantages of studying CA1 is that the pyramidal cell bodies are arranged in a narrow band, which is often visible with a low-power microscope. If the intracellular electrode is placed in this *cell body layer*, the chances of getting a stable impalement are quite high even if the electrode is simply pushed blindly through the slice.

Area CA3 has a similar arrangement to CA1 but contains many recurrent excitatory connections, which can cause spontaneous bouts of neuronal activity, particularly if inhibitory synapses are pharmacologically blocked (as they often are during LTP

experiments). For this reason, CA3 is often removed from slices before recording begins, to prevent the uncontrolled activity from spreading to CA1 and interfering with recordings there (Lynch *et al.*, 1982).

I used rat hippocampal slices to investigate LTP and other forms of synaptic plasticity in area CA1. By using a combination of quantal analysis procedures I tried to find out whether increases in synaptic strength are caused by presynaptic changes, postsynaptic changes, or a combination of both.

### *2.1.2 Rats*

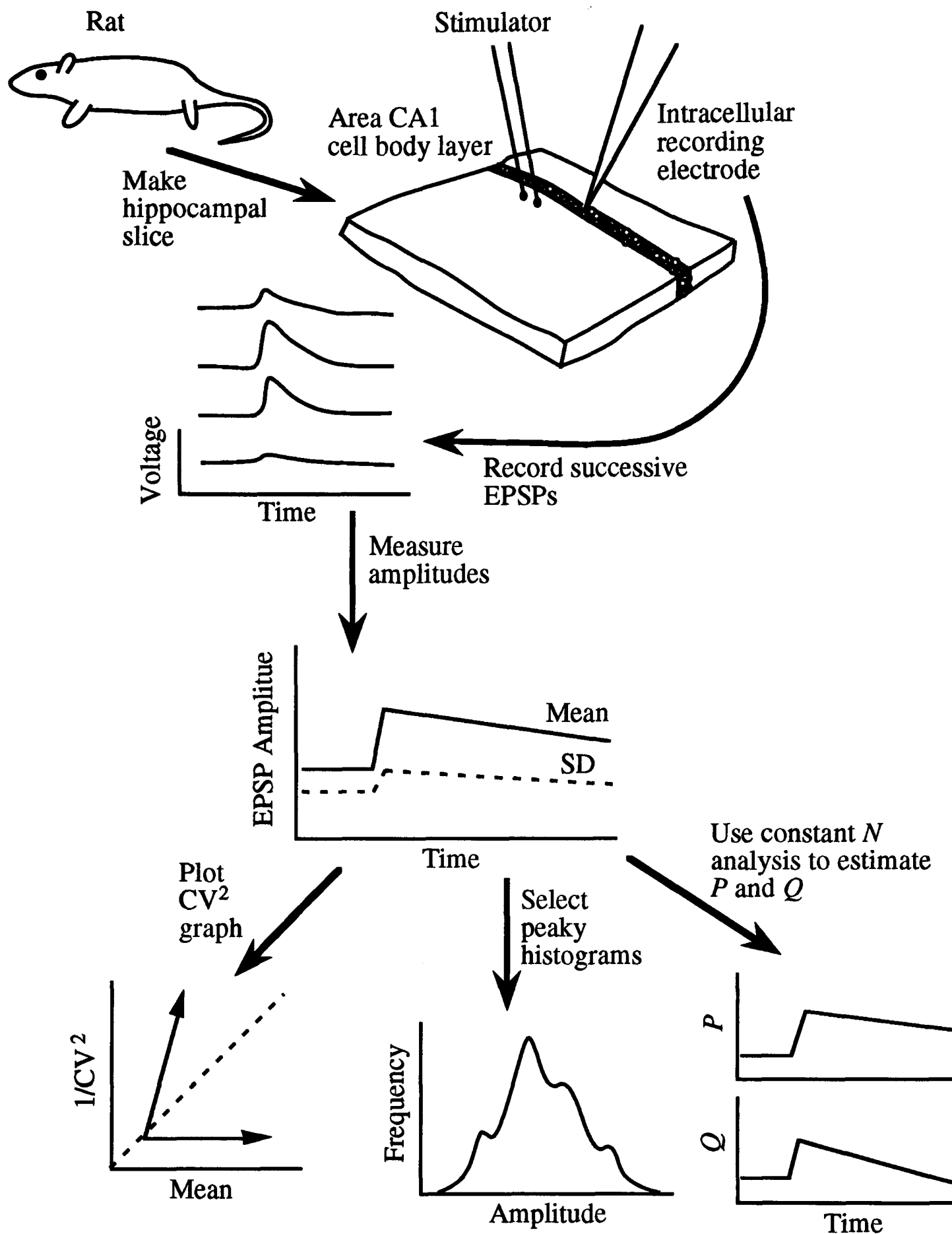
In all experiments I used male albino rats of the Sprague-Dawley strain. They were young adults weighing 100-180g (about 30-60 days old).

### *2.1.3 Artificial cerebrospinal fluid*

The artificial cerebrospinal fluid (ACSF) I used had the following composition (in mM): NaCl 124, NaHCO<sub>3</sub> 26, CaCl<sub>2</sub> 4.0, MgCl<sub>2</sub> 3.0, KCl 2.3, KH<sub>2</sub>PO<sub>4</sub> 1.26, MgSO<sub>4</sub>·7H<sub>2</sub>O 1.0, glucose 10, (all BDH). It was thoroughly gassed with carbogen (composition: 5% CO<sub>2</sub> / 95% O<sub>2</sub>) (BOC).

### *2.1.4 Removing and cooling the brain*

I anaesthetised the rat with halothane inhalation anaesthetic using the following procedure. I poured about 7 ml of anaesthetic (Fluothane, ICI) onto a swab of cotton wool inside a bucket (volume about 5l) with a transparent lid. After a delay of a few minutes to allow the anaesthetic to evaporate, I put the rat into the bucket. When it appeared to be unconscious, I checked to see whether or not it was fully anaesthetised by pinching one hind leg firmly. If there was no withdrawal response, I took it to indicate complete anaesthesia.



**Figure 2.1 Summary of the experimental procedure** I prepared hippocampal slices from young adult rats and kept them *in vitro*. I stimulated Schaffer collaterals using an extracellular metal bipole and recorded the resulting small intracellular excitatory postsynaptic potentials (EPSPs) from pyramidal neurones in the CA1 region. Successive EPSPs were stored on disk for later analysis. I induced changes in EPSP amplitudes using a variety of methods. After the experiment, the amplitudes of successive EPSPs were measured and the mean and SD of these plotted against time. In order to determine which quantal parameters had changed, I used three quantal analysis procedures:  $1/CV^2$  graphs, amplitude frequency histograms and constant  $N$  analysis. Details are given in the text.

To minimise ischaemic and anoxic damage, I removed the brain as quickly as reasonably possible (typically in about 2 minutes) using the following procedure. I decapitated the rat with a purpose-built guillotine and put the head onto a sheet of tissue paper on top of a bed of ice to keep it cool. I exposed the skull by using a scalpel (Swann Morton) to make a single cut along the mid-line of the head, from the bridge of the nose to the back of the head and then easing the skin apart. I then made a series of cross-hatch cuts across the surface of the skull to disrupt the connective tissue and make removal of the skull easier.

Using a syringe, I passed ice-cold (1-2 °C) ACSF over the surface of the skull to cool the brain underneath. I then prised off pieces of the skull with a pair of rongeurs until the cerebellum and most of the cortex was exposed. I poured on more ice-cold ACSF to cool the brain further. I cut away the cerebellum and frontal lobe with a scalpel and then gently scooped out the remaining middle portion with a spatula. This I put in a beaker of ice-cold ACSF for a few minutes, allowing it to cool down thoroughly.

### *2.1.5 Cutting hippocampal slices*

I removed the brain from the ice-cold ACSF and put it onto a cold plastic cutting block covered with a circle of filter paper (Whatman) soaked in ACSF. Using a razor blade (Campden Instruments), I made a cut parallel to the mid-line but about 1mm displaced from it. I then kept the smaller portion. I removed excess fluid with a dry piece of filter paper and glued the brain tissue by its cut surface onto a Teflon cutting block (Campden Instruments) using cyanoacrylate adhesive (RS Components). I waited 5-10s for the glue to set before immersing the cutting block and brain in a small bath of ice-cold ACSF to which I added small cubes of frozen ACSF to keep the solution cold.

I transferred the bath to a vibrating microtome (Vibroslice, Campden Instruments). Cutting from the top (i.e. the side of the brain), I removed the uppermost 2-3mm until I could see the hippocampal formation clearly. Then I cut a series of 6 slices, each

400 $\mu$ m thick, which I stored in ice-cold ACSF. Using a pipette, I transferred them to a petri dish of ice-cold ACSF and trimmed them to size with a scalpel. I made four cuts: one to remove region CA3, one to remove the neocortex, and two to remove tissue posterior and ventral to the dentate gyrus, including the entorhinal cortex. The resulting slice was rectangular and measured about 2-3mm by 3-5mm. After all six slices were trimmed, I used a fine paint brush to transfer them to the recording chamber, where they were left to recover for at least an hour.

## 2.2 Recording

### 2.2.1 *Intracellular-electrode versus patch-clamp recording methods*

The original method for making electrical recordings from inside neurones was to use a sharp glass *intracellular electrode*. These usually have tip diameters of less than 1  $\mu$ m and are used to penetrate the cell and record fluctuations in voltage between the cell interior and the extracellular medium. Now many researchers—including most of the ones involved in quantal analysis—prefer to use the newer whole-cell *patch-clamp* recording technique. This method was developed by Neher & Sakmann (1976) and was first applied to tissue slices by Edwards *et al.* (1989). It uses a blunter electrode with a tip diameter of around 2-3 $\mu$ m. Rather than impaling the cell, this is sealed tightly to the outer surface of the cell membrane, which is then ruptured to allow access to the cell interior (Hamill *et al.*, 1981). This arrangement gives better conductance between the cell and the electrode and also greatly reduces the amount of current that leaks out from around the edge of the electrode. As a result, levels of high-frequency recording noise are much lower than with intracellular electrodes.

But the advantages of patch-clamp recording may have been overestimated. As a method for investigating the behaviour of single ion channels in excised patches of cell membrane it is unrivalled, but as a quantal analysis tool it may have little more to offer than intracellular-electrode voltage recordings. One problem is that the volume

of the solution inside the electrode is very much larger than the volume of the cell's cytoplasm, which therefore loses many important constituents by diffusion. This happens very slowly during intracellular-electrode recordings but the broad tip of the patch-clamp electrode allows it to have effects within minutes. Cellular constituents essential for LTP induction, for example, are lost after a patch-clamp electrode has been attached to a neurone for more than about 30 minutes (Malinow & Tsien, 1990; Rosenmund & Westbrook, 1993).

Another important difference is that patch-clamp electrodes are usually used in '*voltage clamp*' mode, in which the membrane is held at a constant potential and measurements are made of the changes in current flow across it. One consequence of this is that any change in the series resistance of the electrode will have large effects on the currents measured. In contrast, the effects this has on voltage recordings are minimal. Another potential problem is that synaptic currents are attenuated as they pass along dendrites, which results in imperfect voltage clamp at the synapse and a reduction in the measured current at the recording site. The effect is most acute (Major, 1993) when the synaptic currents are fast (as they can be at glutamatergic synapses (e.g. Silver *et al.*, 1992)) and when synapses lie some distance from the cell body (as Schaffer-collateral connections in area CA1 do). These considerations may partly explain why some quantal analysis studies have used patch-clamp recording but failed to detect clear quantal peaks (Malinow & Tsien, 1990; Bekkers & Stevens, 1990).

Another possible explanation is that excessive background recording noise is not the only factor obscuring the peaks. Larkman *et al.* (1991) showed that the quantal size can change with time and that this effect might be at least as important as background noise. By taking into account such *non-stationarity*, they were able to produce amplitude frequency histograms with clear peaks using voltage data recorded with intracellular electrodes from hippocampal slices. My project is a continuation of this

work. I used the same recording and analysis methods and applied them to synaptic potentiation in the hippocampus.

### 2.2.2 *The recording chamber*

I used an *interface*-type recording chamber, so called because the slices are maintained at the interface between warm ACSF and warm, humidified carbogen gas. In my experiments, the slices lay on a strip of lens tissue (Whatman) about 1 centimetre wide and were bathed in a constant stream of ACSF supplied by a drip feed. A peristaltic pump (Philip Harris Scientific) delivered the ACSF at a rate of about 0.1-0.2 ml / minute. During the recovery period the ACSF had an identical composition to that given above (section 2.1.3). The lens tissue lay across a fine nylon mesh platform (about 5 centimetres in diameter) and the ends of the lens tissue rested in a surrounding circular 'moat'. Excess ACSF ran into this and was drawn off by the peristaltic pump.

The temperature of the recording chamber was maintained at  $34\pm 1^{\circ}\text{C}$  by a thermostatically-controlled water-bath. Carbogen gas was bubbled through a column of water and then through the water bath to humidify and warm it. From here, it was passed over the slices to ensure a constant supply of oxygen. The recording chamber rested on an air table (Newport, USA) to minimise vibration.

After the recovery period, the composition of the ACSF passed over the slices was changed to include (in addition to the substances already listed) 0.5mM glutamine (Sigma), 50 $\mu\text{M}$  picrotoxin (Sigma) and 5 $\mu\text{M}$  glycine (BDH). Glycine is needed at low concentrations for NMDA receptor activity (Johnson & Ascher, 1987). Picrotoxin blocks GABA<sub>A</sub> receptors, ionotropic receptors that respond to the inhibitory neurotransmitter  $\gamma$ -amino butyric acid (GABA). The inhibitory postsynaptic potentials (*IPSPs*) that these generate produce low-frequency noise and can also affect the measured amplitude of EPSPs. Using picrotoxin has the unfortunate side-effect of making the slice prone to bouts of spontaneous *epileptiform* activity. The relatively

high  $Mg^{2+}$  concentration helps to counteract this by suppressing neurotransmitter release. Glutamine is converted into glutamate neurotransmitter inside glutamatergic neurones and was included in the hope that it would reduce the amount of synaptic depression caused by glutamate depletion during prolonged stimulation. For some experiments I also added  $100\mu M$  of the NMDA receptor antagonist, D,L-2-amino-5-phosphonovalerate (AP5) (Tocris Neuramin) to the ACSF. This is indicated in the text or figure legends where appropriate.

### *2.2.3 Recording*

I made electrodes from standard-wall glass tubing with an inset filament (outer diameter 1.2mm, inner diameter 0.69mm) (World Precision Instruments, USA) on a horizontal electrode puller (Mecanex, Switzerland). The electrodes were fairly blunt by intracellular recording standards and usually had DC tip resistances of 40-60M $\Omega$  (measured in ACSF) when they were filled with an electrolyte solution containing 2M potassium methyl sulphate and 5mM potassium chloride. I inserted a silver/silver chloride (Ag/AgCl) wire into the electrode. This was connected via a 'headstage' to an amplifier (Axoprobe-1A, Axon Instruments, USA). The headstage was mounted on a piezoelectric stepper motor (Digitimer), which, in turn, was mounted on a 'micromanipulator' (Leitz). Using a low-power (x10) stereo-microscope (Wild Heerbrugg) and the micromanipulator, I lowered the electrode tip onto the surface of a slice as near as possible to the CA1 cell body layer. The resistance and capacitance of the electrode were compensated for using the amplifier's bridge-balance and capacitance compensation controls. Using the stepper motor, which was controlled with a small laptop computer (New Brain or Epson), I pushed the electrode slowly through the slice in 4 $\mu m$  steps.

When the electrode tip enters a pyramidal cell body, the measured potential becomes negative (typically -40 to -70mV) and action potentials can be seen on the oscilloscope trace. By applying a negative holding current (usually less than 1nA)

through the tip of the electrode, it is often possible to stop the action potentials and return the cell to near its *resting potential* of about -70 to -80 mV. When I had done this, I usually waited for several minutes to make sure that the resting potential did not show any large fluctuations or significant drift. When I was sure that the impalement was reasonably stable, I began to record.

I stimulated incoming axons with a bipolar wire stimulator made of two intertwined silver wires, each coated in Teflon insulation (Clark Electrical Instruments) and cut at the tip to reveal the bare metal. Using a second micromanipulator (Leitz), I positioned the stimulator on the surface of the slice in the *stratum radiatum*, a layer of the hippocampus composed mainly of dendrites and axons that lies immediately below (that is, on the ventral, or apical side of) the cell body layer. I placed the stimulator on the side of the *stratum radiatum* that lay closest to the cell body layer at various distances from the recording site. Placing it a long way from the electrode reduces the chance that the impalement will be lost if the stimulator is moved and might also increase the chances of stimulating a single incoming axon rather than multiple axons. However, depending on the health of the slice, it is sometimes difficult to elicit a postsynaptic response at all if the stimulator and electrode are far apart. Though I could not see the axons or synapses I was stimulating, the intention was to stimulate synapses on the *proximal apical* dendrites of the cell, which, because they lie close to the cell body, ought to give the best signal-to-noise ratio.

Using extracellular stimulation is not ideal. It is possible, for example, for more than one incoming axon to be stimulated. Because of this, I discarded excitatory postsynaptic potentials (EPSPs) that looked as if they might have arisen in this way and moved the stimulator tip around the slice in search of more suitable examples. The criteria were that the rising phase had to seem smooth and fairly short (10-90% rise time usually less than 5ms) and that the EPSP had to appear in a reasonably stepwise manner as I increased the stimulating voltage. This gave the best chance of eliciting EPSPs that arose from the stimulation of a single axon.

Another disadvantage of extracellular stimulation is that axons might not be stimulated reliably. Even if they are, action potential propagation failure is quite likely in a hippocampal slice, which always contains a proportion of unhealthy tissue. For this reason, I also discarded EPSPs that showed a high proportion of failures. Nevertheless, unreliable stimulation was still a potential problem and it affects the results of quantal analysis. I will discuss the possible relevance of this to my results in Chapter Four.

The stimulator's power supply (Digitimer) allowed the voltage and duration of each pulse to be controlled. The duration was always kept to the minimum (0.02ms) and the voltage adjusted to elicit the smallest response in the postsynaptic cell that could be reliably seen above the background noise. The stimulation voltage was typically about 10V but could be much higher, depending on the cleanliness of the stimulator tip. I gave test stimuli, or *trials*, at rates between 0.2 and 4Hz. These are given in the text and figures where appropriate. In most experiments I used a rate of 1Hz<sup>1</sup>.

The timing of stimulations and recordings was co-ordinated with a timing box (Digitimer). The voltage recordings were filtered at 1kHz, digitised at 5kHz using an analogue-to-digital converter (Cambridge Electronic Design). At each stimulus, I collected a *sweep* of voltage data 50-200ms long using a computer program ('Sigavg', Cambridge Electronic Design) running on a personal computer (IBM PC-AT) and stored it on disk for later analysis. In each sweep, a brief 5mV calibration pulse (Omnicol 2001, World Precision Instruments, USA) and several milliseconds of baseline period preceded the stimulus artefact and the resulting EPSP<sup>2</sup>.

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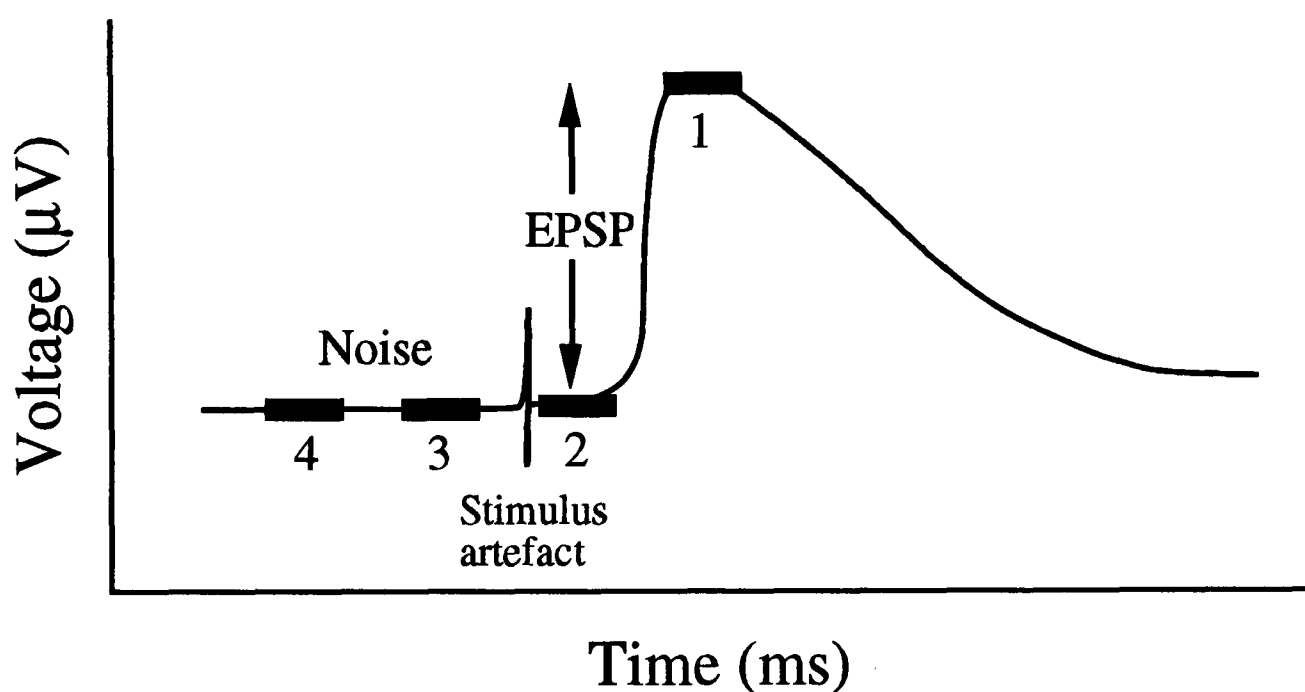
<sup>1</sup>In order to reduce the chance of constant-frequency noise sources (such as mains pick-up) producing any systematic offsets in the recorded data, I never used exact integer frequencies. The rate was therefore slightly less than 1Hz (about 0.95Hz). This is taken into account whenever I have converted trial numbers into times or durations.

<sup>2</sup>I have removed the stimulus artefacts from all the experimental EPSP waveforms shown in this thesis.

## 2.3 Quantal analysis

### 2.3.1 EPSP amplitude measurement

EPSP amplitudes were measured automatically using a computer program (written by Ken Stratford, University Laboratory of Physiology, Oxford). Figure 2.2 shows how the amplitude of the EPSP and noise in each sweep was calculated.



**Figure 2.2** *Measurement of EPSP and noise amplitudes* A computer program calculated the amplitude of the baseline noise and the EPSP in each sweep. EPSP amplitudes were calculated as the difference in the mean voltages between a period of 1.6-2.0ms straddling the EPSP peak (1) and a similar period at its base (2). The noise amplitude was calculated in the same way using zones of the same length and separation positioned in the baseline region (3 & 4).

Using the average waveform of all the sweeps collected for a particular EPSP, I selected two zones of 1.6-2.0ms in length, one straddling the EPSP peak and the other in the baseline region. The amplitude of the EPSP in each sweep was calculated as the difference between the mean voltage in these two zones. The shorter the time interval between the zones, the less the underlying background noise interferes with the measurements so, where possible, I positioned the baseline zone between the stimulus artefact and the foot of the EPSP. When the latency between the stimulus artefact and the rising phase of the EPSP was less than 1.6ms, I positioned it immediately before

the stimulus artefact. The amplitude of the baseline noise was measured using two zones positioned in the baseline period. These were always identical in length and separation to the zones used to measure the EPSP amplitude.

The locations of the zones were always constant for any particular EPSP. If the EPSP showed small changes in rise time or latency during the recording, the zones were selected as a compromise that could satisfactorily measure all the sweeps. I discarded any EPSPs that showed large changes in rise-time or latency. The amplitudes of the EPSP and noise in successive sweeps were stored on disk for analysis.

When the amplitudes of successive sweeps are plotted against time, they usually vary widely from trial to trial so, when looking for trends, it is more useful to bin the amplitudes into *epochs* of, for example, 100 successive trials and then plot the mean EPSP amplitude (hereafter referred to simply as *mean*) for each epoch. When the data are binned in this way, the standard deviation of the EPSP amplitude fluctuations (referred to from now on as *SD*) can also be calculated for each point. The mean and SD can give important information about what is happening at the synapse, particularly when the EPSP amplitude is changing.

### 2.3.2 $1/CV^2$ graphs

One way of using the mean and SD is to calculate the coefficient of variation (*CV*, equal to  $SD/mean$ ). In this case we are interested in the SD of the noise-free amplitude fluctuations, which can be calculated by subtracting the SD of the baseline recording noise from the SD of the EPSP<sup>3</sup>. By making certain assumptions about the nature of transmission, the CV can give useful information about which quantal parameters have contributed to any change in mean (del Castillo & Katz, 1954b, Clements, 1990). Box 2.1 describes the basic theory behind this. Malinow and Tsien

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<sup>3</sup>This is a simplification. In fact, the amount subtracted from the measured SD is usually a bit less than the SD of the baseline noise (see Chapter Three).

(1990) were the first to use this method to determine the locus of the change underlying LTP.

### **Box 2.1 The theory of $1/CV^2$ graphs**

This type of analysis assumes that transmission at the synapse or synapses in question is quantal and that it obeys simple binomial statistics (see Box 1.1). This means that all  $N$  release sites have the same release probability,  $P$ .

In this case, the mean EPSP amplitude,  $E = NPQ$  .....(1)

where  $Q$  is one quantum of postsynaptic response.

It can also be shown that  $SD = Q\sqrt{NP(1-P)}$

So  $SD^2 = NP(1-P)Q^2$  .....(2)

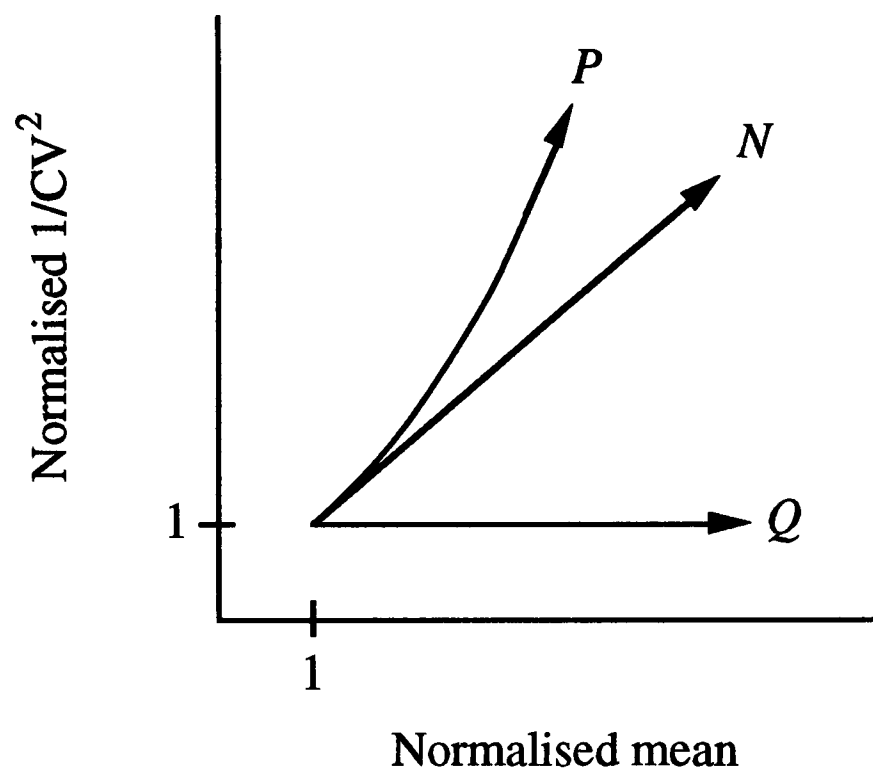
And, by definition:  $\frac{1}{CV^2} = \frac{E^2}{SD^2}$  .....(3)

So, combining equations (1), (2) and (3):  $\frac{1}{CV^2} = \frac{(NPQ)^2}{NP(1-P)Q^2} = \frac{NP}{(1-P)}$

Thus  $E$  increases linearly with any increase in  $N$ ,  $P$  or  $Q$ . In contrast,  $\frac{1}{CV^2}$  behaves differently depending on which parameter is changing: it is non-linearly related to  $P$ , linearly related to  $N$  and independent of  $Q$ . A graph of  $\frac{1}{CV^2}$  against mean therefore shows a steep upwardly curving trajectory if only  $P$  increases, a straight diagonal line if only  $N$  increases and a horizontal line if only  $Q$  increases. Changes in more than one parameter produce intermediate trajectories.

One way to represent CV information is to plot  $1/CV^2$  against the mean (these are usually both normalised to their starting values). The trajectory of the change in  $1/CV^2$  as the mean increases can give information about which quantal parameter or combination of parameters is changing (Figure 2.3). To do this, I usually compared the trajectory with the *diagonal*, an imaginary line for which normalised  $1/CV^2 =$

normalised mean. Trajectories that are much steeper than the diagonal and curve upwards indicate an increase in  $P$ , trajectories along the diagonal indicate an increase in  $N$  and trajectories well below the diagonal indicate an increase in  $Q$ . Intermediate trajectories can indicate changes in combinations of two or all of these three parameters. Note that if Poisson statistics apply (i.e.  $N \gg P$ ) then a change in quantal content ( $M$ , equal to  $NP$ ) will give a trajectory along the diagonal and a change in  $Q$  will produce a horizontal trajectory.



**Figure 2.3** *1/CV<sup>2</sup> graph trajectories predicted by simple binomial theory* If transmission is quantal and obeys simple binomial statistics then an increase in release probability,  $P$ , will produce a steep, upwardly curving trajectory. Increases in the number of release sites,  $N$ , will result in a straight diagonal line. An increase in the quantal size,  $Q$ , will give a horizontal trajectory.

$1/CV^2$  analysis does have shortcomings. Transmitter release is a complex biological process and is therefore very unlikely to conform perfectly to simple binomial statistics. The more it strays from the assumptions of this model, the less accurate the analysis becomes. In extreme cases it can be misleading. For example, if potentiation comes about by a change in  $Q$  alone but the amount by which  $Q$  rises is very different at each release site, the  $1/CV^2$  graph can show a steep diagonal trajectory. This would conventionally be interpreted as a change in  $P$  or  $N$ . Similarly, changes in  $P$  alone, if

they are very unequal, can lead to a shallow  $1/CV^2$  graph trajectory, which would normally be seen as indicating a change predominantly in  $Q$  (Korn *et al.*, 1991; Faber & Korn, 1991; Stratford, 1992).

However, the trajectories of  $1/CV^2$  graphs are reasonably robust to deviations from simple binomial statistics. They are only seriously misleading when the change in mean is small and differences in  $P$  or  $Q$  are extreme (Korn *et al.*, 1991; Faber & Korn, 1991). In a wide range of less extreme cases, the trajectory does not deviate much from the predictions of simple binomial theory (Malinow & Tsien, 1990; Stratford, 1992).

Another difficulty is that  $1/CV^2$  graphs with trajectories that are neither very steep nor very close to the horizontal are difficult to interpret. These are normally thought of as indicating an increase in more than one quantal parameter. But determining which parameters have increased and in what proportion is practically impossible, particularly when you consider possible deviations from simple binomial behaviour.

I will discuss these difficulties and their relevance to my experiments in Chapter Four. Nevertheless, it is clear that  $1/CV^2$  graphs are potentially misleading and do not necessarily give accurate quantitative information. For these reasons I used two other quantal analysis procedures in addition to  $1/CV^2$  analysis.

### *2.3.3 Amplitude frequency histograms*

The second method I used was to analyse amplitude frequency histograms. This involved a procedure developed by Larkman *et al.* (1991). A computer program (written by Ken Stratford, University Laboratory of Physiology, Oxford) is used to plot EPSP amplitude frequency histograms. The width of the histogram bins affects the location of peaks and can even obscure them all together so the data are usually divided into very fine bins ( $5\mu V$  wide) and the resulting histogram is smoothed by the

computer using a moving Gaussian filter. Individual histograms need different amounts of smoothing so the program allows this to be adjusted.

Sometimes a histogram composed of all of the sweeps recorded from a particular EPSP shows clear quantal peaks but this is rare. More often the histogram is almost smooth. There are a number of possible reasons:

1. The quantal size is too small relative to the background recording noise.
2. The quantal size is roughly the same at each release site but tends to change during the recording.
3. The quantal size is different at each release site.
4. Transmission is not quantal.

Background noise comes from a number of sources: thermal and electrical noise from the amplifier and other pieces of recording equipment; interference from electrical appliances such as fluorescent lights; vibration; ion channel and synaptic noise from within the slice itself. The noise that comes from the recording equipment is unavoidable but very small compared to the other sources. I took steps to minimise electrical noise by switching off fluorescent lights and unnecessary electrical equipment. I also carried out most experiments at night, when general activity in the laboratory was at a minimum. The recording chamber rested on an air table to reduce the effects of vibration and the slice was bathed in ACSF containing high concentrations (usually 4 mM) of  $Mg^{2+}$  to suppress background synaptic activity.

Despite these steps, histograms that contained all of the recorded sweeps rarely showed clear peaks. Interestingly, even quantal analysis studies by groups that used patch-clamp recording methods often failed to produce histograms with clear peaks (Malinow & Tsien, 1990; Bekkers & Stevens, 1990), even though this ought to provide lower levels of recording noise than the intracellular-electrode methods I

used. This suggests either that quanta are very small indeed or that excessive recording noise is not the only reason that they cannot be seen.

Larkman *et al.* (1991) addressed the second of the possibilities listed above: that the quantal size changes with time. This would cause the peaks to smear and merge into one another, eventually becoming completely indistinct. But if this change is slow enough, short stretches of data might give peaky histograms, particularly if the change is not continual but saltatory, with periods of stability interspersed with periods of relatively rapid change.

What reasons are there to expect such non-stationarity? The first is that hippocampal synapses readily show both increases and decreases in strength (which is the reason most neuroscientists study them in the first place). Our experiments necessarily involved quite rapid stimulation (0.1-5Hz) and such rates—particularly ones at the higher end of this range—can cause changes in synaptic strength (see Chapter Three), some of which may involve changes in quantal size.

Changes in the quality of the electrode impalement (for example, the leak current between the electrode and the cell membrane) could also cause apparent changes in quantal size. Changes in the field potential (the collective electrical response of the population of neurones in the vicinity of the electrode tip) can interfere with this type of analysis too. In this case, the quantal size would not change because the peaks would all move together, but they would nevertheless become smeared.

Larkman *et al.* (1991) elicited EPSPs in area CA1 of hippocampal slices at rates of 0.5-5Hz. Under these conditions, most EPSPs show a gradual depression (see Chapter Three). They searched the EPSP amplitude data and in many cases found a region or series of regions that produced histograms with clear, approximately equally spaced peaks. Quantal sizes were in the range 84-197 $\mu$ V and successive histograms usually showed slightly different quantal sizes, which suggests that changes in quantal size can obscure the underlying process when all the data are plotted together. The main

change, however, was in the mean number of quanta per trial (i.e. the quantal content), which suggests that the depression was largely presynaptic. This is good evidence for quantal transmission and non-stationarity in quantal size (the second of the options listed above).

I used the same method. I searched long runs of data for regions that gave histograms with clear, evenly spaced peaks. A computer program (written by Ken Stratford, University Laboratory of Physiology) allowed these to be plotted on a screen. I then located the peaks by eye and the computer calculated their mean separation. I used this value as a measure of the quantal size for that particular period of data. As a safeguard against peaks that arise by statistical sampling artefact, I routinely subdivided all peaky histograms into two equal halves and checked to see that both halves contained peaks that aligned with each other and with the summed histogram.

One criticism of this procedure is that data are selected subjectively on the basis of histogram peakiness. This approach not does necessarily identify histograms that give the most accurate representation of the underlying transmission process. For example, even data sets with smooth distributions can have regions within them that produce peaky histograms (Clements, 1991; Stratford, 1992). Any experimental histogram contains a finite sample of data so there is always a chance that peaks in it might not accurately reflect the true underlying distribution, which could even be completely smooth.

Stratford *et al.* (1993) have assessed the reliability of peaky histograms in my data and other similar data. In favourable cases, the chances of the peaks having arisen by chance from a smooth distribution are very small. More generally, histograms that appear by eye to have clear, evenly spaced peaks are usually unlikely to have arisen by chance. I will discuss this point in more detail in Chapter Three.

Even when histogram analysis is reliable, it allows only certain parts of the data to be investigated; it is blind to periods of the recording that, for whatever reason, do not

give peaky histograms. So, although histograms give quantitative information that the qualitative  $1/CV^2$  method cannot provide, this information is patchy and may exclude large sections of the data.

### 2.3.4 Constant N analysis

In order to make better use of all of the recorded data, we decided to try to make quantitative estimates of changes in the quantal parameters,  $N$ ,  $P$  and  $Q$ , using the information contained in the mean and SD of the EPSP and the way these changed with time.

#### **Box 2.2 Calculating N from a histogram**

Assume that transmission conforms to simple binomial statistics (see Chapter One). In this case  $E = NPQ$  .....(1)

and  $SD^2 = NP(1 - P)Q^2$  .....(2)

Rearranging equation (1)  $P = \frac{E}{NQ}$

Substituting this into equation (2),  $SD^2 = EQ - \frac{E^2}{N}$

$$\text{So } N = \frac{E^2}{EQ - SD^2}$$

This allows us to calculate  $N$  from the mean ( $E$ ), standard deviation ( $SD$ ) and peak separation ( $Q$ ) of a histogram.

For example, if a particular histogram gives values of  $E=250\mu\text{V}$ ,  $SD=114\mu\text{V}$  and  $Q=100\mu\text{V}$  then  $N = \frac{62500}{25000 - 12996} = 5.2$

So, to the nearest integer,  $N = 5$ .

To do this we assumed that transmission at these synapses conforms to simple binomial statistics (see Chapter One). The first step was to estimate  $N$  for the EPSP being analysing. We took one histogram (usually the one judged to be the most reliable) and measured the quantal size from it. This gives a value of  $Q$  for the period of data it spans. By combining this with the mean and SD for this period, we could calculate  $N$  (see Box 2.2). Because we relate  $N$  to the number of release sites (see Chapter One), we also assume that it is an integer and therefore round it off to the nearest whole number.

The second major assumption is that this value of  $N$  is constant for all data recorded from a particular EPSP, even after potentiation. This meant that we could use the value of  $N$  already calculated to estimate the values of  $P$  and  $Q$  for all regions of data on the basis of the mean and SD alone (see Box 2.3).

### **Box 2.3 Calculating $Q$ and $P$ from the mean and $SD$**

We know that  $SD^2 = EQ - \frac{E^2}{N}$  (see Box 2.1).

Rearranging this,  $Q = \frac{SD^2}{E} + \frac{E}{N}$

Continuing the example from Box 2.1, let us say that the mean of the EPSP has increased to  $600\mu\text{V}$  and the  $SD$  to  $134\mu\text{V}$ . If we assume that  $N$  has not changed then  $Q = \frac{134^2}{600} + \frac{600}{5} = 150\mu\text{V}$ .

We also know that  $P = \frac{E}{NQ}$  (see Box 2.1).

Thus  $P = \frac{600}{750} = 0.8$

Why did we assume that  $N$  is constant and what is the justification for doing so? The only information about transmission statistics that we have for regions of data that do not give peaky histograms, are their mean and SD. It is impossible to calculate the

values of all three binomial parameters ( $N$ ,  $P$  and  $Q$ ) from these two pieces of information. In order to do this calculation we are forced to make one of the three variables constant.  $N$  is usually thought to correspond to the number of presynaptic vesicle release sites (see Chapter One) and this seems to be the most likely of the three quantal parameters to be constant over periods of a few minutes. There are other reasons for thinking that the assumption of constant  $N$  is reasonable. One is that, for my data, the results of constant  $N$  analysis agree well with the results of histogram analysis. I will discuss the assumption of constant  $N$  in more detail in Chapter Four.

### *2.3.5 Combining the three analysis procedures*

I drew my conclusions about which quantal parameter or parameters had changed from the results of all three analysis procedures ( $1/CV^2$ , histograms and constant  $N$ ). Each one on its own is potentially misleading, but in different ways. In particular, the validity of histogram analysis rests only on the reliability of the histograms used. The other two methods depend (to different extents) on the quite different assumption that the transmission process approximates to simple binomial statistics. Therefore, when all three analysis methods agree, the reliability of the results are much greater than when only one of these methods is used on its own. I will return to this point in Chapter Four.

# *Chapter Three*

## General Properties

This chapter describes some of the features of pyramidal neurones in area CA1 of the hippocampus, concentrating in particular on transmission at the glutamatergic synapses made onto these cells by Schaffer collateral axons.

### 3.1 Pyramidal neurones

Pyramidal neurones are the main projection cells of the neocortex and hippocampus: their axons usually carry signals to relatively distant sites before making synapses onto other neurones. This is in contrast to non-pyramidal cells, which make most of their connections locally. The name 'pyramidal' comes from the roughly conical shape of their cell bodies. Several short basal dendrites, one longer apical dendrite and a thinner axon emerge from the cell body. The great majority of excitatory synapses seem to connect to the dendrites via postsynaptic dendritic spines (see Chapter One).

One of the principle excitatory inputs to pyramidal neurones in area CA1 of the hippocampus comes from Schaffer collateral axons of other pyramidal neurones in area CA3 (see Chapter One). Along the length of each CA3 axon are swellings or varicosities, at which *en passant* synapses are formed with the dendrites of CA1 cells (Andersen, 1966a,b). Each presynaptic axon can form multiple connections with a single postsynaptic cell, often, though not always, localised to a small part of the dendritic tree (Sorra & Harris, 1993). In most cases, a single presynaptic bouton is thought to contain one vesicle release site (see Chapter One).

## 3.2 Short-term changes in EPSP amplitudes

In order to characterise the EPSPs I was studying, I carried out a number of experiments to investigate their behaviour during constant stimulation and two forms of brief synaptic enhancement: paired-pulse facilitation and post-tetanic potentiation. As in most areas of synaptic physiology, study of these was pioneered at the neuromuscular junction (NMJ).

### 3.2.1 Short-term changes at the NMJ

Even over timescales of seconds and below, synapses are mercurial structures. Postsynaptic responses to successive stimuli can rise and fall not only because of the randomness of synaptic transmission but also as a result of changes in the strength of the synapse. These transient effects are shaped by the synapse's previous activity. Sometimes a stimulus can cause the response to later stimuli to be reduced (*depression*). In other cases the response to later stimuli is increased and the effect is called *facilitation*, *augmentation* or *potentiation*, depending on how long it lasts.

Facilitation is the briefest of the three effects. A single stimulus will facilitate the response to a second stimulus that arrives a few milliseconds later, but if the gap between the two is more than about a second<sup>1</sup>, there is no noticeable effect (Feng, 1941; Eccles *et al*, 1941; Mallart & Martin, 1967). This is known as paired-pulse facilitation (*PPF*).

PPF is caused by an increase in probability of vesicle release from the presynaptic terminal (del Castillo & Katz, 1954b; Zucker, 1973). This seems to happen when the second stimulus arrives before the neurone has been able to clear away all of the presynaptic calcium ( $\text{Ca}^{2+}$ ) that entered during the first stimulus (Katz & Miledi,

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<sup>1</sup>Exactly how long the effect lasts depends on the type of NMJ preparation and its temperature.

1965, 1968; Rahamimoff, 1968). When the second dose of  $\text{Ca}^{2+}$  flows into the cell, it adds to this residual amount, producing a higher final concentration than during the first stimulus. Even a small amount of residual  $\text{Ca}^{2+}$  can cause quite large facilitation because the amount of transmitter release goes up as roughly the fourth power of the presynaptic  $\text{Ca}^{2+}$  concentration (Dodge & Rahamimoff, 1967). As the interval between the stimuli increases, there is more time for the cell to remove  $\text{Ca}^{2+}$  from the presynaptic terminal so the effect reduces. This idea is known as the *residual calcium hypothesis*.

Potentiation is longer-lasting than facilitation and is seen after a tetanus, when it is known as post-tetanic potentiation (*PTP*). The decay of PTP with time has two exponential components, augmentation, which decays in about 30s (Magleby & Zengel, 1976a, b) and potentiation proper, which usually lasts for a few minutes (Feng, 1941; Liley & North, 1953). Like PPF, PTP seems to be due to an increase in presynaptic transmitter release (Liley, 1956) and some researchers have suggested that it may be caused by a build up of residual  $\text{Ca}^{2+}$  in the presynaptic terminal (Rosenthal, 1969; Magleby & Zengel, 1975). The reason that it lasts longer than PPF may be that the greater amount of residual  $\text{Ca}^{2+}$  that presumably accumulates during a tetanus causes a slower stage of the  $\text{Ca}^{2+}$  removal process to become saturated<sup>2</sup>.

Sometimes a single stimulus can cause the response to a second stimulus to be depressed rather than enhanced. This effect typically happens at stimulus separations of a second or two (Eccles *et al.*, 1941) and seems to be caused by a presynaptic reduction in transmitter release, possibly as a result of a temporary depletion in the number of vesicles available for release (Thies, 1965). Prolonged stimulation results in greater, longer-lasting depression (Feng, 1941). This effect is also caused by a

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<sup>2</sup>Although  $\text{Ca}^{2+}$  is not the whole story (Miledi & Thies, 1971). Sodium ( $\text{Na}^+$ ) ions and second messengers also seem to play roles in PTP (Birks & Cohen, 1968; Erulkar & Rahamimoff, 1978; Mislner & Hurlbut, 1983).

reduction in presynaptic transmitter release (del Castillo & Katz, 1954b) and could be due to a chronic run-down of the presynaptic vesicle store.

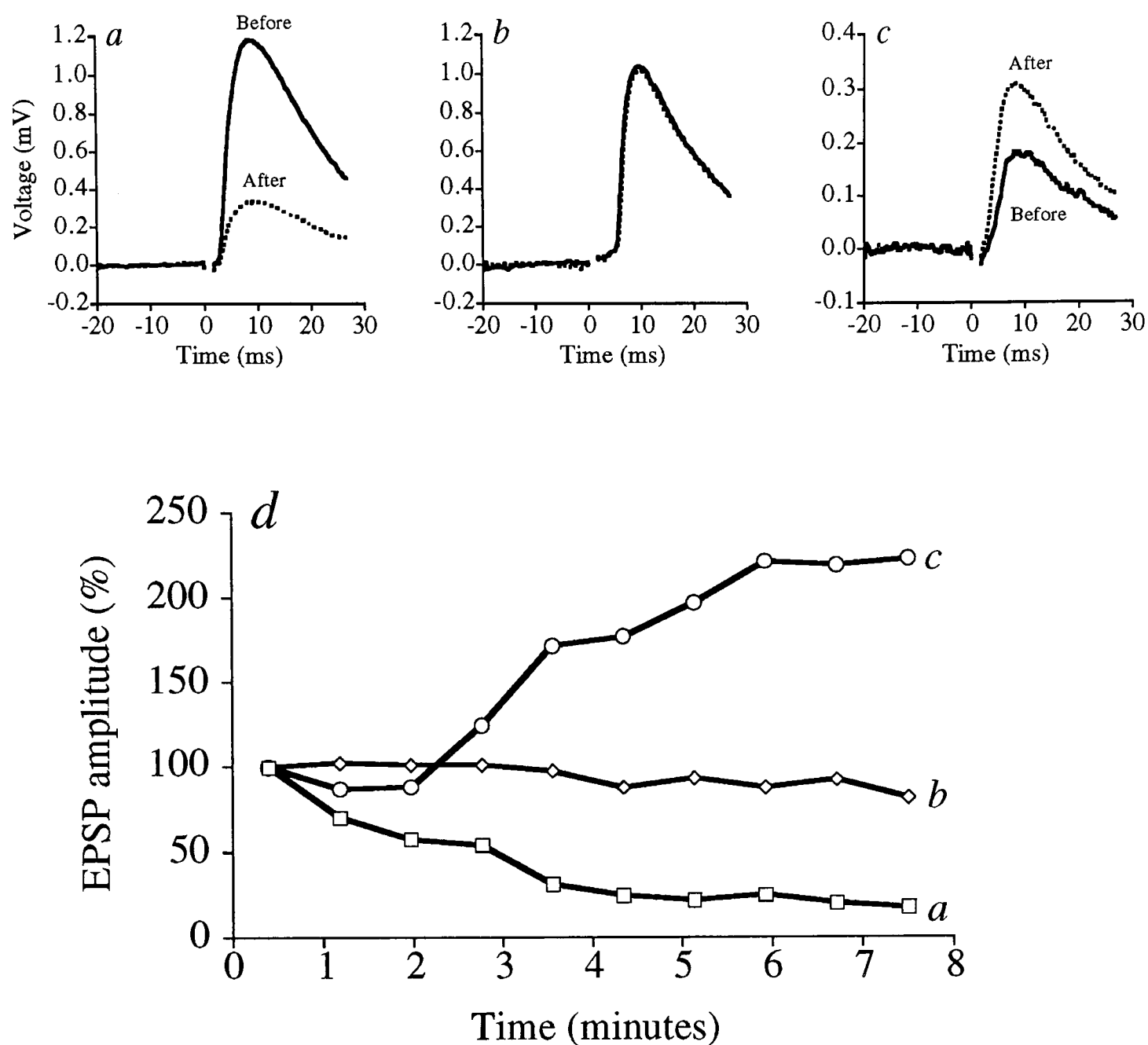
At any particular synapse, all these effects are at play and the overall balance of the competing forces determines the synapse's strength at a given instant (Zucker, 1989).

### *3.2.2 Hippocampal EPSPs get smaller during constant stimulation*

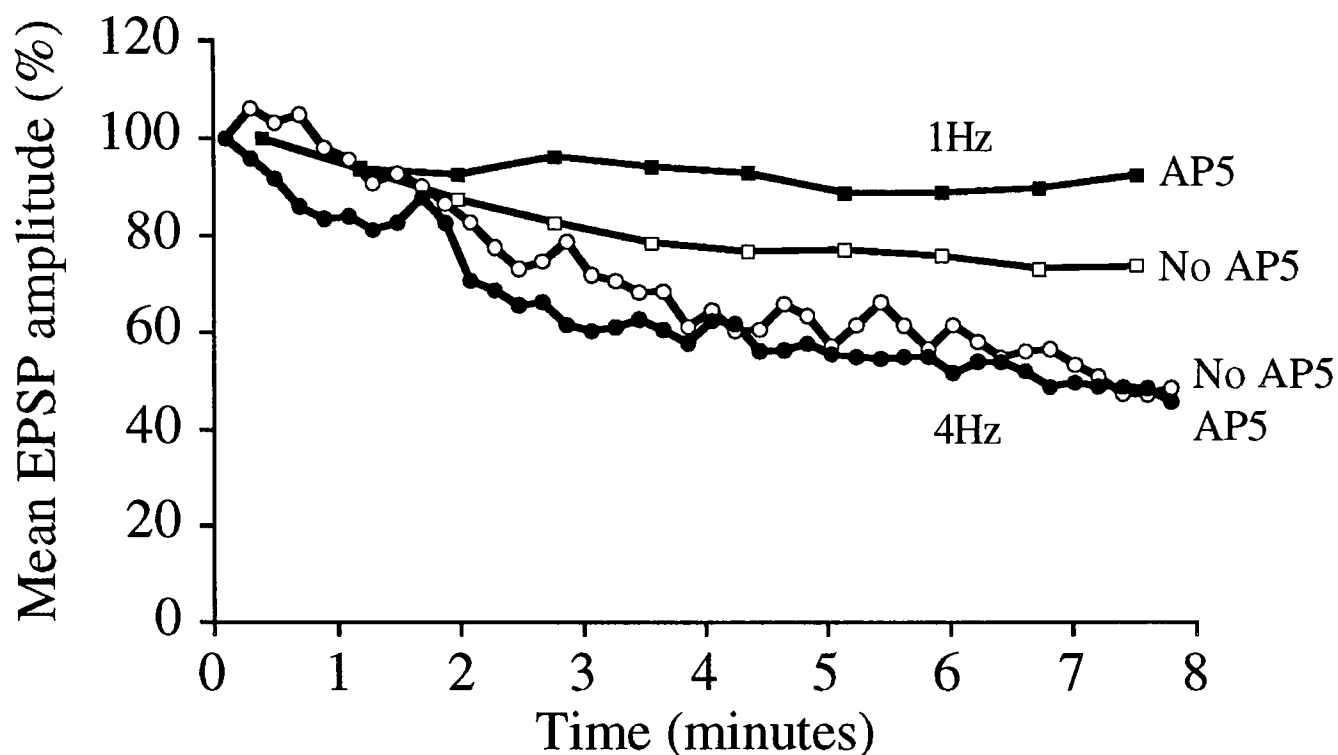
When I continuously stimulated synapses in the CA1 region of hippocampal slices at rates of 1-4Hz, the EPSPs usually showed a gradual decrease in amplitude over a period of minutes. This is superficially similar to the depression seen after continual stimulation of an NMJ preparation. Individual EPSPs show very different behaviour. Most decrease in size (Figure 3.1a), some stay roughly constant (Figure 3.1b) and a few even increase in size (Figure 3.1c). The time courses of three EPSPs that each showed a very different response to constant stimulation are shown in Figure 3.1d.

On average, EPSPs stimulated at constant rates of 1-4Hz become depressed and higher rates of stimulation lead to more rapid depression (Figure 3.2). After about 8 minutes, EPSPs stimulated at 4Hz depressed, on average, about twice as much as those stimulated at 1Hz.

Figure 3.2 also shows the effect of the NMDA receptor antagonist, 2-amino-5-phosphonovalerate (AP5), on the rate of depression. At 1Hz it appears to slow the rate of depression. However, because individual EPSPs behave so differently, the SDs of many of the points on the graph are large, particularly at later times. After nearly 8 minutes of stimulation at 1Hz, for example, EPSPs decline to  $93\pm 31\%$  in the presence of AP5 and to  $74\pm 22\%$  in its absence. This difference is only just statistically significant (Student's t test gives  $p=0.032$ ) but it is in broad agreement with the finding that 1Hz stimulation of these synapses can result in AP5-sensitive long-term depression (*LTD*) measured at a test stimulation rate of 0.1Hz (Dudek & Bear, 1992; Mulkey & Malenka, 1992).



**Figure 3.1** *Individual EPSPs behave differently during constant stimulation* (a) Waveforms of an EPSP stimulated at 4Hz for 2,000 trials. The solid line shows the mean waveform of the first 200 trials and the dotted line shows the last 200 trials. In this and most other EPSPs, the stimulus artefact has been removed. Like most EPSPs, this one reduced in amplitude as it was stimulated. (b) Another EPSP stimulated at the same rate for the same length of time. This example shows almost no change in amplitude. (c) A third EPSP stimulated in the same way. In this case the mean amplitude of the EPSP increased, which is rare (in these experiments, only 1 EPSP out of 47). (d) The time courses of the EPSPs shown in a, b and c. They illustrate the wide variety of behaviour seen during constant stimulation. Each point represents the mean amplitude of 200 consecutive trials normalised by the mean amplitude of the first 200 trials and expressed as a percentage.



**Figure 3.2** *The average rate of EPSP decline depends on the stimulation rate* The mean time courses of EPSP amplitudes stimulated at 1 or 4Hz in the presence or absence of the NMDA receptor antagonist, AP5. Normalised time courses of individual EPSPs were calculated in a similar way to those in Figure 3.2d but with each point representing 50 (rather than 200) consecutive trials and normalised to the mean amplitude during the first 50 (rather than 200) trials. Each line on the graph shows the mean normalised time course of between 13 and 28 EPSPs. On average, EPSPs that were stimulated at 4Hz showed a slight initial enhancement, before depressing to  $49 \pm 29\%$  of their initial amplitude after 2,000 trials (open circles; 28 EPSPs; mean initial amplitude  $616 \pm 247 \mu\text{V}$ ). EPSPs stimulated in the presence of the NMDA receptor antagonist, AP5, (closed circles; 19 EPSPs;  $679 \pm 305 \mu\text{V}$ ) showed no initial enhancement but depressed steadily to  $46 \pm 40\%$  of their initial amplitude. The rate of depression at 1Hz is substantially less (open squares; 27 EPSPs;  $878 \pm 312 \mu\text{V}$ ), EPSPs falling to  $74 \pm 22\%$  of their initial amplitude after 500 trials. At 1Hz, AP5 seems to reduce this decline (closed squares; 13 EPSPs;  $868 \pm 353 \mu\text{V}$ ), EPSPs falling only as far as  $93 \pm 31\%$  of their initial amplitudes.

At 4Hz, the average EPSP amplitude in the absence of AP5 climbed slightly during the first minute or so, whereas it fell during the same period in the presence of AP5. After 200 trials (50 seconds) the average amplitude in AP5 is  $86\pm 14\%$  of the starting amplitude and in the control EPSPs it was  $105\pm 30\%$ . This difference is significant (Student's t test gives  $p=0.014$ ). It seems then, that AP5 blocks a brief enhancement that counteracts the effects of depression during the early period of 4Hz stimulation. Thereafter, the EPSPs exposed to AP5 decline less rapidly than the controls. This seems to be a similar effect to that seen at 1Hz, i.e. an inhibition of depression. After 2000 trials (about 8 minutes) the two groups of EPSPs had, on average, depressed by almost identical amounts. In the presence of AP5 the amplitude was  $46\pm 40\%$  of the starting amplitude. In the control EPSPs it was  $49\pm 29\%$ . It would have been interesting to see if the average amplitude of the control EPSPs falls significantly below the amplitude of the AP5 group during even longer stimulation.

This depression could, in principle, be either presynaptic or postsynaptic. Prolonged stimulation might, for example, cause depletion of transmitter-containing vesicles in the presynaptic cell, leading to a reduction in the release probability, as appears to be the case at the NMJ. Another possibility is that presynaptic metabotropic glutamate receptors might be involved (see Chapter One). Alternatively, the depression could be a result of desensitisation of postsynaptic AMPA receptors. The ambient concentration of extracellular glutamate in the brain is estimated to be 2-4 $\mu$ M but continued stimulation of slices could easily raise this two- or three-fold, which might cause AMPA receptors to desensitise (Mayer and Vyclicky, 1989; Tang *et al.*, 1989; Trussel and Fischbach, 1989) and the postsynaptic response to be reduced.

Larkman *et al.* (1991) showed that most of the depression seen during constant stimulation of hippocampal neurones is also due to a presynaptic change. More recent work by them has shown that there can also be a substantial change in quantal size, particularly during the early stages of stimulation (Larkman, Stratford & Jack, in preparation).

### 3.2.3 Paired-pulse facilitation

At some hippocampal synapses, when one stimulus is closely followed by another, the response to the second is, on average, larger than the first (Andersen, 1960a; Lømo, 1971). This effect is similar in duration and amplitude to PPF seen in NMJ preparations. The optimum stimulus interval for recording PPF from the CA1 region of hippocampal slices is usually about 50ms and quantal analysis shows that PPF here is presynaptic (Hess *et al.*, 1987; Foster & McNaughton, 1991; Kullmann & Nicoll, 1992; Liao *et al.*, 1992).

The residual calcium hypothesis, which seems to explain PPF at the NMJ (see above), is usually thought to explain the phenomenon at these hippocampal synapses too (Zucker, 1989). However, the CNS has the additional complication of inhibitory synapses, which can become rapidly depressed when they are stimulated repeatedly (McCarren & Alger, 1985)<sup>3</sup>. Nathan *et al.* (1990; Nathan & Lambert, 1991) have studied PPF in extracellular and large intracellular EPSPs recorded from CA1. These EPSPs are made up of inhibitory as well as excitatory components and they concluded that PPF here can be explained by a brief depression of the inhibitory GABAergic component rather than an enhancement of the excitatory portion.

Many researchers studying PPF do not block GABAergic synapses in their preparations (Hess *et al.*, 1987; Muller & Lynch, 1988, 1989; Voronin & Kuhnt, 1990; Foster & McNaughton, 1991) so these effects might have contributed to the facilitation they saw. But other groups see significant PPF even in the presence of picrotoxin, which abolishes fast GABAergic transmission (Sastry *et al.*, 1986; Kullmann & Nicoll, 1992; Lio *et al.* , 1992) so there is at least a component of PPF

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<sup>3</sup>This effect is mediated by presynaptic GABA<sub>B</sub> receptors that inhibit further GABA release from the presynaptic terminal (Deisz & Princek, 1989).

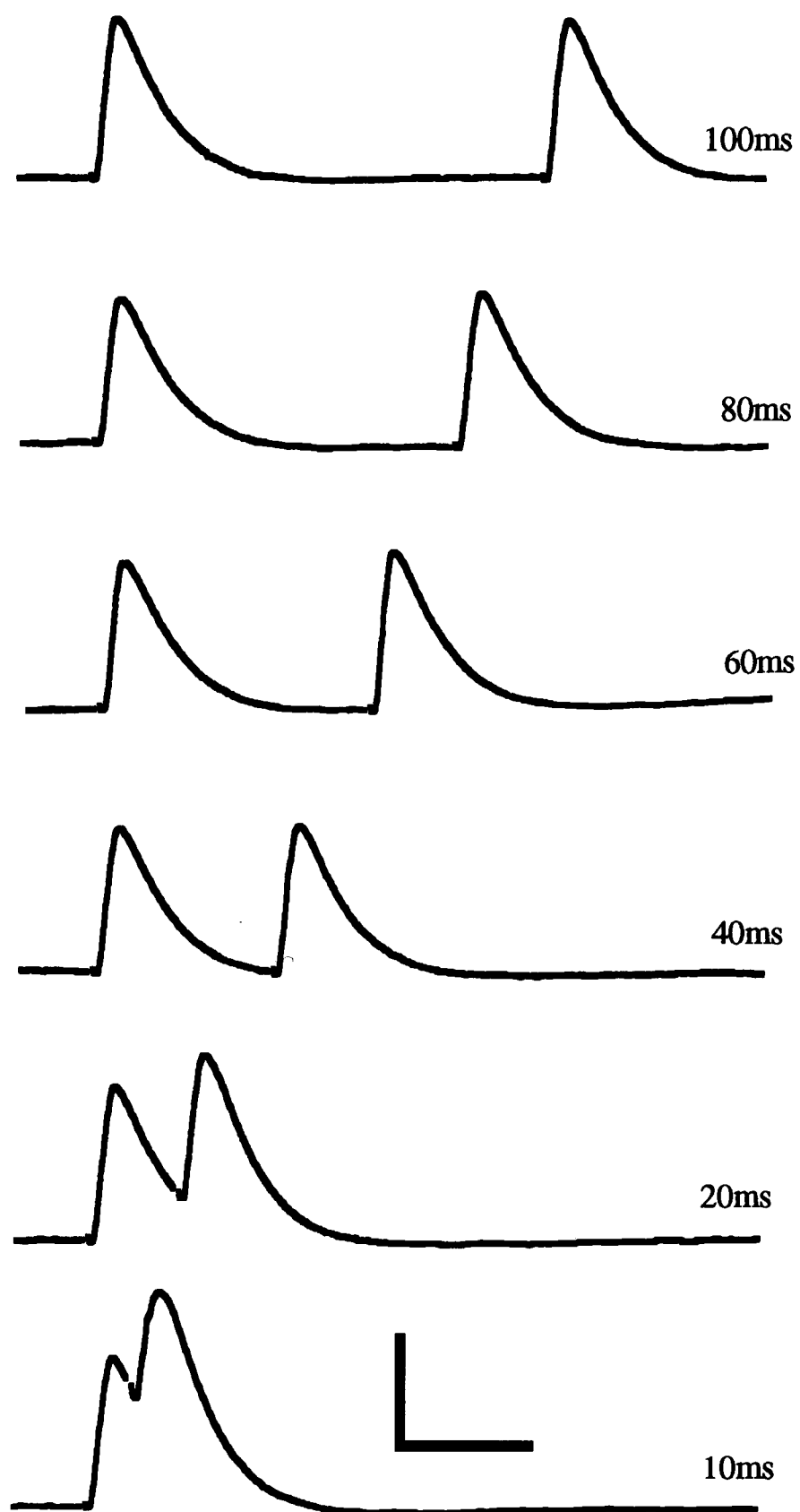
that does not depend on the inhibitory circuitry of the hippocampus and the residual calcium hypothesis appears to explain this effect (Hess & Kuhnt, 1992).

In a preliminary study, I evoked 10 different minimal (i.e. putatively single-axon) and compound (i.e. multi-axon) EPSPs at paired-pulse intervals of 10-100ms. These EPSPs were all recorded in  $\text{Ca}^{2+}$  and magnesium ( $\text{Mg}^{2+}$ ) concentrations of 2.5mM. None of these showed any noticeable paired-pulse facilitation. Figure 3.3 shows a typical example. It is a compound EPSP with an amplitude of about 3mV and shows no significant enhancement or depression at various stimulus intervals between 10 and 100ms.

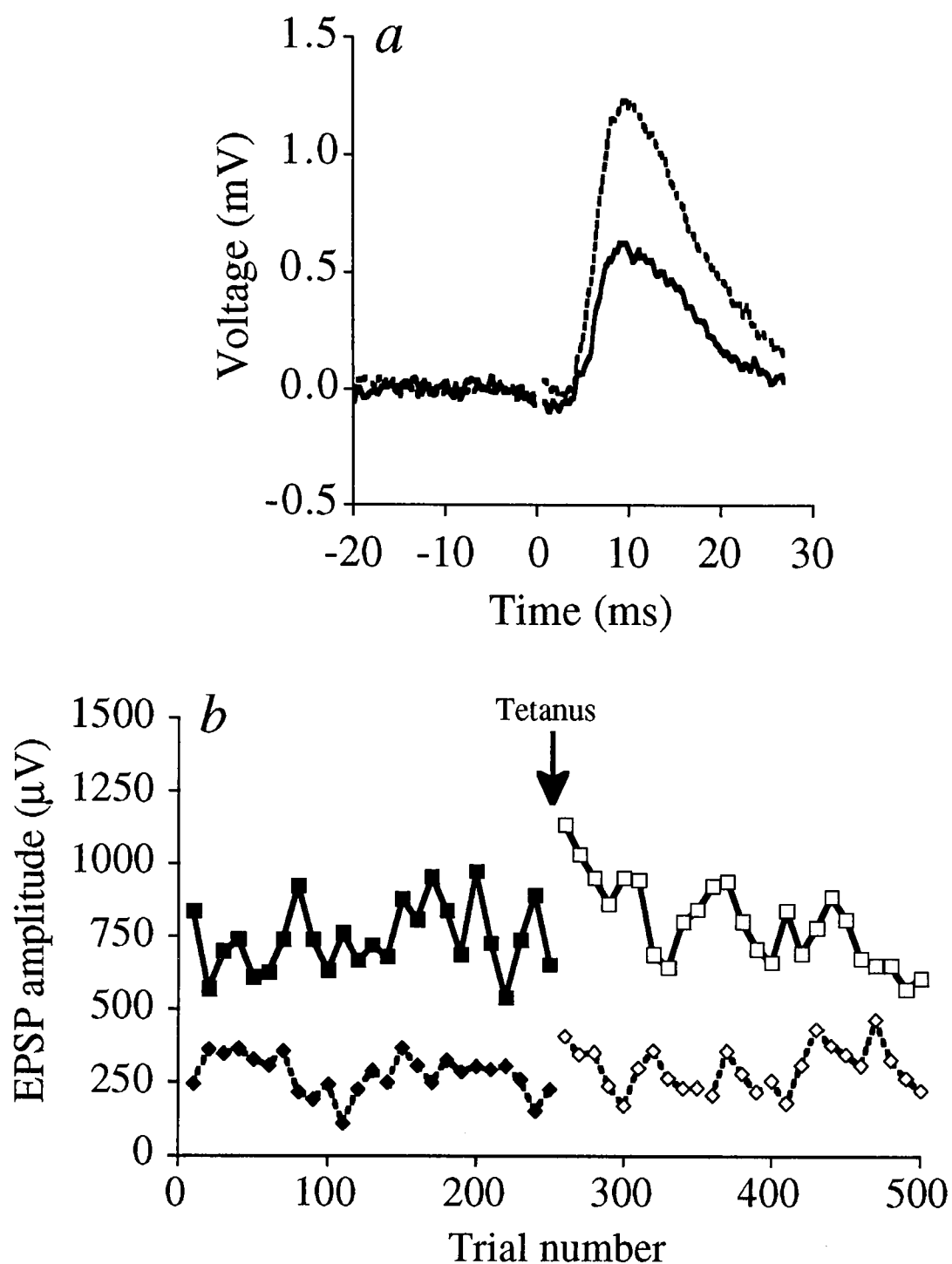
#### *3.2.4 Post-tetanic potentiation*

When a tetanus too weak to result in LTP is applied to hippocampal synapses, they can show PTP, which, in this brain area, usually lasts for less than a minute (Andersen *et al.*, 1966b; Deadwyler *et al.*, 1975). Like PTP at the NMJ, this seems to be due to an increase in transmitter release (McNaughton, 1982; Anwyl *et al.*, 1989). PTP in the hippocampus is particularly interesting to researchers studying LTP because both PTP and LTP can be elicited by a brief tetanus. This means that the two effects might be expected to share common mechanisms. Although this now seems unlikely, PTP is often thought to accompany the early stages of LTP and might therefore interfere with its analysis.

Some researchers (McNaughton, 1982; Racine & Milgram, 1983) have reported that hippocampal PTP has two components, one that decays in a matter of seconds and another that lasts for several minutes. By analogy with the results obtained from NMJ preparations, these were naturally assumed to correspond to augmentation and potentiation. However, it now seems likely that the longer-lasting component was short-term potentiation (STP), a very different form of synaptic enhancement that I will describe in detail in Chapter Five. I gave 100 or 200Hz tetani for 1 or 2s to 10 separate EPSPs but none of them showed large PTP. These EPSPs were all recorded



**Figure 3.3** *EPSPs did not show paired-pulse facilitation* A compound EPSP of about 3mV in amplitude that did not show any noticeable paired-pulse facilitation at stimulus separations of 100, 80, 60, 40, 20 and 10ms. Paired pulses were given at 1Hz. Scale bar: 2mV, 30ms.



**Figure 3.4** *EPSPs showed little post-tetanic potentiation* (a) Average EPSP waveform of 10 trials immediately before and 10 trials immediately (<1s) after a tetanus of 100Hz for 1s. Test stimulation was at 1Hz. This EPSP, and nine others that I examined showed only a small amount of potentiation. (b) The time course of the change in amplitude of this EPSP. Each point represents the average of 10 trials. Filled squares represent pre-tetanus points and open squares represent post-tetanus points. The enhancement was just under two-fold initially but this was not far outside the natural baseline variation in EPSP amplitude and it declined back to baseline in 30-60s.

in 100 $\mu$ M AP5 to prevent LTP and in Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations of 2.5mM. Figure 3.4 shows data from one of these. The delay between the end of the tetanus and the first post-tetanus test stimulus was less than a second so it is unlikely that any PTP that might have been present would have subsided much during this time.

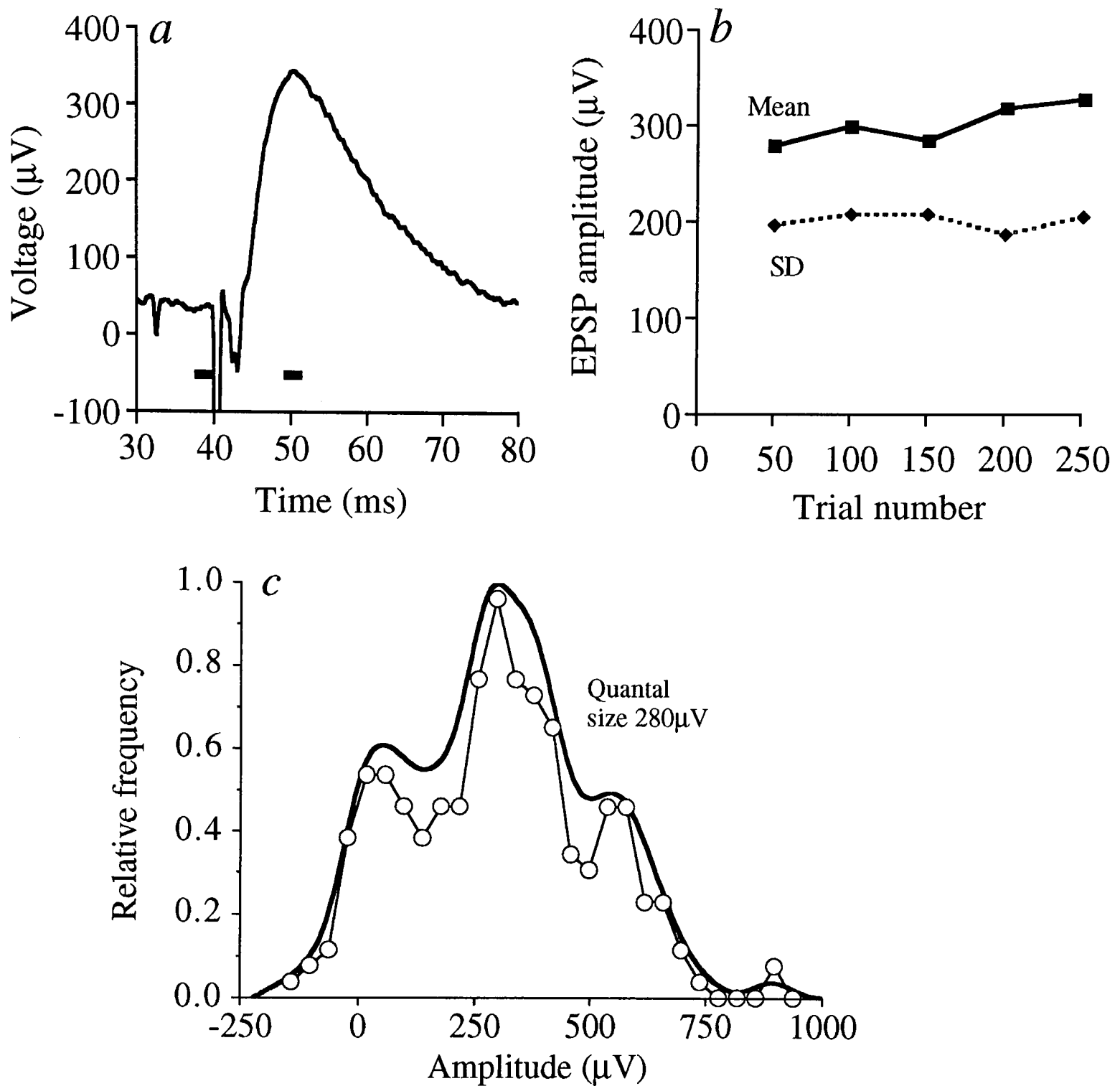
The absence of PPF and virtual absence of PTP in the EPSPs I studied is at odds with most other reports in the literature. PPF, in particular, is routinely used for assessing the reliability of quantal analysis procedures because it is thought to be entirely presynaptic (e.g. Kullmann & Nicoll, 1992; Liao *et al.*, 1992; Manabe *et al.*, 1993). I will discuss possible reasons for this finding in Chapter Four.

### 3.3 Synaptic transmission

The results shown in this section (Figures 3.5, 3.6 and 3.7) are not my own. They are the work of Alan Larkman, Ken Stratford and Julian Jack of the University Laboratory of Physiology, Oxford. I have acknowledged the person responsible for individual parts of these results in the text and figure legends. I show them because they involve data similar to my own and because my experiments were an extension of these ones and are largely dependent on their conclusions. These data have appeared in a paper (Larkman *et al.*, 1992).

#### 3.3.1 *Transmission is quantal*

Figure 3.5 shows data from an EPSP (Figure 3.5a) recorded from area CA1 of a hippocampal slice by Alan Larkman. The cell was stimulated 250 times at 0.1Hz and, when the responses were binned into epochs of 50 consecutive trials (Figure 3.5b), the mean and SD showed no substantial change over time. A histogram composed of all 250 trials (Figure 3.5c) shows three clear peaks with a mean spacing of 280 $\mu$ V. This EPSP therefore appears to show quantal transmission.

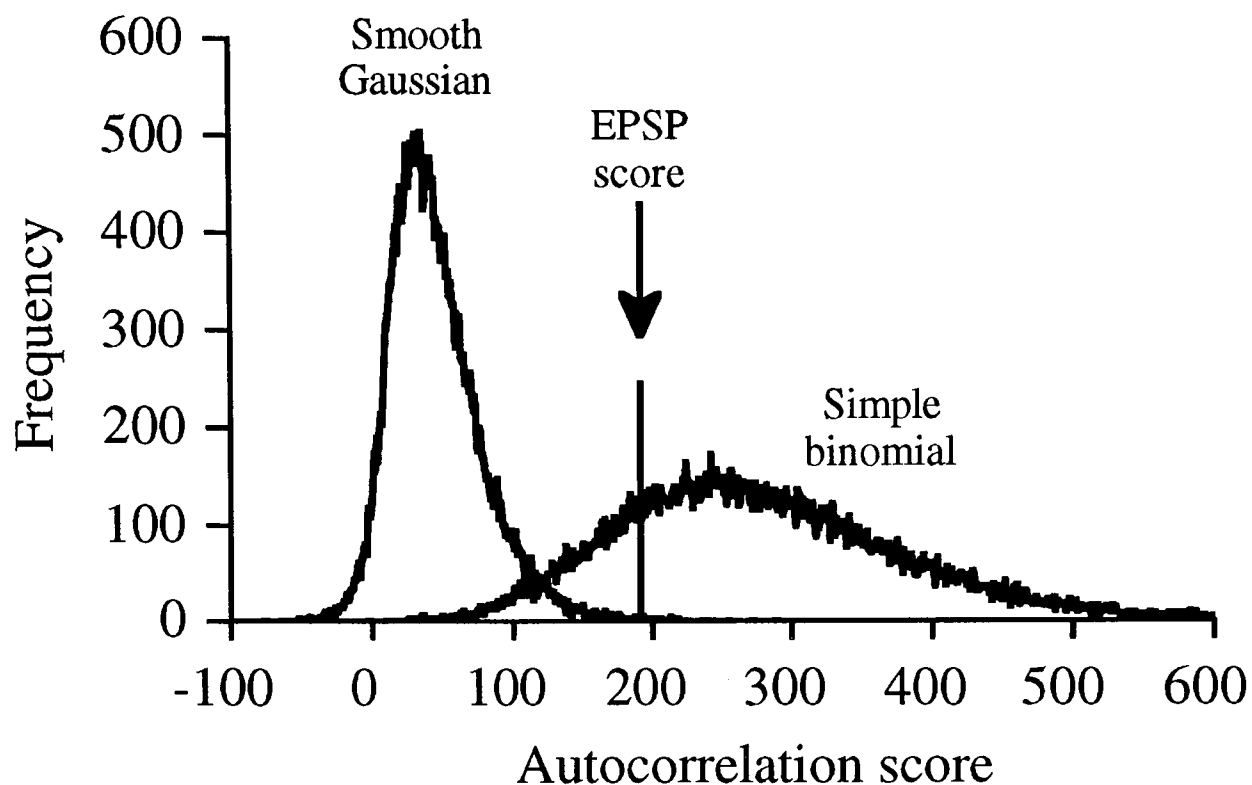


**Figure 3.5** *Quantal analysis of an EPSP* (a) The averaged waveform of all 250 trials evoked at a constant 0.1Hz. The 10-90% rise time is 3.6ms. The amplitude of the response to each stimulation was measured by a computer program that calculated the mean voltage during a 2ms period straddling the peak and subtracted the mean voltage during another 2ms period at the base of the EPSP (bars). (b) The mean (filled squares) and SD (filled diamonds) of this EPSP binned into epochs of 50 consecutive trials. The mean increased slightly during the recording period. (c) A histogram composed of all 250 trials and divided into bins of 40µV (open circles) shown beneath the same histogram binned at 5µV and smoothed with a moving Gaussian filter (see Chapter Two). The relative scale of the unsmoothed histogram has been reduced slightly for clarity. The mean peak spacing is about 280µV. The relative heights of the peaks are well described by a simple binomial distribution with  $N=3$  and  $P=0.34$ . Data courtesy of Alan Larkman, University Laboratory of Physiology, Oxford.

However, the mere existence of a peaky histogram is not necessarily good evidence for quantal behaviour. As I described briefly in Chapter Two, any histogram that contains a finite number of trials (i.e. all empirical histograms) are potentially misleading. The effects of sampling error mean that the shape of a histogram might not be an accurate reflection of the underlying distribution. Even data sets with smooth distributions, for example, can have regions within them that produce peaky histograms (Clements, 1991; Stratford, 1992). This argument has been used to suggest that the behaviour of these synapses might not be quantal at all (Clements, 1991). It is therefore important to assess the likelihood that histogram peaks could have arisen by chance from an underlying non-quantal distribution.

One objective measure of histogram peakiness is *autocorrelation* (Magleby & Miller, 1981; Stratford, 1992, Stratford *et al.*, 1993). This is usually used to study variables that change with time. It gives a measure of how well the variable correlates with previous values of itself and is therefore useful in detecting periodicity. It can be used in a similar way to measure how distinct and equally spaced the peaks in an amplitude histogram are. Ken Stratford used this method together with computer-based Monte Carlo simulations to assess the likelihood that the peaks in the histogram in Figure 3.5 arose by chance from a smooth Gaussian distribution. Figure 3.6 shows the results. The experimental histogram had an AC score of 190 (arbitrary units). This was typical of histograms taken from a simulated simple binomial distribution but very rare (less than 1 in 1,000) among histograms taken from a smooth Gaussian distribution.

This does not mean that transmission at this synapse or group of synapses precisely matches the parameters of the particular simple binomial simulation used. In fact, histograms sampled from the simple binomial generator had, on average, better AC scores than the experimental histogram (which itself is a good example of its kind) so the model is obviously inaccurate in some ways. One likely source of error is that the simulation did not include some of the complicating factors that might blur the peaks



**Figure 3.6** *The peaks in Figure 3.5c are very unlikely to be statistical artefacts* ‘Monte Carlo’-type computer simulations were used to determine the distribution of AC scores of histograms randomly drawn from either smooth Gaussian or simple binomial data. First, the AC score of the experimental histogram was calculated and found to be 190 (arbitrary units). Next, 50,000 histograms, each composed of 250 trials, were randomly sampled from a smooth Gaussian distribution whose mean and SD matched those of the experimental histogram. The AC score for each of these was calculated. They averaged  $38 \pm 28$  and less than 1 in 1,000 matched or exceeded the AC score of the experimental histogram. Finally, 50,000 histograms were sampled from data generated by a computer simulation of simple-binomial transmission similar to that suggested by the experimental histogram. The AC scores of these histograms averaged  $270 \pm 97$ . The AC score of the experimental histogram is therefore more typical of this quantal distribution than it is of the smooth Gaussian data. Data courtesy of Ken Stratford, University Laboratory of Physiology, Oxford.

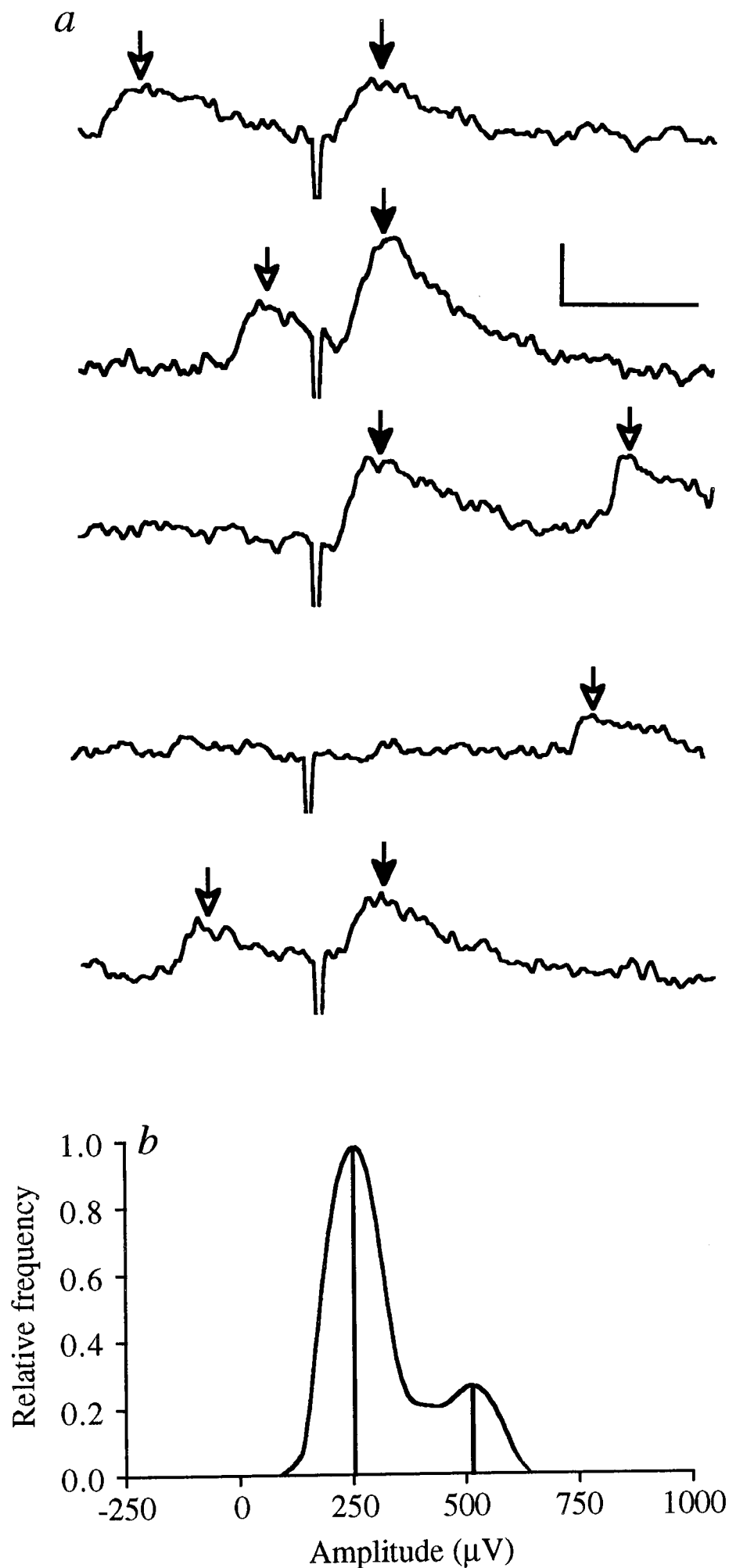
of experimental histograms, such as small changes in quantal size with time or slight variability in quantal size from one release site to another. What this procedure does demonstrate is that, in all likelihood, the experimental histogram came from an underlying distribution that is more like peaky simple binomial data than smooth Gaussian data. It shows, in other words, that transmission is almost certainly quantal.

Larkman, Stratford and Jack have analysed a large number of amplitude histograms, including some of mine. Their overall conclusion is that histograms with clear, roughly equally spaced peaks are unlikely to have arisen by sampling artefact from smooth distributions and the peak spacing in such histograms can provide a reliable estimate of quantal size.

### *3.3.2 Spontaneous EPSP amplitudes agree well with the quantal size*

Another way to verify an estimate of quantal size from a histogram of evoked responses is to compare it with the amplitude of spontaneous 'miniature' postsynaptic responses. This is routine at the neuromuscular junction (NMJ) but is extremely difficult in the CNS, partly because the lower signal-to-noise ratio makes spontaneous EPSPs difficult to detect reliably. For the EPSP illustrated in Figure 3.5, the ratio of the quantal size to the noise SD was about 4, which is unusually high (values of 2 or 3 are more typical in our experiments). This favourable signal-to-noise ratio allowed small spontaneous events to be seen in the brief sweeps of data collected on either side of the evoked response.

In all, 34 spontaneous EPSPs were found in the 250 sweeps. Figure 3.7*a* shows some examples of these. Their amplitudes were measured and a histogram plotted (Figure 3.7*b*). This shows a peak at about 250 $\mu$ V and another at 500 $\mu$ V, which agrees reasonably well with the quantal size of 280 $\mu$ V measured from the histogram of evoked responses in Figure 3.5*c*. The difference is small enough to be accounted for by statistical sampling error. This close agreement suggests that the spontaneous



**Figure 3.7 Analysis of spontaneous EPSPs** (a) Spontaneous EPSPs (open arrows) could often be seen in the 100ms-long sweeps of data collected on either side of the stimulus artefact and evoked EPSP (filled arrows). Scale bars: 500 $\mu$ V and 20ms. The sweeps are not consecutive and have been selected to show the clearest examples of spontaneous EPSPs. (b) A histogram composed of all 34 spontaneous EPSPs that were found. There are two peaks, one at 250 $\mu$ V and the other at 500 $\mu$ V, suggesting a quantal size of about 250 $\mu$ V. This agrees reasonably well with the quantal size estimated from the histogram of evoked responses shown in Figure 3.5c. Data courtesy of Alan Larkman, University Laboratory of Physiology, Oxford.

EPSPs are indeed 'miniature' events (i.e. most of them are composed of only one quantum) and also helps to confirm the quantal size estimated from the histogram of evoked EPSPs.

It may seem surprising at first sight that the size of spontaneous miniature EPSPs should agree so well with the quantal size of an evoked response. After all, the evoked response comes from (putatively) a single axon and a very small number (<20) of associated release sites. On the other hand, spontaneous events can, in principle, arise from any one of a vast number of synapses (about 20,000) scattered all over the neurone, each of which might have a different quantal size.

Taken on their own, the results shown in Figure 3.7 suggest a number of possible situations:

1. Spontaneous events come from sites all over the cell but these all have similar quantal sizes.
2. Spontaneous events from the synapse or synapses giving rise to the evoked EPSP are much larger than at any other sites on the cell, which are all below the threshold for detection (in this case, about  $100\mu\text{V}$ ).
3. The rate of spontaneous events is much higher at the synapse or synapses giving rise to the evoked EPSP than at any other sites on this neurone.

Option 1 seems unlikely for several reasons. One is that these synapses are plastic and show independent changes in quantal size (see Chapters Four and Five) so the chances of all synapses on a given cell having the same quantal size would seem to be virtually zero. For similar reasons, a situation in which any single synapse or group of synapses has a quantal size much larger than the rest (option 2) appears to be extremely unlikely to arise. A continuous range of different quantal sizes all over the

cell would be more in keeping with the idea that information is stored in the different weights of these connections.

This view agrees with patch clamp recordings of spontaneous miniature EPSCs from pyramidal cells in areas CA3 (McBain & Dingledine, 1992; Jonas *et al.*, 1993) and CA1 (Bekkers *et al.*, 1990; Manabe *et al.*, 1992; Malgaroli & Tsien, 1992; Raastad *et al.*, 1992; Otmakhov *et al.*, 1993). These show that spontaneous miniature EPSCs, which are thought to be single quanta, vary greatly in amplitude. In a single cell, the range typically covers all amplitudes from the lower detection limit (about 2pA) up to about 20pA, with a few responses even larger than this. The distribution is skewed and the most common amplitude is usually about 5pA. Of course, some of this variation is likely to be caused by dendritic attenuation of synaptic currents (see Chapter Two), which will be greater for synapses further away from the cell body. However, Bekkers *et al.* (Bekkers & Stevens, 1989; Bekkers *et al.*, 1990) showed that this is unlikely to account for all of the variation. Using hippocampal neurones in culture and hippocampal slices, they encouraged preferential discharge of spontaneous events from a closely spaced group of synapses by applying a sucrose solution. The resulting spontaneous EPSCs showed a similarly wide range of amplitudes. This means that synapses on the same neurone seem to show a continuous range of different quantal sizes, which rules out possibilities 1 and 2, above.

A more plausible explanation, then, is that most of the spontaneous events come from the same synapses that produce the evoked EPSP (option 3). It seems likely that the stimulation itself might encourage these synapses to show more spontaneous activity than others on the same neurone. This idea is supported by the fact that we often notice a rise in the rate of spontaneous EPSPs as soon as stimulation begins and this declines after stimulation has stopped. Similar observations have been made during whole-cell recording from CA1 pyramidal cells (Manabe *et al.*, 1992) and at the NMJ

(Rahamimoff & Yaari, 1973). The effect might be enhanced by our use of high  $Mg^{2+}$  concentrations to suppress general spontaneous activity.

### 3.3.3 *Quantal variance is low*

How can this be reconciled with the existence of histograms with clear, evenly spaced peaks, such as the one in Figure 3.5? If such high quantal variances exists at connections made by a single axon then peaks in histograms ought not to arise: the high variance of each peak would make them blur into one another. In most of the experiments described above, however, the spontaneous EPSCs are likely to have come from many hundreds or thousands of individual synapses, which would be expected to have a range of different quantal sizes. This is true even when sucrose is applied to a smaller group of closely grouped synapses (Bekkers & Stevens, 1989; Bekkers *et al.*, 1990).

The existence of peaky histograms such as the one shown in Figure 3.5, however, strongly suggests that the quantal size at synapses activated by the same incoming axon are roughly equal. We often see clear peaks at responses corresponding to the release of up to 5-10 quanta, which means that quantal variance at individual synapses must be low. As I mentioned in Chapter One, similar results were reported after experiments in the spinal cord, where the quantal variance is less than 5%. This compares to about 30% at the NMJ, which means that peaks corresponding to more than about 4 or 5 quanta cannot easily be picked out by eye (e.g. Boyd & Martin, 1956). This might be because of the different ways in which the quantal size appears to be set at peripheral and central synapses. At the NMJ it depends mainly on the number of transmitter molecules in each vesicle, but in the CNS the number of postsynaptic receptors seems to be the limiting factor (see Chapter One).

There are good grounds for thinking that synapses made by a single axon might have similar transmission properties. One factor that appears to strongly influence the release probability and quantal size of a synapse seems to be its recent history of

activity (see section 3.2). Release sites activated by the same axon will have been subjected to the same amount of presynaptic activity and are also likely to have experienced similar amounts of postsynaptic activity, particularly if they are closely spaced on the dendritic tree. As a result, they might be expected to have similar release probabilities and quantal sizes. This idea is consistent with an anatomical study showing that groups of synapses connecting the same two cells are morphologically more similar than groups of synapses between different pairs of cells (Sorra & Harris, 1993).

Several recent patch-clamp studies support the notion that transmission at single-axon inputs is quantal and that quantal variance is low. Edwards *et al.* (1990) made recordings of evoked responses at inhibitory, GABAergic synapses in the dentate gyrus. They saw histograms with clear, equally spaced peaks. The number of peaks, but not their separation could be influenced by extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  levels. The peak separation could be increased by depolarising the cell. There was little variation in quantal size and they calculated that one quantum involves the opening of less than 30 ion channels, which is consistent with the idea that, in the central nervous system (CNS), quantal size is determined by the number of postsynaptic receptors (see Chapter One).

Stern *et al.* (1992) obtained similar results for excitatory transmission in visual cortical neurones. Like GABAergic synapses, these gave histograms with clear, evenly-spaced peaks. They estimated that each quantum is the result of the opening of as few as 10-20 non-NMDA-receptor channels. This poses potential problems for explaining the low quantal variance seen. When individual channels are activated, they open and close in a random manner. If such a low number are involved, this random behaviour might be expected to become significant and could increase the quantal variance. Kullmann (1993) has suggested that this can be explained if most available receptors are bound by transmitter and individual channels open with high probability.

Jonas *et al.* (1993) showed that the range of spontaneous EPSC amplitudes in CA3 pyramidal neurones show a similarly wide distribution to those reported by other groups (see above). Nevertheless they saw clear examples of quantal behaviour and low quantal variance in single-axon evoked responses at mossy fibre synapses. They estimated that the probability of the peaks in their histograms having arisen from non-quantal distributions were in the range  $<0.001-0.094$ .

Three quantal analysis studies of LTP in area CA1 have also demonstrated quantal transmission with low quantal variance. Malinow (1991) recorded from individual CA3-CA1 neurone pairs. Histograms of CA1 cell responses to firing of the connected CA3 cell showed clear quantal peaks that did not get obviously wider at higher quantal contents. Kullmann & Nicoll (1992) used maximum entropy noise deconvolution (MEND) (see Chapter One) and demonstrated that paired-pulse facilitation (a presynaptic phenomenon, see section 3.2.3, above) altered the peak heights of their histograms but not the peak spacing. On the other hand, changes in membrane potential of the neurone produced changes only in quantal size, as expected. Liao *et al.* (1992) recorded similar histograms. They too showed that changes in membrane potential altered the peak spacing and PPF did not. They found that removal of noisy sweeps by an objective method increased the sharpness of the histogram peaks. This would not be expected to happen if the peaks were statistical artefacts.

Thus data from my own and other groups strongly suggest that transmission at a variety of synapses in the CNS, including those between Schaffer collateral axons and pyramidal neurones in area CA1 of the hippocampus, is quantal and that quantal variance is low.

### *3.3.4 Noise reduction*

Conventional quantal analysis theory, based largely on observations of transmission at the neuromuscular junction (see Chapter One), predicts that the SD of the

individual peaks within a histogram will be equal to the SD of the background noise (which is the same for all peaks) plus a contribution from quantal variance (which is greater for peaks on the right-hand side of the histogram). This leads to two expectations. The first is that the peaks on the left-hand side of the histogram will be narrower and sharper than those to the right. The second is that even the narrowest peaks will have an SD at least equal to (but probably greater than) the SD of the background noise.

In most of our histograms, one or both of these expectations turn out to be wrong. Most have right-hand peaks with SDs very similar to those on the left, which suggests that quantal variance is low. In some cases, the peaks actually appear to get sharper towards the right-hand side of the histogram (apparent *negative quantal variance*). Also, the SDs of individual peaks are usually substantially lower than the SD of the noise. Larkman *et al.* (1991) found that the variance that gave optimum MLE (maximum likelihood estimation, see Chapter One) solutions to their histograms ( $53 \pm 17 \mu\text{V}$ ) was almost always lower than that of the measured background noise ( $96 \pm 39 \mu\text{V}$ ). Some mismatch might be expected because the histograms were selected for the clarity of their peaks but the size of the difference suggests that other factors might be involved.

Stratford (1992) has proposed a possible explanation, which he calls the *noise reduction hypothesis*. This relies on the idea that most spontaneous activity arises from the same release sites that produce the evoked response (see section 3.3.2, above). It also depends on two other main assumptions. The first is that these spontaneous EPSPs make up a significant part of the background noise. The second is that after a release site has discharged a vesicle of transmitter, there is a period of a few milliseconds during which the release probability of that site (and only that site) temporarily falls to zero. This is known as a *refractory period* and might happen, for example, while the site is being reloaded with a fresh vesicle.

The hypothesis proposes the following picture of events at the synapse. The background noise is measured in the baseline period at the beginning of each sweep, during which all of the release sites are available to contribute spontaneous activity to the noise. During the period of the sweep after the stimulus, however, only those sites at which it did not evoke release are available to contribute to the noise (because sites that did release a vesicle will be in their refractory periods). Similarly, spontaneous events that happen immediately before the stimulus will reduce the number of release sites available to respond and therefore reduce the mean amplitude of the evoked response. Thus, on average, large-amplitude evoked EPSPs produce sweeps that are less noisy in the period after the stimulus and sweeps that are noisy in the period before the stimulus result in small-amplitude evoked EPSPs. The result of both effects is the same: the larger the EPSP, the lower the noise due to spontaneous release so right-hand histogram peaks (which correspond to larger amplitudes) end up sharper than those on the left.

The effect of noise reduction would therefore counteract the effects of quantal variance and tend to make histogram peaks, particularly those on the right-hand side, sharper than they would otherwise be. If the effect of noise reduction is greater than the effect of quantal variance then we would expect to see both apparent negative quantal variance and histogram peaks that are sharper than the noise peak. The fact that we do suggests not only that the true quantal variance is low but also that noise reduction might be having an effect at these synapses. These arguments have been developed in greater detail in a forthcoming publication (Jack *et al.*, 1994).

### *3.3.5 Transmission is well approximated by simple binomial statistics*

If the individual synaptic connections made by a single axon onto a postsynaptic neurone all tend to show similar quantal sizes and release probabilities (see section 3.3.3, above), then transmission might be well approximated by simple binomial statistics (see Chapter One).

Two of the methods I used,  $1/CV^2$  graphs and constant  $N$  analysis, are based on the assumption that simple binomial statistics apply (although  $1/CV^2$  analysis is relatively robust to a range of models). A forthcoming paper from our group (Larkman, Stratford & Jack, in preparation) describes an extension of the work in Larkman *et al.*, 1991. It demonstrates that simple binomial statistics provide a good approximation of the trial-to-trial fluctuations seen in EPSP amplitude at synapses in area CA1 of hippocampal slices. For example, some EPSPs show substantial reductions in quantal size (interspersed with occasional small increases) when they are stimulated at a constant rate of 3 or 5Hz.  $1/CV^2$  graphs from these EPSPs have shallow gradients and histograms containing large numbers of trials are not peaky. However, constant  $N$  analysis can be used to estimate the quantal size for each epoch of data on the basis of its mean and SD (see Chapter Two). If the amplitude of each trial is then normalised by (i.e. divided by) the quantal size calculated for that epoch, the effects of the change in quantal size can be compensated for. After this has been done, histograms containing large numbers of trials show clear peaks and  $1/CV^2$  plots become steep and curve upwards.

This is good evidence that reasonably accurate quantitative estimates of parameters such as the quantal size can be made by assuming that transmission at these synapses obeys simple binomial statistics and that the number of release sites remains constant. To some extent, my data depend on this finding, but they also lend support to it, as I will discuss in Chapter Four.

### 3.4 Conclusions

Data from my own and other groups shows that synaptic transmission at Schaffer collateral connections onto CA1 pyramidal neurones is, in favourable cases at least, quantal with low quantal variance. In most cases, the quantal peaks in histograms composed of all recorded trials are obscured by non-stationarity in quantal size. In

order to carry out a full quantal analysis, it is therefore necessary to select regions of data that give peaky histograms.

These EPSPs depress when they are stimulated continually at frequencies of 1-4Hz. The rate of depression appears to be influenced by AP5, suggesting that NMDA receptors are partially involved. In my hands, these EPSPs do not show PPF or much PTP. I will discuss possible reasons for this in Chapter Four.

# Chapter Four

## Long-Term Potentiation

### 4.1 Introduction

How is long-term potentiation (LTP) of synaptic transmission expressed? Does the presynaptic cell release more neurotransmitter or does the postsynaptic cell become more sensitive to the chemical message? Perhaps both types of change occur, possibly with different time courses. This question has been the subject of much research and a lively, occasionally rancorous, debate over the last ten years (see Chapter One).

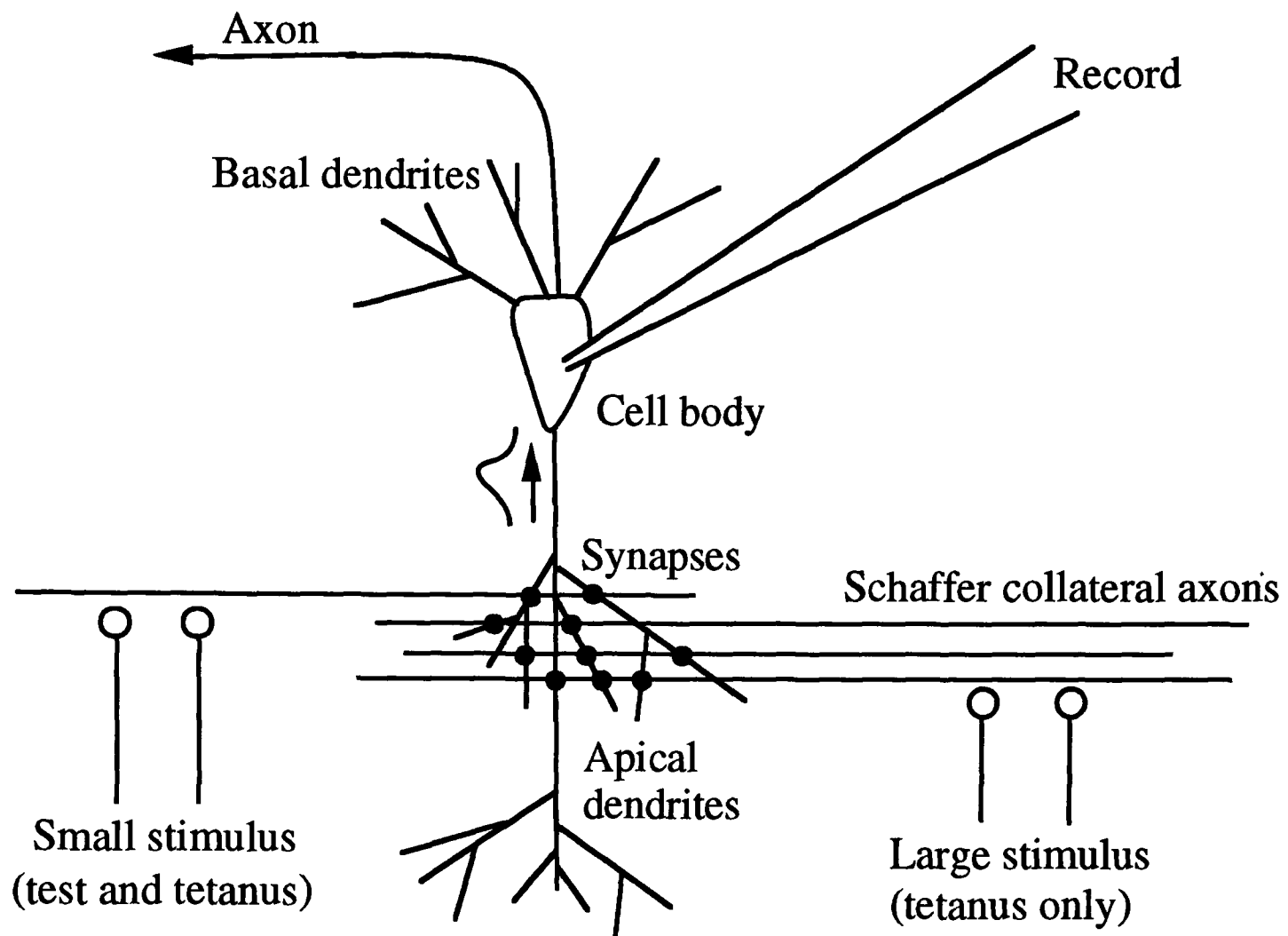
One approach is to use quantal analysis. This can, in principle, measure the relative contributions of presynaptic and postsynaptic neurones to any change in synaptic strength. Unfortunately there are serious technical difficulties associated with quantal analysis of synaptic transmission in the central nervous system (CNS) (see Chapters One and Three).

This chapter describes how I used the quantal analysis methods described in Chapter Two to study LTP in the CA1 region of rat hippocampal slices. These results have been published as a paper (Larkman *et al.*, 1992).

### 4.2 Methods

I prepared hippocampal slices from young adult rats and made intracellular voltage recordings from the cell bodies of pyramidal neurones in area CA1. The slices were bathed in artificial cerebrospinal fluid (ACSF) of the composition given in Chapter Two. In most experiments calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) ions were both present at 4mM, but in some experiments I lowered the concentrations of both to 2.5mM. I evoked small (usually  $<1\text{mV}$ ) excitatory postsynaptic potentials (EPSPs) by

stimulating Schaffer collateral axons with a metal bipole placed on the surface of the slice. Further details are given in Chapter Two.



**Figure 4.1** *Diagram of the recording arrangement* I made intracellular voltage recordings from the cell bodies of pyramidal neurones in the CA1 area of rat hippocampal slices and elicited a small test EPSP by stimulating Schaffer collateral axons with a bipolar wire stimulator. The stimulator was positioned to give the best chance of stimulating axons that made synapses onto the portion of the neurone's apical dendrites nearest to the cell body. During the tetani, this was paired with a second, much larger EPSP that I elicited using a separate bipole positioned on the other side of the slice.

Figure 4.1 is a diagram of the stimulating and recording arrangement. I evoked test stimuli at 1Hz, usually for 500 trials, after which I stopped stimulation. There was then a one-minute pause before I applied a series of 5 brief tetani. Each tetanus consisted of 20 stimuli given at 100Hz and they were separated by 15s intervals. During tetanic stimulation (but not during test stimulation), I elicited the small test EPSP together with a second, much larger EPSP (~10mV) that usually caused the cell to fire action potentials during each tetanus. This was in the hope that the neurone

would depolarise enough to trigger LTP induction (see Chapter One). I stimulated the larger EPSP with a second bipole (that had a separate power supply) placed on the other side of the slice, roughly opposite the first bipole. Following the last tetanus, there was another one-minute pause, after which I resumed 1Hz test stimulation of the small EPSP alone.

I repeated this procedure for 159 different EPSPs in 103 neurones. A total of 27 of these EPSPs (17 neurones) were recorded in ACSF containing 2.5mM  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .

I applied three different quantal analysis methods to EPSPs that showed LTP:

1.  $1/\text{CV}^2$  graphs
2. Amplitude frequency histograms
3. Constant  $N$  analysis

All three methods are described in Chapter Two.

Peaky histograms seemed to be most common in regions of data for which the trajectory of the  $1/\text{CV}^2$  graph was steep. This is not surprising: a steep  $1/\text{CV}^2$  trajectory indicates that any synaptic changes are mostly in release probability or the number of release sites (which would not obscure histogram peaks) rather than in quantal size (which would). For this reason, the  $1/\text{CV}^2$  graphs were used to guide the search for peaky histograms. Regions of data were selected on the basis of the steepness of the corresponding part of the  $1/\text{CV}^2$  graph. There were then minor alterations of the histograms to achieve the maximum amount of peakiness. This helped to reduced the amount of subjective data selection used in producing the histograms since the steepness of the  $1/\text{CV}^2$  graph is a measure not directly related to histogram peakiness.

## 4.3 Results

### *4.3.1 A small proportion of cells showed LTP*

Of the 159 EPSPs I recorded, 91 (57%) showed no significant increase in amplitude. Another 37 (23%) were too contaminated with polysynaptic events or electrical noise for a reliable quantal analysis. (Most of these EPSPs also did not show any significant increase in EPSP amplitude, although it was often difficult to pick out the original EPSP among the polysynaptic events.) Another 6 (4%) showed brief enhancements that were too small to be analysed reliably. None of these EPSPs, a total of 134 (84%), were analysed further.

Twenty five EPSPs (16%) showed a substantial increase in amplitude and had few enough polysynaptic events to be analysed. Of these, 12 (8%) showed short-term potentiation (STP) and are described in Chapter Five. The rest of this chapter is concerned with the 13 EPSPs (8%) that showed LTP, which I defined as a significant enhancement lasting for 15 minutes or longer.

These 13 EPSPs had a wide variety of initial amplitudes. The mean peak amplitude during the 500 pre-tetanus trials ranged from 85 to 957 $\mu$ V and averaged  $567\pm 234\mu$ V. In all 13 EPSPs, potentiation was apparent as soon as I resumed test stimulation and was usually maximal then (but not always, e.g. see Figure 4.2a). On average, the mean of the 500 trials immediately after LTP induction was  $182\pm 28\%$  of the mean before induction. The amount of potentiation was not correlated with the initial amplitude of the EPSPs. In most cases, potentiation lasted for between 20 and 40 minutes. One EPSP showed potentiation that lasted for 8 minutes with minimal decrement whereupon the impalement was lost; this EPSP was included in the analysis. Potentiation of two of the EPSPs lasted for over 60 minutes before the impalements were lost.

Table 4.1 shows information about  $\text{Ca}^{2+}$  concentrations, EPSP rise times and latencies, resting potentials, and the SD of both the noise and the EPSP for all 13 examples of LTP. There was no substantial change in resting potential after LTP induction. Immediately before the tetanus, the mean resting potential of all 13 cells was  $73 \pm 7$  mV. Immediately after the tetanus, it was  $73 \pm 8$  mV. In only two cells did the resting potential change by more than 2 mV, rising (i.e. becoming less negative) by 4 mV in one (L6) and falling by 3 mV in the other (L12). This suggests that there were no large changes in the input resistances of the cells. The amount of potentiation did not correlate with resting potential.

EPSP waveforms were digitised at 5 kHz so rise times and latencies were measured to an accuracy of  $\pm 0.2$  ms. Most EPSPs showed no significant change in rise time following LTP induction although there were some exceptions (L3, L5 & L12). In some cases the rise time fell but the overall tendency was for it to increase slightly after the tetanus. The mean 10-90% rise time for all 13 EPSPs increased from  $2.8 \pm 0.8$  to  $3.0 \pm 1.0$  ms. Latencies remained virtually constant for all but two EPSPs (L3 & L4). The average before the tetanus was  $3.9 \pm 0.9$  ms and afterwards it was  $3.8 \pm 0.9$  ms.

There was a tendency for the SD of the noise to increase slightly following the tetanus. This rose from  $95 \pm 27 \mu\text{V}$  to  $105 \pm 30 \mu\text{V}$  after LTP induction. Only two EPSPs showed large changes in noise (L8 & L10). In contrast, there was a substantial increase in the SD of EPSP amplitudes, from  $250 \pm 82$  to  $348 \pm 132 \mu\text{V}$ . There was no significant correlation between the SD of the noise and either the quantal size or the SD of the EPSP amplitude both before and after LTP induction.

EPSP number	[Ca <sup>2+</sup> ] (mM)	10-90% before/after		Latency before/after		Rest. Pot. before/after		Noise SD before/after		EPSP SD before/after	
L1	2.5	2.2	2.4	5.2	5.2	-83	-84	80	90	129	166
L2	2.5	2.8	2.8	3.4	3.4	-69	-70	145	126	243	286
L3	2.5	3.6	4.6	5.8	5.2	-72	-72	110	108	299	288
L4	4.0	3.6	3.4	5.2	4.6	-75	-77	84	86	127	220
L5	4.0	3.4	2.6	3.6	3.8	-77	-77	72	73	347	389
L6	4.0	2.2	2.0	4.4	4.4	-66	-62	96	87	239	311
L7	4.0	1.4	1.4	3.6	3.6	-66	-64	45	54	172	196
L8	4.0	4.4	4.8	2.8	2.6	-82	-80	83	145	263	428
L9	4.0	2.4	2.2	2.8	3.0	-66	-65	113	105	357	445
L10	4.0	2.2	2.4	3.8	4.0	-83	-84	85	155	254	494
L11	4.0	2.2	2.6	2.2	2.2	-61	-61	147	150	261	441
L12	4.0	3.6	4.4	3.8	4.0	-82	-85	79	85	393	635
L13	4.0	2.8	3.2	3.8	3.8	-70	-69	98	97	170	229
Average	—	2.8	3.0	3.9	3.8	-73	-73	95	105	250	348
±SD	—	±0.8	±1.0	±0.9	±0.9	±7	±8	±27	±30	±82	±132

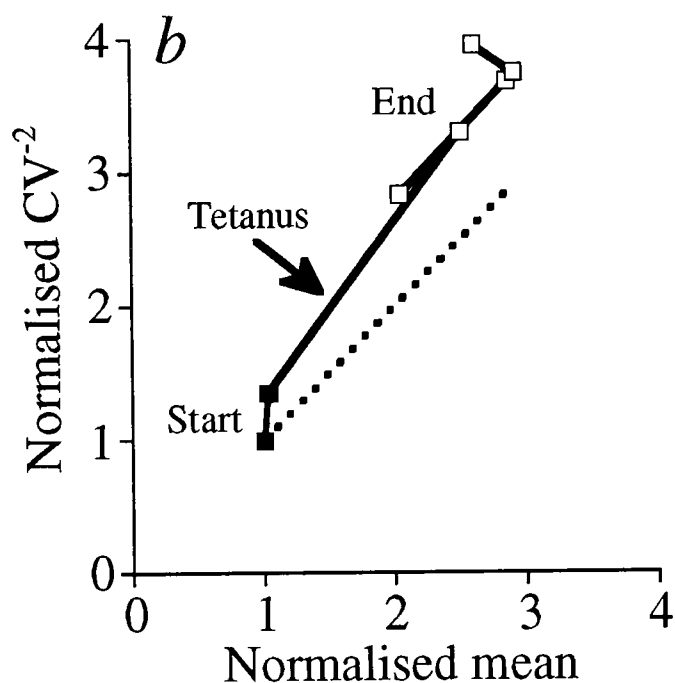
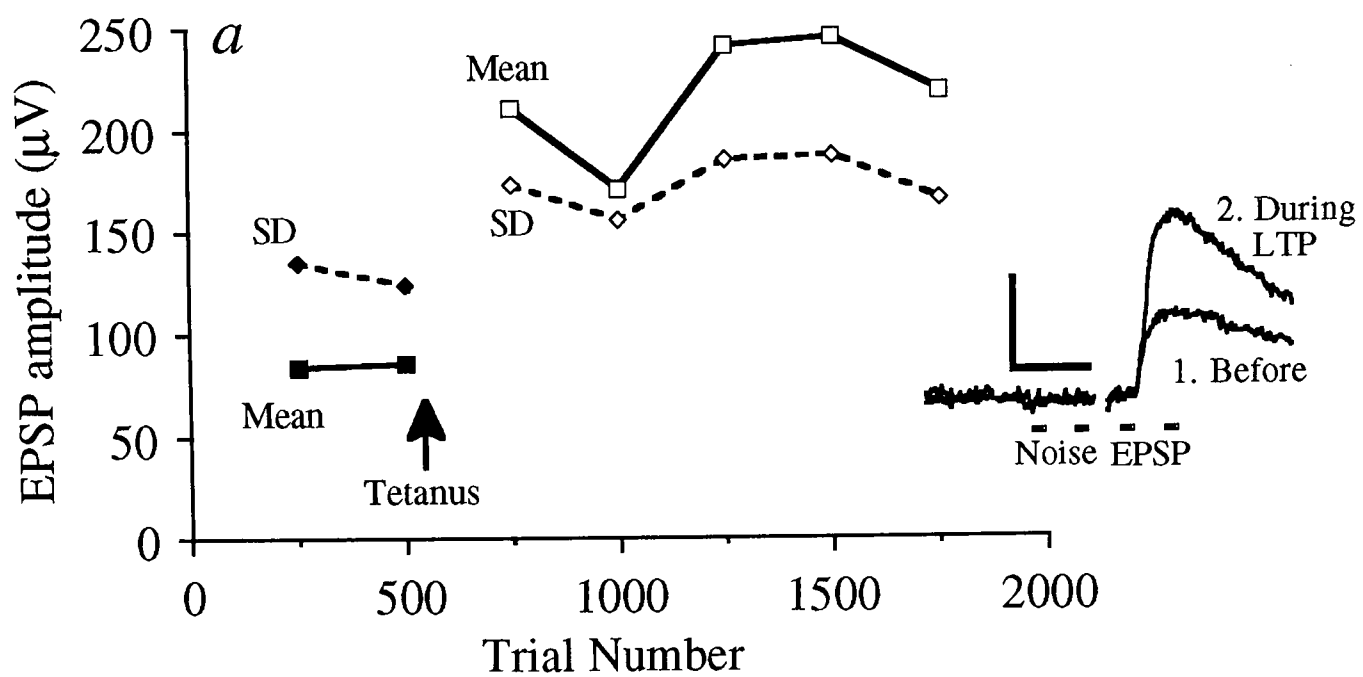
**Table 4.1 Properties of all 13 EPSPs** [Ca<sup>2+</sup>] shows the concentration of calcium present in the ACSF in mM. **10-90%** rise times and **latencies** for the mean waveforms of 500 trials before and after LTP induction are given in ms. Sweeps were digitised at 5kHz so these measurements are accurate to ±0.2ms. The **resting potential** of the neurones immediately before and immediately after tetanus are given in mV. In some cases the recorded potential has been corrected for estimated changes in the tip potential of the electrode so the absolute values are approximate only. However, assuming that no significant change in tip potential accompanied the tetanus, the relative values before and after should be accurate. The SD of the baseline **noise** and the **EPSP** during the 500 trials before and after LTP induction are given in µV.

### 4.3.2 An example of LTP that involved an increase in $P$

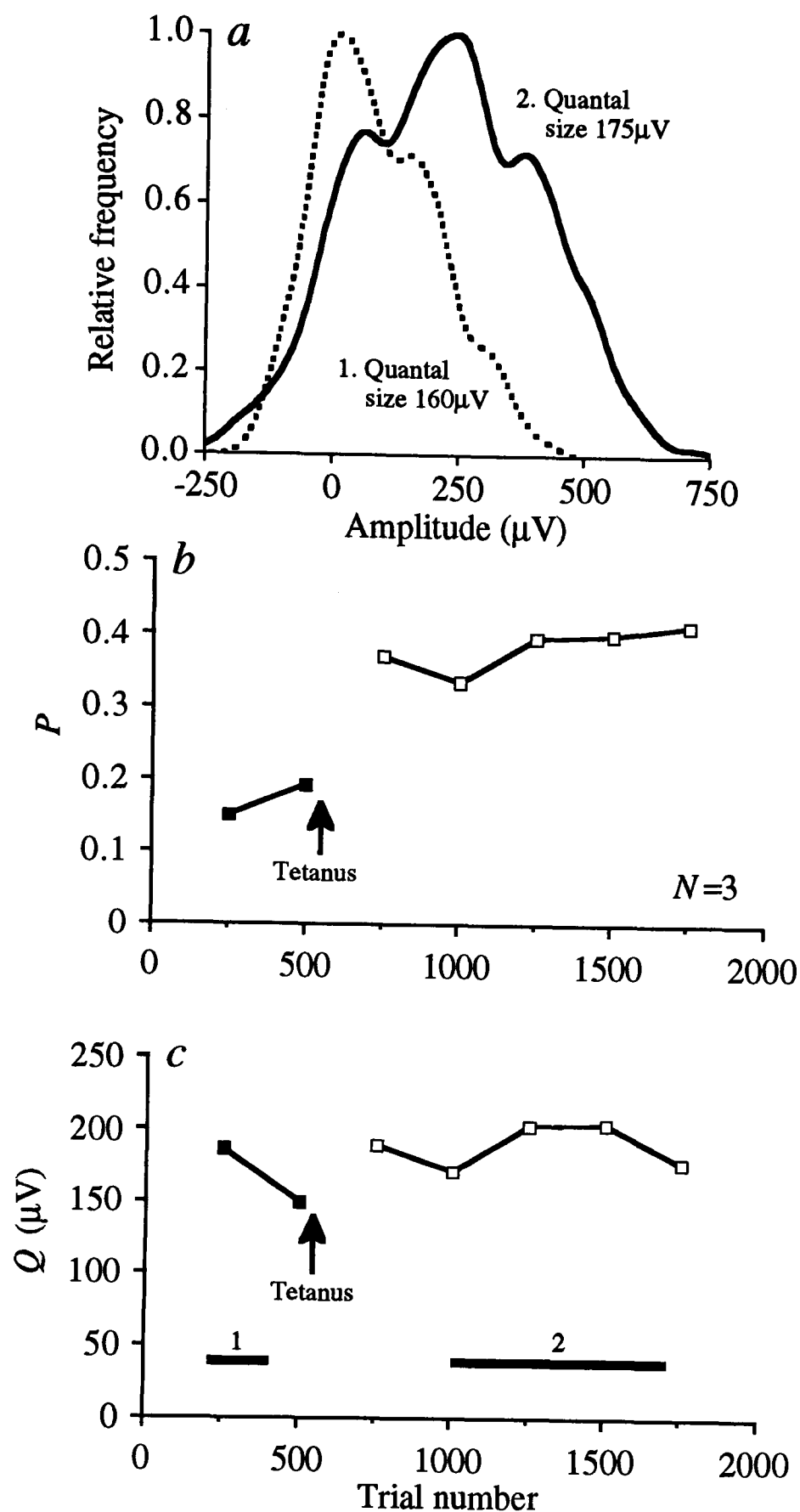
Figure 4.2 shows data from an EPSP (L1) with an amplitude that was initially less than  $100\mu\text{V}$  and increased more than two-fold after the tetanus (Figure 4.2 *a*). The increase in SD was more modest and, as a result, the  $1/\text{CV}^2$  graph has a trajectory (Figure 4.2 *b*) that is steeper than the diagonal (dotted line in Figure 4.2 *b*). Within the assumptions implicit in a  $1/\text{CV}^2$  analysis (see Chapter Two), this is typical of a change in release probability rather than the number of release sites and is incompatible with a substantial change in quantal size. However, the exact proportions cannot be quantified using this type of analysis.

One way of getting quantitative information is to look at amplitude frequency histograms. Figure 4.3*a* shows histograms plotted from regions of data before (dotted line) and after (solid line) LTP induction. There is only a small increase in quantal size from  $160$  to  $175\mu\text{V}$ . The relative peak heights, however, change substantially, with the leftmost peak (in this case representing failures) becoming smaller and those to the right (representing responses composed of one or two quanta) becoming larger. This indicates an increase in either release probability or the number of release sites (or both), which agrees well with the results from the  $1/\text{CV}^2$  graph.

Assuming a simple binomial release process, the mean, SD and quantal size of the pre-tetanus histogram can be used to calculate the number of release sites,  $N$  (see Chapter Two). The data for this histogram give  $N=3.2$ . Rounding to the nearest whole number,  $N=3$ . By making the further assumption that  $N$  is constant throughout the recording period, constant  $N$  analysis can be used to calculate the values of release probability ( $P$ ) and quantal size ( $Q$ ) for all periods of data from the EPSP mean and SD alone. The results show a sustained rise in  $P$  after LTP induction (Figure 4.3*b*) but no substantial change in  $Q$  (Figure 4.3*c*). This agrees with the results of both the



**Figure 4.2** *An EPSP that showed a predominantly presynaptic form of LTP* (a) The mean and standard deviation (SD) of the EPSP amplitude (L1) for the first 2,000 trials evoked at 1Hz (about 30 minutes). The mean amplitude increased more than two-fold while the increase in SD was much less marked. Each point represents 250 trials. Closed points show pre-tetanus data and open points post-tetanus data. The inset shows the average EPSP waveform of all 500 trials before LTP induction (1) and the 500 trials immediately after LTP induction (2). The stimulus artefact has been removed. Horizontal bars mark the zones used for measuring the amplitudes of the noise and the EPSP during each sweep. Scale bars: 100 $\mu$ V and 10ms. (b) A  $1/CV^2$  graph of all 2,000 trials. The trajectory is steep and lies above the diagonal (dotted line).



**Figure 4.3** *Quantal analysis of the EPSP shown in Figure 4.2* (a) The dotted line shows a histogram plotted from 200 pre-tetanus trials (selected from the 500 available). The quantal size, measured from the mean peak separation, is  $160\mu\text{V}$ . The solid line shows a histogram plotted from 700 post-tetanus trials (selected from the 1,500 available). This gives a quantal size of  $175\mu\text{V}$ . Such a small increase in quantal size cannot account for all of the increase in EPSP amplitude. However, the relative heights of the histogram peaks also changed, which suggests a significant presynaptic enhancement. (b) Assuming simple binomial release statistics, the value of  $N$  for the region of data used to construct the pre-tetanus histogram is  $N=3$  (to the nearest integer). According to constant  $N$  analysis,  $P$  shows a two-fold increase after the tetanus. This accounts for most of the change in EPSP amplitude. (c) Similar calculations for  $Q$  show no substantial change. Bars mark the regions of data used to plot the histograms in a.

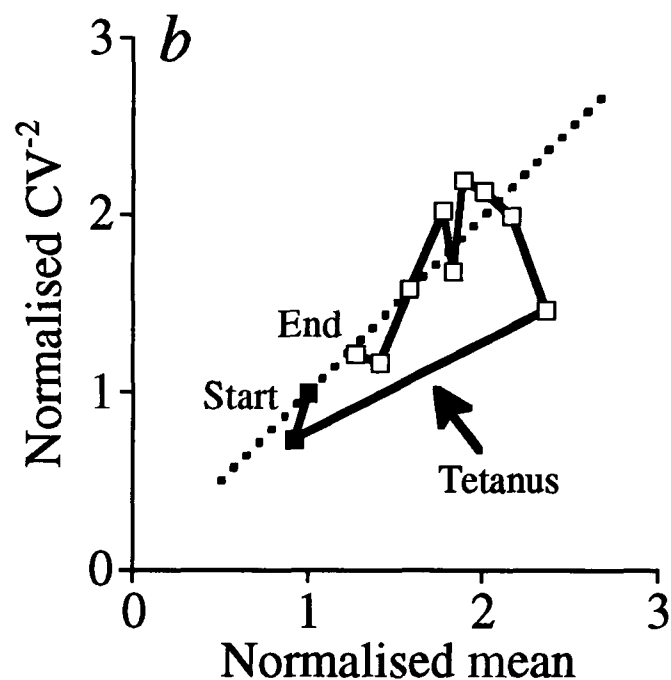
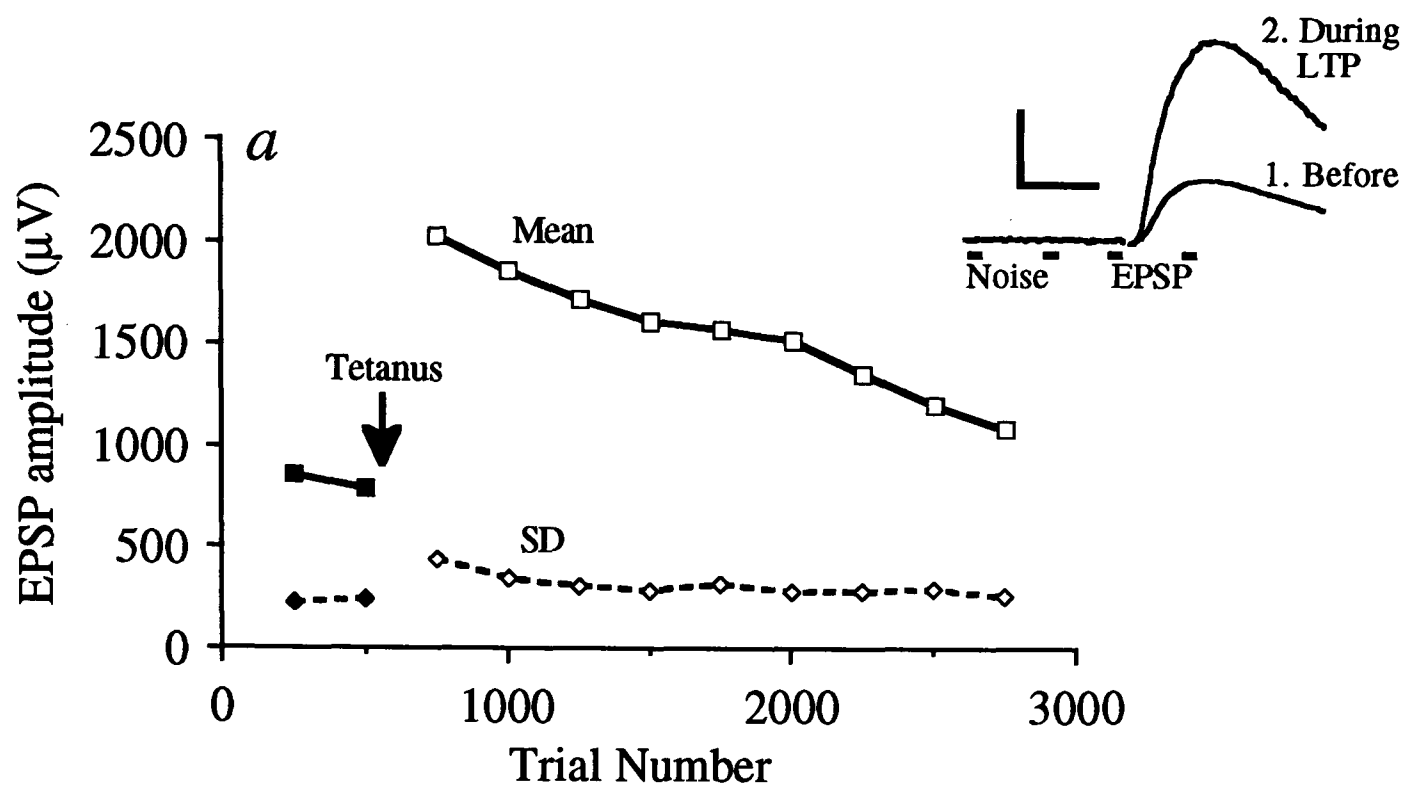
$1/CV^2$  graph (which relies on some of the same assumptions) and the histograms (which rely on different assumptions).

The bars in Figure 4.3c show the regions of data from which I plotted the histograms in *a*. I calculated the value of  $N$  from the first histogram, so the pre-tetanus value of  $Q$  inevitably agrees well with the quantal size estimated from this histogram. However, the post-tetanus value of  $Q$  also agrees well with the quantal size from the second histogram. This suggests that constant  $N$  analysis gives a reasonably accurate quantitative measure of changes in  $P$  and  $Q$ , which indicates that simple binomial statistics provide a good description of the behaviour of this EPSP and makes a substantial change in  $N$  unlikely.

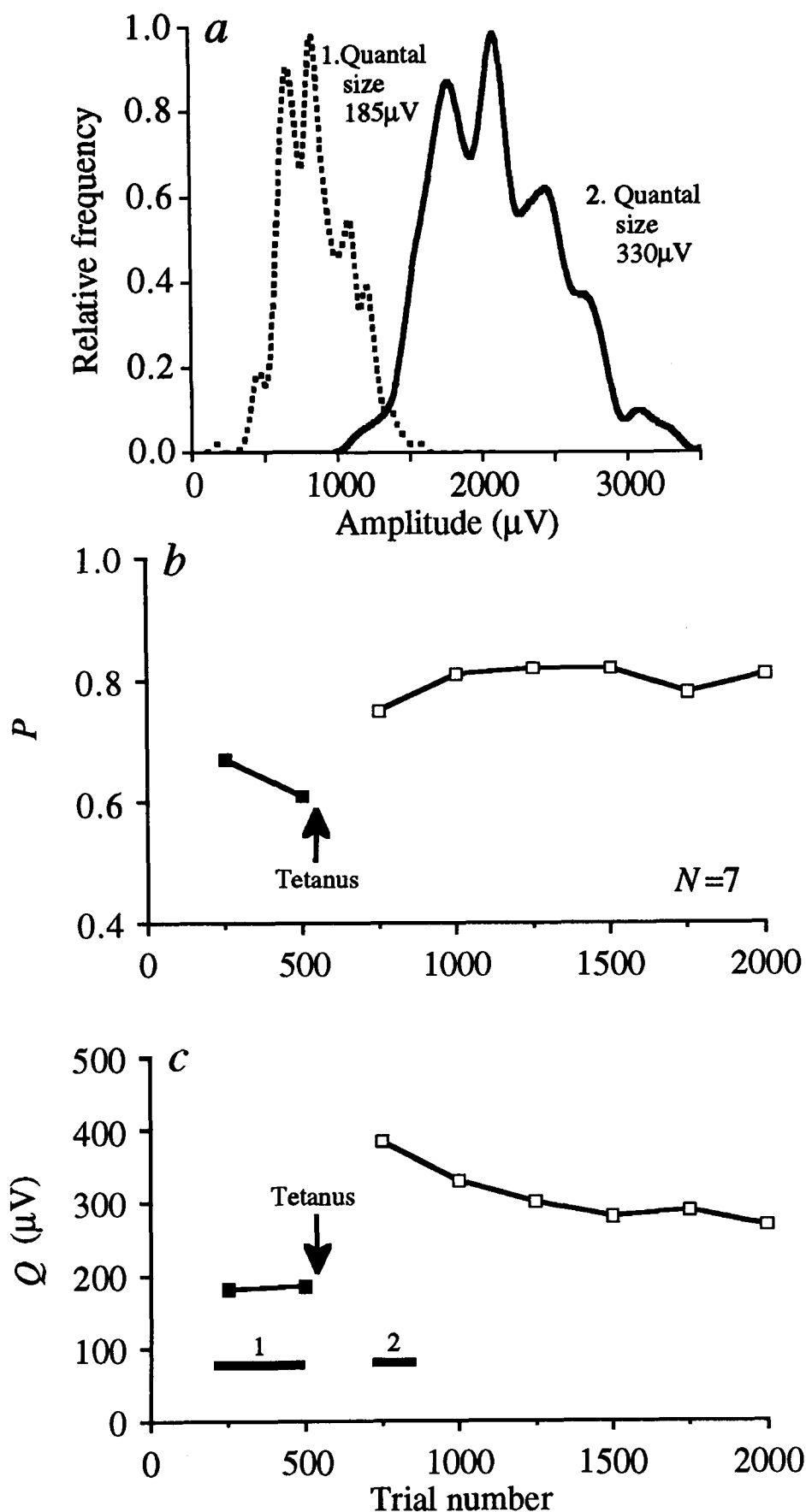
The results of all three analysis procedures taken together suggest that this EPSP potentiated by showing an increase mainly in release probability. Large changes in the number of release sites or in quantal size are unlikely.

### *4.3.3 An example of LTP that involved increases in P and Q*

Figure 4.4 shows data from an EPSP (L8) that was initially quite large (about  $800\mu V$ ) and increased in amplitude by about 2.5 times after the tetanus. It then declined slowly over the next 35 minutes or so (Figure 4.4a). After the tetanus, there was also a modest increase in the SD. The  $1/CV^2$  graph (Figure 4.4b) has a trajectory that is neither horizontal nor steep, but half-way between these two extremes. This suggests one of two possibilities. Potentiation might be due to some combination of changes in release probability, number of release sites and quantal size. Alternatively, it might be that only one of these variables changed and that the trajectory of the  $1/CV^2$  graph was inconclusive because transmission at these synapses did not conform well to simple binomial statistics. The first point after the tetanus lies well below the diagonal but later points lie close to the diagonal. This might indicate that the potentiation had a slow-developing presynaptic component.



**Figure 4.4 An EPSP that showed presynaptic and postsynaptic changes** (a) The mean and SD of the EPSP amplitude (L8) for the first 2,750 trials evoked at 1Hz (just under 45 minutes). The mean amplitude increased about 2.5 times and was accompanied by a modest increase in the SD. Each point represents 250 trials. The inset shows the average EPSP waveform of all 500 trials before LTP induction (1) and the 500 trials immediately after LTP induction (2). Scale bars: 1,000 $\mu$ V and 10 ms. (b) A  $1/CV^2$  graph of all 2,750 trials. The trajectory is neither horizontal nor very steep and above the diagonal (dotted line) but lies between these two extremes.



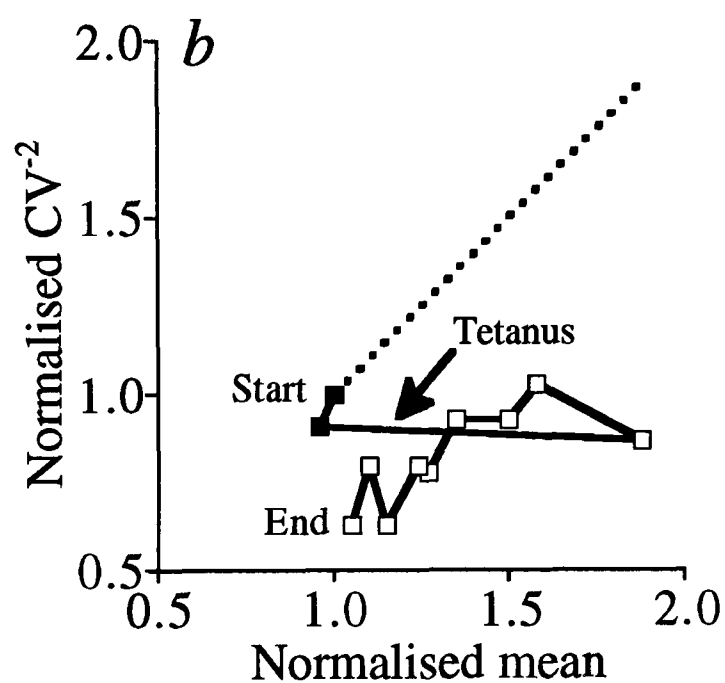
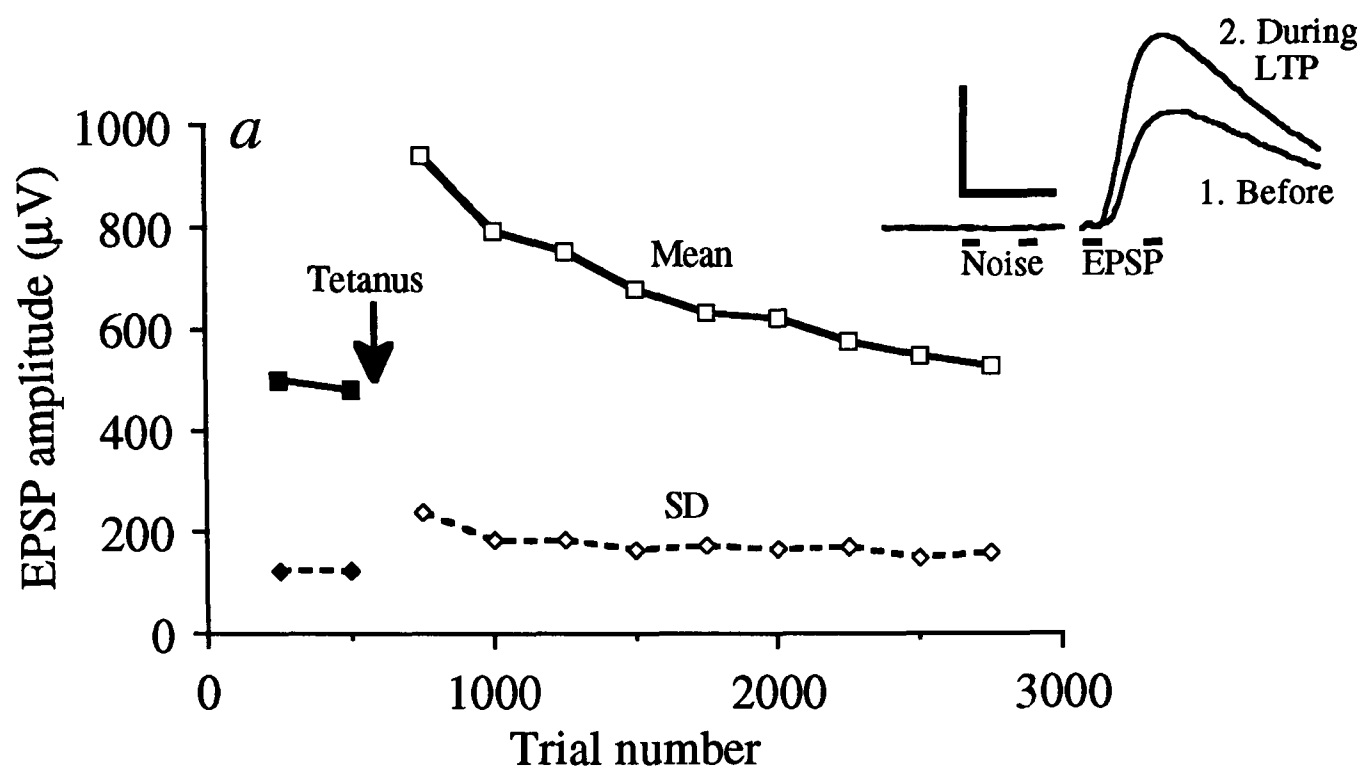
**Figure 4.5** *Quantal analysis of the EPSP shown in Figure 4.4* (a) The dotted line shows a histogram plotted from 300 pre-tetanus trials. The quantal size is  $185\mu\text{V}$ . The solid line shows a histogram plotted from 150 post-tetanus trials, which gives a quantal size of  $330\mu\text{V}$ . This increase can account for some, but not all, of the potentiation. The relative heights of the peaks also changed slightly. (b) Assuming simple binomial release statistics,  $N=7$  (to the nearest integer). Using this value, constant  $N$  analysis showed that  $P$  increased significantly after the tetanus. (c) Similar calculations for  $Q$  also show a substantial increase. Bars mark the regions of data used to plot the histograms in a.

A more complete quantal analysis for this EPSP is shown in Figure 4.5. A histogram of 300 trials recorded before the tetanus (dotted line in Figure 4.5a) has a quantal size of  $185\mu\text{V}$ . Another one plotted from 150 trials just after the tetanus has a quantal size of  $300\mu\text{V}$ . This increase can account for some, but not all, of the potentiation. Note that as well as a change in the peak spacing there has also been a small shift in the relative heights of the peaks, with responses composed of more quanta becoming slightly more frequent and those containing fewer quanta slightly less so.

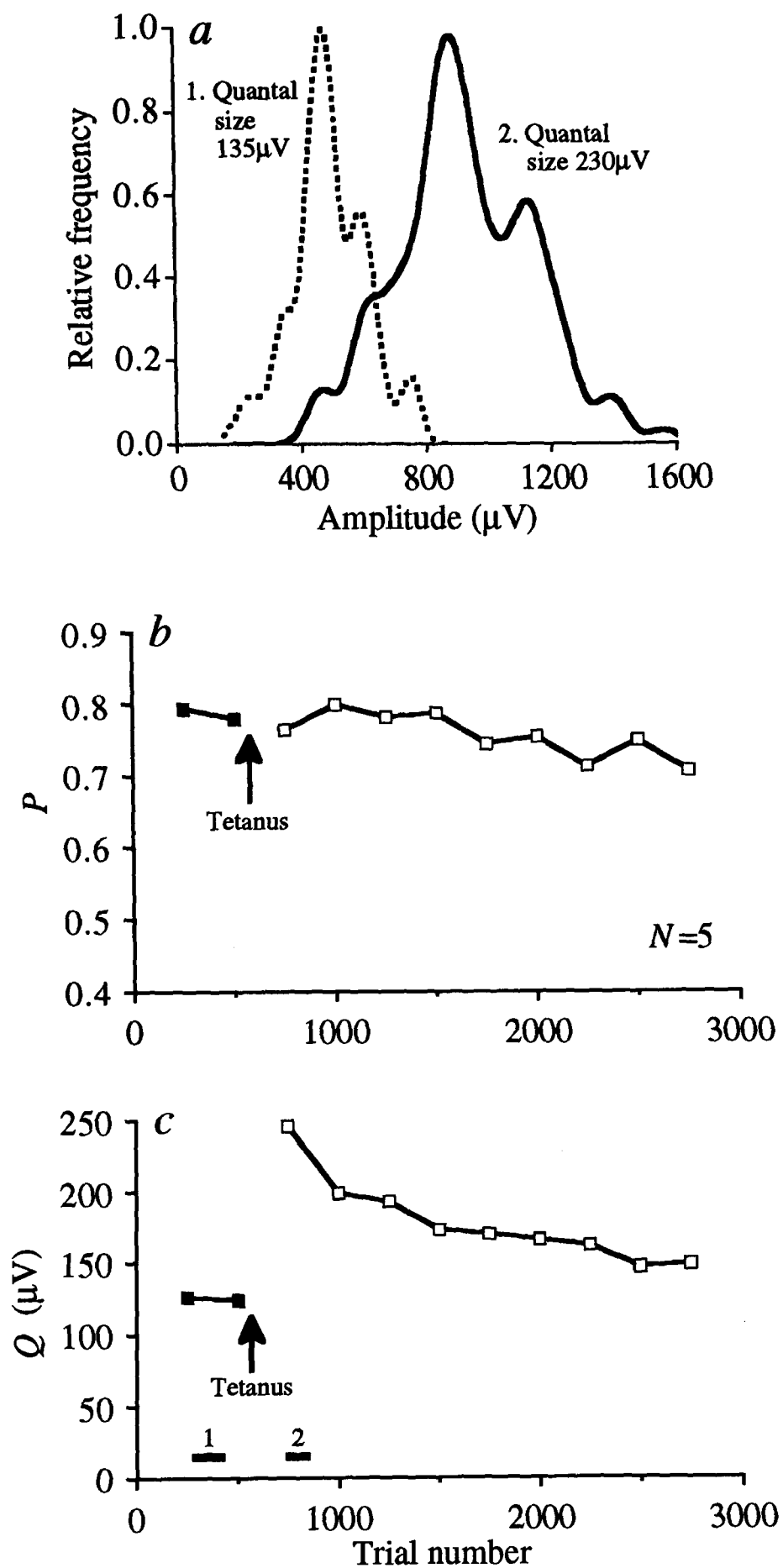
Using the quantal size estimated from the pre-tetanus histogram and assuming simple binomial statistics,  $N=7$  for this period of data. Using this value of  $N$  for all of the data, constant  $N$  analysis indicates that there were significant increases in both  $P$  (Figure 4.5b) and  $Q$  (Figure 4.5c) after the tetanus. The value of  $Q$  is maximal immediately after the tetanus but  $P$  continues to rise during the 250 trials immediately after the tetanus. The quantal size of the post-tetanus histogram agrees reasonably well with the value of  $Q$  estimated by constant  $N$  analysis for the same period. This suggests that transmission was well described by simple binomial statistics and that there were no large changes in  $N$ . So, for this EPSP, the quantal analysis results suggest that potentiation was caused by increases in both  $P$  and  $Q$ . A substantial increase in  $N$  is less likely.

#### *4.3.4 An example of LTP that involved an increase in $Q$*

Figure 4.6 shows an EPSP (L4) that expressed yet another form of LTP. This was initially  $500\mu\text{V}$  in amplitude and rose to about  $900\mu\text{V}$  immediately after the tetanus. It then declined slowly over the next 35 minutes (Figure 4.6a). The SD approximately doubled and, as a result, the trajectory of the  $1/CV^2$  graph is virtually horizontal (Figure 4.6c). This suggests that the potentiation is largely or exclusively caused by an increase in quantal size.



**Figure 4.6** *An EPSP that showed a predominantly postsynaptic form of LTP* (a) The mean and SD of the EPSP amplitude (L4) for the first 3,000 trials evoked at 1Hz (just over 45 minutes). Both increased nearly two-fold. Each point represents 250 trials. The inset shows the average EPSP waveform of all 500 trials before LTP induction (1) and the 500 trials immediately after LTP induction (2). Scale bars: 500µV and 10 ms. (b) A  $1/CV^2$  graph of all 3,000 trials. The trajectory is horizontal and well below the diagonal (dotted line).



**Figure 4.7** *Quantal analysis of the EPSP shown in Figure 4.6* (a) The dotted line shows a histogram plotted from 100 pre-tetanus trials. This has a quantal size of  $135\mu\text{V}$ . The solid line shows a histogram, plotted from 80 post-tetanus trials, which has a quantal size of  $230\mu\text{V}$ . This 70% increase in quantal size accounts for most of the potentiation. In contrast, the relative heights of the peaks remain virtually the same, indicating that there was no significant presynaptic change. (b) Assuming simple binomial release statistics,  $N=5$  (to the nearest integer). Using this value, constant  $N$  analysis shows that  $P$  drifted downwards throughout the recording period but showed no significant change immediately after the tetanus. (c) Similar calculations for  $Q$  show an increase of almost two-fold. Bars mark the regions of data used to plot the histograms in a.

Histogram results agree with the  $1/CV^2$  graph. These show an increase in quantal size from  $135\mu V$  before the tetanus (dotted line in Figure 4.7a) to  $230\mu V$  shortly afterwards (solid line in Figure 4.7a). There is no discernible change in the relative heights of the histogram peaks, which suggests that neither release probability nor the number of release sites have changed significantly.

Assuming simple binomial release statistics, the mean, SD and quantal size of the pre-tetanus histogram give, to the nearest whole number,  $N=5$ . Constant  $N$  analysis shows that  $P$  declines slowly during the recording period but shows no significant change immediately after the tetanus (Figure 4.7b). In contrast,  $Q$  almost doubles (Figure 4.7c) and this change can account for most or all of the potentiation. The bars in Figure 4.7c show the regions of data I used to plot the histograms in Figure 4.7a. The value of  $Q$  during the period used to plot the post-tetanus histogram agrees well with the quantal size measured from this histogram. This suggests that, once again, constant  $N$  analysis gave a reasonably accurate quantitative measure of changes in  $P$  and  $Q$ .

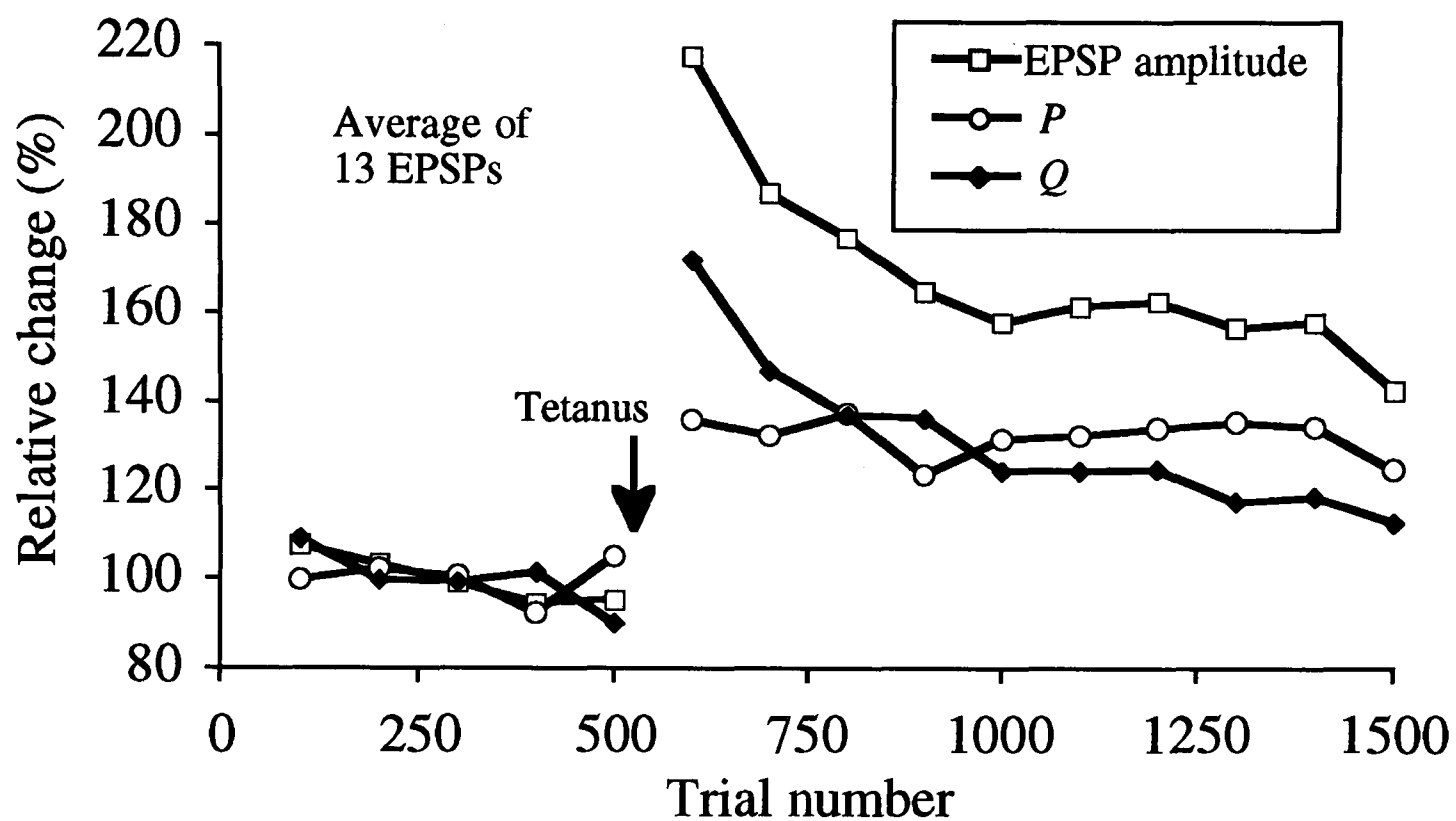
#### *4.3.5 An overview of all 13 EPSPs that showed LTP*

So it seems that LTP at these synapses can be expressed as an increase in release probability or quantal size, or both. Under the conditions I used, individual EPSPs showed a great deal of variability in the relative proportions but both types of change usually contributed to the potentiation.

Table 4.2 gives the amplitude, duration,  $1/CV^2$  graph gradient, and values of  $N$ ,  $P$  and  $Q$  for all 13 EPSPs. The range of  $N$  values was 3-12 and the average was  $7.0\pm 2.4$ . Mean  $P$  values during the 500 trials before LTP induction ranged from 0.13 to 0.79 and averaged  $0.47\pm 0.22$ . Initial values of  $Q$  ranged from 120 to  $293\mu V$  and averaged  $201\pm 58\mu V$ .

EPSP number	Amplitude		Duration (minutes)	1/CV <sup>2</sup> grad.	N	P		Q	
	before/after					before/after		before/after	
L1	85	191	>32	1.72	3	0.17	0.35	168	180
L2	486	802	63	1.36	9	0.35	0.51	154	176
L3	400	717	39	3.52	6	0.24	0.52	282	231
L4	492	867	36	0.09	5	0.79	0.78	125	223
L5	411	658	35	0.56	12	0.13	0.19	261	299
L6	526	970	47	0.60	5	0.51	0.64	206	307
L7	373	503	15	0.46	5	0.50	0.48	150	208
L8	823	1939	41	0.51	7	0.66	0.78	180	355
L9	654	1007	>63	0.56	9	0.28	0.32	259	347
L10	932	1837	>8	-0.18	5	0.76	0.77	243	480
L11	540	1097	19	0.57	8	0.39	0.51	173	272
L12	957	1759	18	-0.18	9	0.56	0.59	293	504
L13	686	1076	28	0.23	8	0.72	0.75	120	181
Average	567	1033	—	0.76	7.0	0.47	0.55	201	289
±SD	±234	±505	—	±0.95	±2.4	±0.22	±0.18	±58	±104

**Table 4.2 Results of quantal analysis for all 13 EPSPs** Mean amplitudes for the 500-trial periods before and after LTP induction are given in  $\mu\text{V}$ . Durations are the approximate time it took the EPSP amplitudes to return to baseline. '>' indicates that the EPSP was still potentiated when the impalement was lost. 1/CV<sup>2</sup> gradients are for graphs of 1/CV<sup>2</sup> against mean, binned at 250 trials, and normalised to the first 250 trials. The gradient is the change in 1/CV<sup>2</sup> that accompanied LTP induction divided by the change in mean over the same period. A value of zero indicates a horizontal trajectory, 1 indicates a trajectory along the diagonal and a value >1 indicates a trajectory steeper than the diagonal. Values for N, P and Q (in  $\mu\text{V}$ ) are binomial parameters calculated using constant N analysis. In the cases of P and Q mean values for the 500-trials immediately before and after LTP induction are shown.



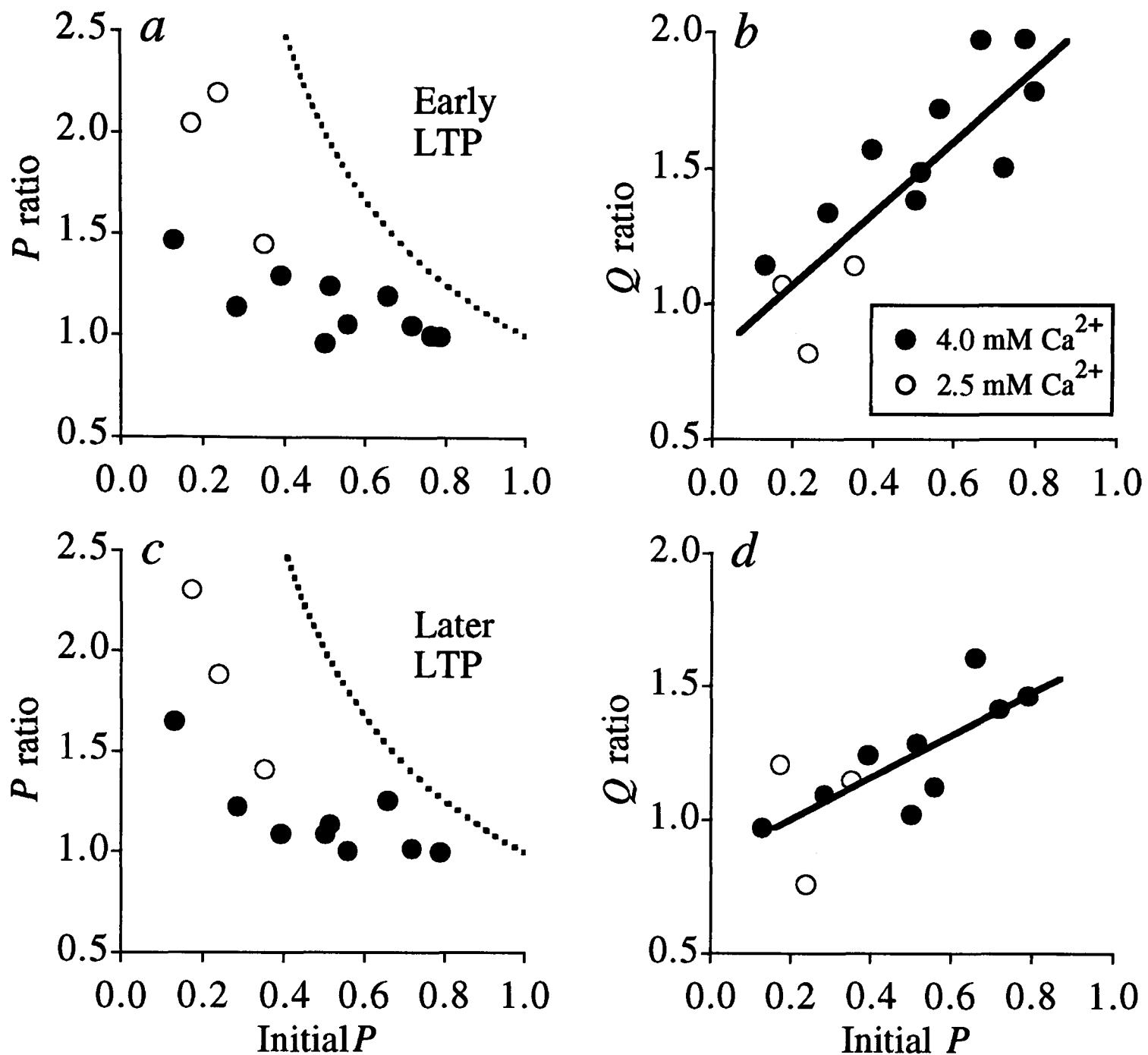
**Figure 4.8** *The average time courses of changes in mean EPSP amplitude, P and Q for all 13 EPSPs* The EPSP amplitude (open squares) increased, on average, more than two-fold after the tetanus and then declined slowly over the 16-minute post-tetanus period shown. During the first 5 minutes or so, the change in Q (closed diamonds) predominated but this fell. The increase in P (open circles) was initially lower but was also more robust and therefore accounted for most of the potentiation at later times. Each point represents 100 trials and is expressed as a percentage of the mean value during the 500 trials before LTP induction.

I pooled the results of constant  $N$  analysis for all 13 EPSPs. Figure 4.8 shows the average time course of changes in EPSP amplitude,  $P$  and  $Q$  during the pre-tetanus period and the first 16 minutes after LTP induction. On average, the EPSP amplitude increased to just over 200% of the baseline value and then declined slowly. In the first 5 minutes after the tetanus, the change in  $Q$  tended to predominate but this reduced with time and at later times the smaller but more robust change in  $P$  accounted for most of the potentiation.

#### *4.3.6 Changes in $P$ and $Q$ correlate with initial $P$*

Why do individual synapses show such different behaviour and what determines the relative contributions of presynaptic and postsynaptic changes at any given synapse? Figure 4.9 goes some way towards providing an explanation. The ' $P$  ratio' is a measure of the increase in  $P$  during the first 500 trials after LTP induction. This shows a negative correlation with the initial  $P$  (the value of  $P$  for the 500 trials before LTP induction) (Figure 4.9a). In other words, synapses that had low initial release probabilities responded to the tetanus by showing an increase in this probability whereas synapses at which the release probability was already high did not show much change in this value. To some extent this is to be expected because  $P$  can never be greater than one and this imposes an upper limit on the  $P$  ratio (dotted line in Figure 4.9a).

However, the  $Q$  ratio, which is a measure of the post-tetanus increase in  $Q$ , is not restricted in this way and yet shows a strong positive correlation with initial  $P$  (Figure 4.9b). Thus synapses at which initial  $P$  was low showed a predominant increase in  $P$  and those at which  $P$  was already high expressed larger increases in  $Q$ . Those with intermediate values of  $P$  usually showed more equal combinations of both types of change. Synapses that I incubated in ACSF with a lower  $\text{Ca}^{2+}$  concentration (open circles in Figure 4.9a&b) tended to show lower initial  $P$  values and a predominant



**Figure 4.9 Post-tetanus increases in P and Q correlate with initial P**

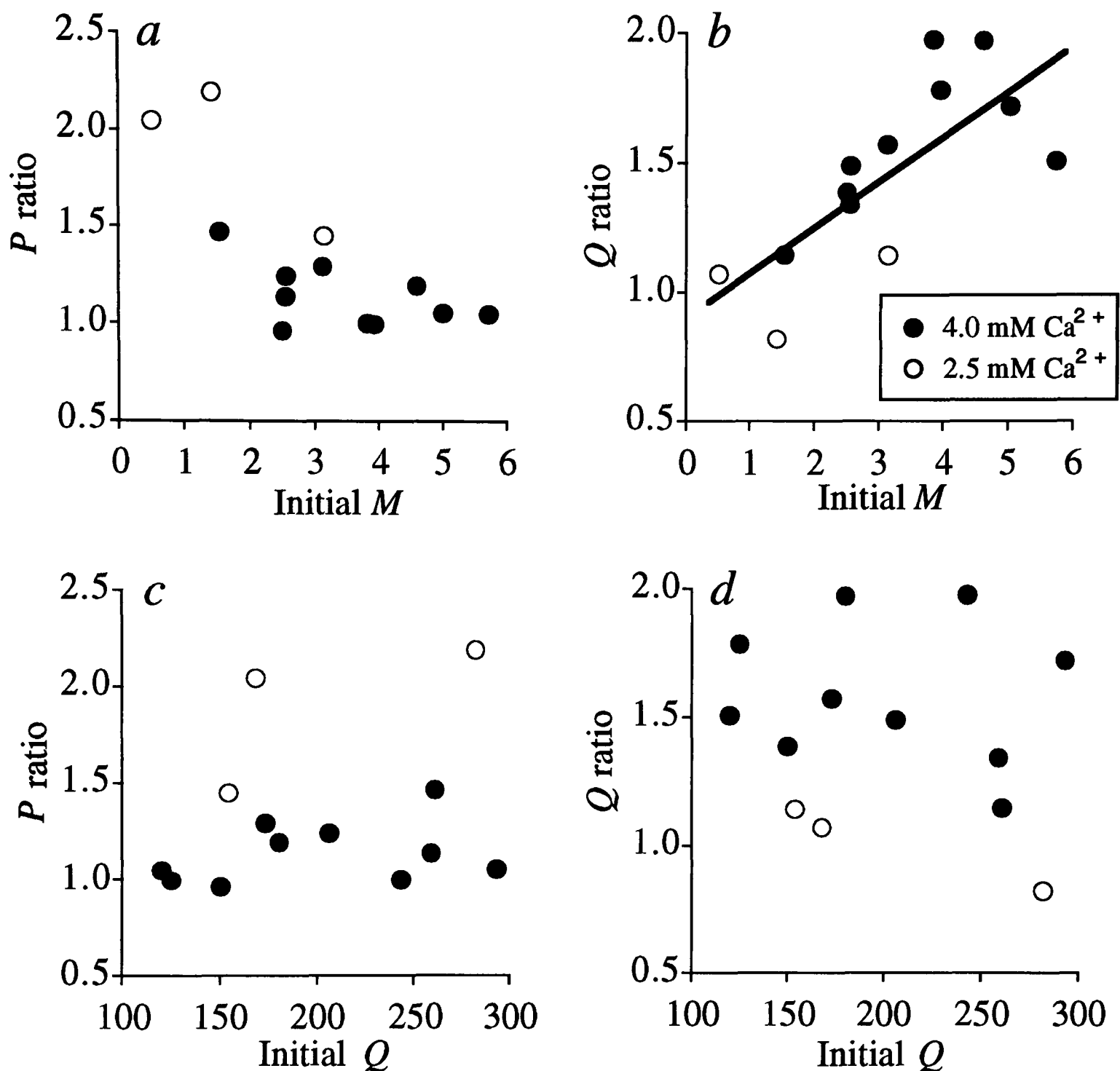
(a) Initial  $P$  is the mean value of  $P$  during the 500-trial pre-tetanus period. The  $P$  ratio is the mean value of  $P$  during the 500 trials immediately after the tetanus divided by initial  $P$ . The  $P$  ratio is negatively correlated with initial  $P$  (linear regression gave correlation coefficient,  $r=-0.73$ ). There is an upper limit on the values of  $P$  ratio (dotted line) because  $P$  can never be greater than one. EPSPs recorded in medium containing 2.5mM  $\text{Ca}^{2+}$  (open circles) tend to show lower initial  $P$  values and larger increases in  $P$  than those recorded in 4mM  $\text{Ca}^{2+}$  (closed circles). (b) The increase in  $Q$  is positively correlated with the pre-tetanus value of  $P$  ( $r=0.85$ ,  $p<0.001$ ). The  $Q$  ratio is the mean value of  $Q$  during the 500 trials immediately post-tetanus divided by the mean value of  $Q$  during the 500-trial pre-tetanus period. EPSPs recorded in a higher  $\text{Ca}^{2+}$  concentration (closed circles) tended to show higher values of initial  $P$  and greater increases in  $Q$  than those recorded in a lower  $\text{Ca}^{2+}$  concentration (open circles). (c) The increases in  $P$  at times 8-16 minutes after the tetanus show a similar correlation with initial  $P$  ( $r=0.76$ ). In this case, I calculated  $P$  ratios as the mean value of  $P$  during the period 500 to 1,000 trials after the tetanus divided by initial  $P$ . (d) The correlation with increases in  $Q$  also holds at times 8-16 minutes after the tetanus ( $r=0.74$ ,  $p<0.006$ ).

change in  $P$ . Synapses incubated in a higher  $\text{Ca}^{2+}$  concentration (closed circles) usually had greater initial  $P$  values and tended to show larger increases in  $Q$ .

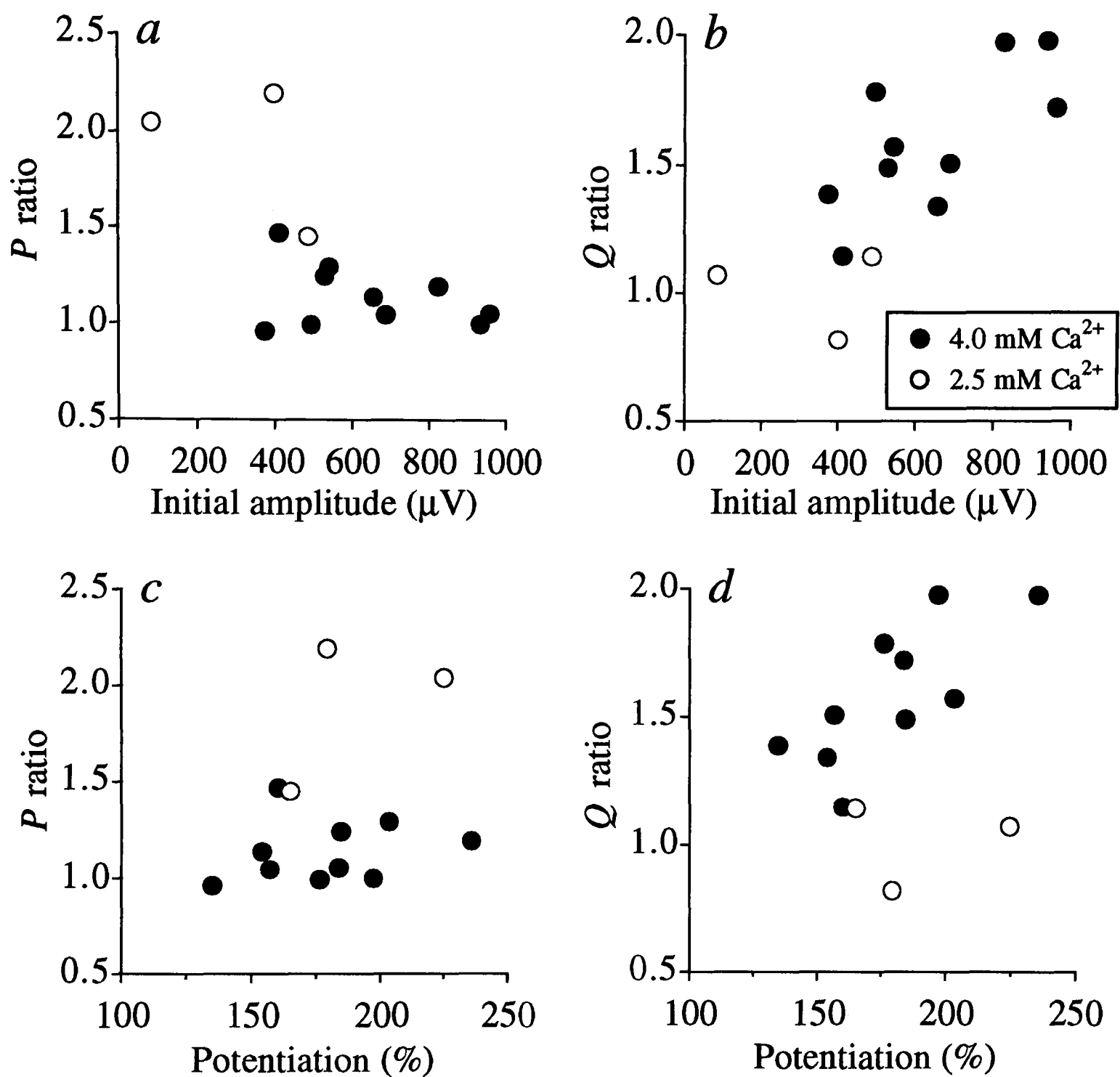
These correlations also hold true at later times after LTP induction. Figure 4.9c&d show similar plots of  $P$  ratios and  $Q$  ratios calculated for trials 500-1,000 (about 8-16 minutes) after LTP induction and plotted against initial  $P$ . The correlation between  $P$  ratio and initial  $P$  is almost identical at early and later times (compare Figure 4.9a&c) because the increase in  $P$  is does not decline much over this period. The correlation between  $Q$  ratio and initial  $P$  also remains at later times but the gradient is reduced (compare Figure 4.9b&d) because the increase in  $Q$  is less robust.

Figure 4.10a&b shows that  $P$  ratios and  $Q$  ratios also correlate with the initial (pre-tetanus) values of the quantal content,  $M$  (equal to  $NP$ ). Neither of them, however, correlate with initial  $Q$  values (Figure 4.10c&d).

Figure 4.11a&b shows that both  $P$  ratios and  $Q$  ratios correlate with the initial amplitude of the EPSP, presumably because synapses with low initial  $P$  values tend to produce smaller EPSPs. Voronin (1991b) found that 'small' LTP (defined as an increase in EPSP amplitude to less than 160% of the baseline value) is mainly postsynaptic while 'large' LTP is predominantly presynaptic. My data do not support this idea: changes in  $P$  and  $Q$  do not show significant correlations with the degree of potentiation (Figure 4.11c&d).



**Figure 4.10** *P ratios and Q ratios for early LTP correlate with initial M but not with initial Q* (a&b) Initial M is the mean quantal content (equal to  $NP$ ) during the 500 trials before tetanus.  $P$  ratios and  $Q$  ratios show similar correlations with initial M as they do for initial  $P$  ( $r=-0.72$  and  $r=0.74$ ,  $p<0.004$ , respectively). (c&d) Initial  $Q$  is the mean value of  $Q$  during the 500 trials before tetanus. Neither  $P$  ratios nor  $Q$  ratios correlate significantly with this ( $r=-0.24$  and  $r=0.17$ ,  $p<0.581$ , respectively).



**Figure 4.11** *P ratios and Q ratios correlate with initial EPSP amplitude but not with the amount of potentiation* (a&b) *P ratios and Q ratios correlate significantly with the mean EPSP amplitudes during the 500 trials before the tetanus ( $r=-0.637$ ,  $p<0.019$  and  $r=0.748$ ,  $p<0.003$ , respectively).* (c&d) *Neither P ratios nor Q ratios correlate significantly with the amount of potentiation seen during the first 500 trials after the tetanus ( $r=0.303$ ,  $p<0.172$  and  $r=0.323$ ,  $p<0.182$ , respectively).*

## 4.4 Discussion

### 4.4.1 *Why was LTP so rare?*

In my experiments, I defined LTP as a significant enhancement lasting longer than 15 minutes. Using this criterion, only 13 (8%) of the 159 EPSPs I studied showed LTP. One possibility is that only a small proportion of synapses express LTP. Another explanation was offered by Redman and his colleagues, who, in an extension of the work I described in Chapter One (Sayer *et al.*, 1989; Sayer *et al.*, 1990), tried to induce LTP in small and single-axon EPSPs recorded from CA1 pyramidal neurones in hippocampal slices. They met with a similarly low success rate: only 7 of 54 EPSPs expressed LTP. Larger EPSPs seemed to potentiate much more reliably so, to explain this, they proposed a curious scheme in which LTP, once induced, is only expressed when a critical level of depolarisation is reached in the postsynaptic cell (Friedlander *et al.*, 1990).

However, neither of these ideas can explain the relative reliability with which LTP can be induced in small and single-fibre EPSCs monitored by whole-cell recording methods (e.g. Malinow & Tsien, 1990; Malinow, 1991; Manabe *et al.*, 1993). One difference in the methods used in these studies is that the depolarisation of the postsynaptic cell was much deeper (typically to  $\sim 0\text{mV}$ ) than it is possible to achieve using an intracellular electrode and much longer (20-60s) than the duration of most tetani. The lower yield of my experiments (and those of Friedlander *et al.*) may be because the methods I used did not provide enough postsynaptic depolarisation at the synapse or synapses mediating the small EPSPs.

#### 4.4.2 LTP was relatively brief

In the first report of LTP, Bliss & Lømo (1973) described a variety of durations between 30 minutes and 10 hours. Since then, the range has extended further in both directions. The whole collection of synaptic enhancements referred to as LTP now includes examples with durations of just a few minutes and others that last for many days. This has caused some wrangling over exactly how durable an increase in synaptic strength has to be before it can properly be described as LTP. Some researchers categorise anything that lasts longer than post-tetanic potentiation (PTP) as LTP, or at least a form of LTP. Others suggest that LTP, by definition is 'non-decremental' (e.g. Ben Ari *et al.*, 1992). This does not specify a time limit, but presumably means that it should not decline significantly within a period of hours, which is the usual length of an *in vitro* slice experiment. Faced with such a variety of definitions, a recent meeting of several eminent researchers in the field (Barnes *et al.*, 1994) decided that LTP should be defined as potentiation that lasts at least one hour.

This is at odds with my definition of LTP as an enhancement lasting for more than 15 minutes. Only two of my EPSPs would pass the criterion of lasting over an hour. However, I saw two forms of potentiation that were clearly distinct on the basis of their time course alone. One typically lasted 2-5 minutes (see Chapter Five) and the other lasted 15 minutes or longer. Associative, NMDA-receptor-dependent forms of potentiation have already been divided into two categories on the basis of their time course and labelled STP and LTP (see Chapter Five), so it is not unreasonable to assign the two types of potentiation I saw to these categories.

In both cases, their durations are at the lower end of the range reported by other groups but there may be good reasons for this. In some respects, the experimental conditions I used were quite different to those used by other researchers. In particular, I used a relatively high rate of test stimulation (1Hz). Most researchers are interested simply in recording the mean amplitude of the postsynaptic response, for which low-

frequency (typically about 0.1Hz) test stimulation is sufficient. But in order to carry out a reliable quantal analysis I needed to collect more trials than such a low rate would allow. As I described in Chapter Three, stimulation at 1Hz or higher can itself cause changes in synaptic strength. Other researchers using relatively high stimulation rates also report LTP that decrements over periods of well under an hour (e.g. Malinow, 1991).

Several minutes of stimulation at 1Hz can produce long-term depression (LTD) in responses stimulated at 0.1Hz (Dudek & Bear, 1992; Mulkey & Malenka, 1992). Perhaps more importantly, other groups have reported that LTP seen at test stimulation rates of 0.05Hz in the CA1 region of intact animals or hippocampal slices can reverse ('depotentiate') in minutes when the stimulation rate is increased to 1-5Hz (Barrionuevo *et al.*, 1980; Staubli & Lynch, 1990; Fujii *et al.*, 1991; Larson *et al.*, 1993). This means that LTP recorded at a test stimulation rate of 1Hz might be expected to be shorter-lasting than LTP recorded at much slower rates. The widely varying durations reported by different groups are an irritating complication in the literature but a limit that arbitrarily divides up the range of reported durations might do an injustice to the likely subtleties of the mechanisms involved. A degree of flexibility is therefore needed in interpreting results that have been arrived at by very different means. For the time being, then, LTP or perhaps 'LTP-like' (Nowicky & Bindman, 1993) is a reasonable term to apply to my data.

#### *4.4.3 There was no PTP*

At first sight it might seem surprising that my results do not show a brief presynaptic enhancement immediately after the tetanus since this type of stimulation is commonly followed by PTP. However, PTP in the CA1 area of hippocampal slices decays in less than a minute at 30°C (Malenka, 1991) and so ought to have been over by the time I resumed test stimulation following the one-minute pause after the tetanus. Also, as I showed in Chapter Three, I saw only very modest PTP under the experimental

conditions I used. The relatively high values of initial  $P$  that many of these EPSPs have is one likely explanation. Seven of the 10 EPSPs that I recorded in 4mM  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  had initial  $P$  values of over 0.5. Given that the theoretical upper limit on  $P$  is one (and that the practical limit may be lower than this), these EPSPs would not be expected to show much presynaptic enhancement.

These high release probabilities do not necessarily reflect the true range of probabilities seen among all CA1 synapses. As I described in Chapter Two, I chose EPSPs by looking for the smallest response that appeared above the background noise on a large proportion of trials. If the response was intermittent, I suspected unreliable stimulation and moved the stimulating bipole elsewhere to evoke another EPSP. This method may well have had the inadvertent effect of selecting EPSPs with high initial release probabilities. Any EPSP with a low release probability would probably have shown a high proportion of transmission failures and I would have interpreted these as possible failures of stimulation. For similar reasons, I might also have unwittingly selected EPSPs with relatively large numbers of release sites and large quantal sizes.

#### *4.4.4 Did I stimulate single presynaptic axons?*

Two of the quantal analysis methods I used— $1/\text{CV}^2$  graphs and constant  $N$  analysis—are based on the assumption that transmission obeys simple binomial statistics (although  $1/\text{CV}^2$  analysis is relatively robust to deviations from the model). This implicitly assumes that release probability and quantal size are the same at all release sites. As I described in Chapter Three, there are good reasons for thinking that single-axon EPSPs might obey simple binomial statistics and the behaviour of small EPSPs in area CA1 of the hippocampus seems to be well described by simple binomial statistics. However, the chances of transmission closely obeying simple binomial statistics is likely to worsen rapidly as the number of axons increases (see Chapter Three).

For this reason, I tried to stimulate only one axon that was connected to the cell from which I was recording. I did this by slowly increasing the stimulation voltage until a reliable postsynaptic response appeared. The relationship between the stimulation voltage and the postsynaptic response often seemed to be step-like, with a small increase in stimulus strength causing the sudden appearance of a postsynaptic response. This suggests that I stimulated single axons but, because I was using extracellular stimulation, it was impossible to be certain.

In this respect, it is interesting to compare my results with those of Sayer *et al.* (1990), who, as I described in Chapter One, studied the response of CA1 pyramidal neurones to the stimulation of individual connected CA3 neurones. They reported response amplitudes of 30 to 665 $\mu$ V, with an average of 131 $\mu$ V. My EPSPs are larger than these, ranging from 85 to 957 $\mu$ V and averaging 557 $\mu$ V. At first sight this suggests that some or all of my EPSPs were produced by stimulating more than one axon. However, some of the difference might be explained by the possibility that I inadvertently selected synapses with high release probabilities and large quantal sizes (see above). Also, Sayer *et al.* used ACSF containing 2mM Ca<sup>2+</sup> (rather than 4mM Ca<sup>2+</sup> used in most of my experiments), which would have reduced transmitter release and hence the average EPSP amplitude. It is also possible that the technical difficulty of their 'dual impalement' recordings led to impalements with lower input resistances than mine. This would have the effect of reducing the measured voltage responses to the same synaptic currents. Given these considerations, the range of amplitudes that I saw are not inconsistent with the idea that they were produced by stimulating single axons, although the possibility that I stimulated multiple axons cannot be ruled out.

It is also interesting to note that the only successful deconvolution of a histogram from a single fibre input to be carried out by Sayer *et al.* (1990) was consistent with a simple binomial description and gave  $N=5$ ,  $P=0.27$  and  $Q=278\mu$ V. These values agree well with my results.

#### *4.4.5 The reliability of $1/CV^2$ analysis*

Analysis of  $1/CV^2$  graphs is based the assumption that transmission obeys simple binomial (or Poisson) statistics. Although it is reasonably robust to deviations from this model, it can be misleading under certain circumstances. Errors can be introduced not only when transmission at the synapses themselves deviates from simple binomial statistics but also when stimulation is unreliable, which would tend to increase the amount of trial-to-trial variation in response amplitude. The method I used to search for EPSPs was designed to reduce the chance of unreliable stimulation but it is impossible to rule it out entirely. However, additional evidence against it having a significant effect comes from the fact that my histograms never had bimodal shapes or large failures peaks (e.g. see Malinow & Tsien, 1990; Kullmann & Nicoll, 1992), which might indicate unreliable stimulation.

$1/CV^2$  graphs are less robust to deviations from simple binomial behaviour when the change in mean is small or the trajectory is neither very steep nor nearly horizontal (see Chapter Two). The changes in mean for the EPSPs analysed here were relatively large (>1.5-fold for all but one EPSP) and trajectories were sometimes either very steep or nearly horizontal. This is strong evidence for the existence of predominantly presynaptic and predominantly postsynaptic forms of LTP. However, most EPSPs showed intermediate trajectories, for which the nature of the underlying change is less certain. One way of checking the robustness of the simple binomial interpretation of these  $1/CV^2$  trajectories is to compare them with the results of histogram and constant  $N$  analyses. I will return to this point after I have discussed the reliability of these other methods.

#### *4.4.6 The reliability of histogram and constant N analysis methods*

A potential source of error in analysis of histograms is that spurious peaks can arise as a result of finite sampling. As I described in Chapter Three, work by Ken Stratford

(University Laboratory of Physiology, Oxford) suggests that, in general, this is unlikely to be the case. However, for individual histograms, this possibility can only be ruled out with a high degree of certainty in a few exceptionally favourable examples, such as the EPSP shown in Figure 3.5.

Constant  $N$  analysis is also open to criticism but of a different kind. It assumes that transmission obeys simple binomial statistics and that  $N$  does not change even after LTP is induced. As I described in Chapters One and Two,  $N$  is usually thought to correspond to the number of release sites, each of which is thought to exist in individual presynaptic boutons and therefore seems the least likely of the three parameters to change over periods of a few minutes. In Chapter Three I briefly described how this approximation appears to give a good quantitative description of synaptic changes during the depression that accompanies constant-frequency stimulation of EPSPs very similar to the ones I recorded.

However, recent results from Nicoll's laboratory (Manabe *et al.*, 1993) have been interpreted as showing that a change in  $N$  underlies LTP, which implies that my constant  $N$  analysis may be inappropriate. Recording compound EPSCs in hippocampal slices, they showed that a variety of manipulations known to affect the presynaptic neurone (e.g. increasing extracellular  $\text{Ca}^{2+}$  concentration) resulted in a  $1/\text{CV}^2$  graph trajectory that was steeper than the diagonal, as predicted by simple binomial theory when  $P$  increases. On the other hand, manipulations known to affect the postsynaptic side (e.g. glutamate receptor antagonists) gave horizontal trajectories, which indicate a change in  $Q$ . Stimulating a greater number of incoming axons produced intermediate trajectories, consistent with an increase in  $N$ . They then induced LTP and showed that the average  $1/\text{CV}^2$  trajectory was close to the diagonal (although slightly below it). This is not inconsistent with my results. The average trajectory for all 13 of my EPSPs is also just below the diagonal ( $1/\text{CV}^2$  gradient =  $0.76 \pm 0.95$ , compared with  $\sim 0.80$ - $0.85$  found by Manabe *et al.*). The results of Manabe *et al.* show less variation between individual EPSCs but this can be

explained by the fact that they elicited compound EPSCs, which would be expected to show a smaller range of behaviours because this method is itself equivalent to averaging the behaviour of a number of individual single-axon EPSCs.

However, whereas I interpreted the average  $1/CV^2$  trajectory as a being due to a combination of changes in  $P$  and  $Q$  (with individual EPSPs sometimes showing predominantly one or the other), Manabe *et al.* attribute it to a change in  $N$ . They justify this on the basis that LTP did not affect PPF, in contrast to manipulations known to affect  $P$ , which did. This reasoning may not be sound because in some cases PPF is reported to affect LTP (see Chapter One) and, even if it does not, this would not rule out possibility that LTP and PPF both affect  $P$  but by different mechanisms. As Manabe *et al.* point out, PPF is thought to affect transmitter release by increasing the concentration of  $Ca^{2+}$  in the presynaptic nerve terminal (see Chapter Three), while LTP might increase transmitter release by affecting some other stage of the process, such as the  $Ca^{2+}$ -sensitivity of the vesicle release machinery. Thus the results (as distinct from the conclusions) of Manabe *et al.* (1993) do not significantly disagree with my own.

Another explanation of the results obtained by Manabe *et al.* is that the change they saw was purely in  $Q$ , but nevertheless gave  $1/CV^2$  graph trajectories well above the horizontal. They used large, multi-axon inputs, presumably with a wide range of  $P$  and  $Q$  values. Under these circumstances, if low- $P$  synapses show little increase in  $Q$  and high- $P$  synapses show much greater increases in  $Q$  (as my data suggests they would), purely postsynaptic changes would be expected to give trajectories just below the diagonal (Stratford, 1992). This is despite the fact that no change in  $N$  is involved. The effect would be compounded if, as the data of Liao *et al.* suggest, synapses at which  $Q$  is initially low show the largest increases in  $Q$  (Stratford, 1992).

Nevertheless, the assumption that  $N$  is constant even after the abrupt change in EPSP amplitude seen when LTP is induced does seem to be less well founded than the assumption that it is constant during stimulation at a fixed frequency. In principle, the

synaptic enhancement could be partly or entirely due to increases in  $N$ . However, LTP caused by an increase in  $N$  seems less likely than forms caused by increases in  $P$  or  $Q$ . Assuming that each presynaptic bouton contains only one release site (see Chapter One), it is difficult to imagine plausible circumstances under which a change in  $N$  could be associative: there can be no conjunction of presynaptic and postsynaptic activity if the presynaptic side does not release neurotransmitter. One possibility is that some individual postsynaptic spines contain only NMDA receptors, which play a small role in low-frequency synaptic transmission. Activation of these during a tetanus might lead to the 'uncovering' of a previously inactive group of AMPA receptors on the postsynaptic side. Although this is a postsynaptic change, conventional  $1/CV^2$  analysis would interpret it as a change in  $N$ . An alternative hypothesis is that different release sites contributing to the same EPSP or EPSC show very different release probabilities, with some very high and others very low (as has been suggested by Hessler *et al.* (1993) and Rosenmund *et al.* (1993)). If the low-probability connections release sufficient transmitter during induction, their release probabilities could become enhanced to levels close to those of the high-probability sites. Although this is an increase in  $P$ , it would look superficially similar to an increase in  $N$ , and the  $1/CV^2$  graph could be close to the diagonal.

However, my data provide strong evidence against large changes in  $N$ . When the quantal amplitude measured from a histogram is compared with the value of  $Q$  estimated for that period of data by constant  $N$  analysis, the reliabilities of both methods of analysis are put to the test. There is a chance that either or both could be wrong, but the chance of them both giving similar wrong answers is much smaller. The agreements between the two were almost always very close. This helps to confirm both that the histograms are true reflections of the underlying transmission process and that the estimates made by constant  $N$  analysis are accurate enough to give useful quantitative results. In particular, it suggests that large changes in  $N$  are unlikely, although it does not rule out the possibility that small changes in  $N$

contributed to the potentiation or that changes in  $N$  occur at later times after LTP induction.

The close agreement between results from histograms and constant  $N$  analysis also suggests that overall deviations from simple binomial statistics are small, at least for regions of data that give peaky histograms. It is possible that simple binomial statistics did not apply during periods of data that did not give peaky histograms. Indeed, the very fact that histograms could not be found suggests that there may have been some deviations. However, given that these periods seemed to be interspersed with other periods during which simple binomial statistics applied, it seems unlikely that these were very large. If this is true then the interpretations I have made of the  $1/CV^2$  plots are also likely to be reasonably accurate.

Thus all three of the analysis methods I used can be potentially misleading in certain circumstances. But when they all agree closely—as they do for almost all of my data—the analysis procedure as a whole becomes much more reliable than when any of these methods is used on its own.

#### *4.4.7 LTP can involve changes in presynaptic and postsynaptic cells*

The results outlined in this chapter show that changes in both release probability and quantal size can underlie LTP for at least the first 15 minutes or so and that both types of change develop rapidly (<1 minute) after induction. Changes in release probability are usually interpreted as being presynaptic and those in quantal size as being postsynaptic (see Chapter Two). Thus both sides of the synapse seem to be able to contribute to LTP, but the relative proportions vary widely between individual synapses.

These results are not necessarily inconsistent with those of Malinow & Tsien (1990) or Bekkers & Stevens (1990), both of whom concluded that LTP expression is wholly or very predominantly presynaptic (see Chapter One). With hindsight, it seems that

most observers of the field at that time were expecting LTP expression to be presynaptic or postsynaptic, but not both. Malinow & Tsien (1990) and Bekkers & Stevens (1990) showed that after LTP induction, the number of failures reduces and  $1/CV^2$  increases. This is good evidence of a presynaptic change but does not rule out an accompanying postsynaptic change. Bekkers & Stevens (1990) found that the  $1/CV^2$  graph trajectory followed the diagonal and concluded that their EPSCs were well described by Poisson statistics, with an increase in  $M$  underlying LTP. An alternative interpretation is that these EPSCs obeyed simple binomial statistics and showed combined changes in  $P$  and  $Q$ . A similar argument holds for the results of Malinow & Tsien (1990) but in their case the increase in  $1/CV^2$  was less than the increase in mean, so the trajectory of the  $1/CV^2$  graph (which they do not show) would be below the diagonal. This argues in favour of a significant postsynaptic contribution.

A later study by Malinow (1991) of individual CA3-CA1 neurone pairs revealed histograms with clear quantal peaks. These showed that a portion of the 10- to 20-fold enhancement he saw was due to a roughly 50% increase in quantal size. This agrees with the idea that LTP can be at least partly postsynaptic. It also raises the question of whether or not the very large potentiations seen by Malinow (1991) and some other researchers who use patch-clamp recording methods are physiologically relevant. As I have discussed above, the induction procedures used in these experiments use more extreme depolarisations for longer periods than either intracellular electrodes or synaptically applied tetani can provide. Thus it could be that more physiologically relevant procedures produce smaller potentiation with more equal presynaptic and postsynaptic components. My results support this idea. Assuming that the presynaptic change seen by Malinow was largely or wholly in  $P$  rather than  $N$ , the synapses that he studied must also have had extremely low initial release probabilities in order to show such large presynaptic enhancements. Among the 13 EPSPs in which I successfully induced LTP (including 3 at a  $Ca^{2+}$  concentration of 2.5mM, the same as Malinow used), the lowest initial release probability was 0.17. Even in theory, this

would be incapable of potentiation of much more than 5-fold. As I have discussed above, it is possible that I inadvertently selected EPSPs with relatively high  $P$  values. It will be interesting to find out the true range of  $P$  values shown by synapses *in vivo* and to what extent this limits the amount of presynaptic enhancement they can show.

After I had carried out these experiments and submitted a paper for publication (Larkman *et al.*, 1992), another group reported conclusions very similar to mine. Kullmann and Nicoll (1992) used maximum entropy noise deconvolution (MEND) (see Chapter One) to analyse histograms of EPSC responses. After induction, they saw abrupt changes in both quantal size and quantal content, with individual EPSCs often showing predominantly one or the other. Later the same year, Liao *et al.* (1992) reported another study that reached essentially the same conclusions.

#### *4.4.8 The locus of the change seems to depend on initial P*

In my experiments, the amounts by which  $P$  and  $Q$  increased during LTP induction seemed to depend on the initial value of  $P$ . This is consistent with the results of Kullmann & Nicoll (1992), who showed that synapses displaying a large amount of paired pulse facilitation (PPF) tend to show an increase in quantal content rather than quantal size. PPF is a presynaptic enhancement (see Chapter Three) so synapses at which the effect is large presumably have relatively low release probabilities and, according to my results would tend to show a predominant increase in this probability. This would be detected in Kullmann & Nicoll's experiments as an increase in quantal content.

Liao *et al.* (1992) showed a correlation between the initial quantal content and the increase in quantal content. This is also consistent with my results, which show a correlation between the increase in  $P$  and initial  $M$ . However, Liao *et al.* also showed that the increase in quantal size is negatively correlated with its initial value. I did not find this, nor did I find that the increase in  $P$  correlated with initial  $Q$ .

Thus the initial 'setting' of the synapse appears to influence the type of potentiation that it will show. My data point to the initial release probability being a particularly important factor.

#### *4.4.9 The effects of Ca<sup>2+</sup> on LTP*

It is not surprising that lowering the concentration of Ca<sup>2+</sup> in the ACSF from 4.0 to 2.5mM caused the average release probability of the EPSPs to fall. The trigger for transmitter release appears to be Ca<sup>2+</sup> flowing into the presynaptic terminal from the extracellular fluid (see Chapter One) and lowering the extracellular Ca<sup>2+</sup> concentration is a well-established way of reducing the amount of transmitter release (Dodge & Rahamimoff, 1967; Malinow & Tsien, 1990). The novel part of my results is that such a change can influence the site of LTP expression. Thus, incubating neurones in lower concentrations of Ca<sup>2+</sup> would be expected to produce predominantly (though perhaps not exclusively) presynaptic LTP. Higher concentrations of Ca<sup>2+</sup>, on the other hand, would bias results towards predominantly postsynaptic forms of LTP.

This makes it tempting to speculate that the very different results reported by various groups investigating the locus of LTP expression might have been caused, in part, by their use of different Ca<sup>2+</sup> concentrations. I gave a brief summary of this work in Chapter One and have mentioned other, more recent studies in this discussion. All of the studies I have cited that found LTP to be mostly or wholly presynaptic used Ca<sup>2+</sup> concentrations of between 1.2 and 2.5mM. On the other hand, most of the studies that reported postsynaptic changes used Ca<sup>2+</sup> concentrations of between 3.0 and 4.0mM (Baudry *et al.*, 1980; Lynch *et al.*, 1982; Kauer *et al.*, 1988b; Muller *et al.*, 1988a; Muller & Lynch, 1988). This may not be the whole explanation because some groups find predominantly postsynaptic changes even in lower Ca<sup>2+</sup> concentrations (Foster & McNaughton, 1991; Manabe *et al.*, 1992). In Chapter Seven I will discuss the possible implications that this might have for the nature of LTP expression *in vivo* .

However, in light of my results it would be sensible to pay close attention to the incubation conditions—in particular, the  $\text{Ca}^{2+}$  concentration—when interpreting results of studies on the locus of LTP expression.

# *Chapter Five*

## Short-Term Potentiation

### 5.1 Introduction

#### *5.1.1 Short-term potentiation*

Short-term potentiation (STP) has received a small fraction of the attention lavished on its more durable relative, long-term potentiation (LTP). One reason is that its brevity seems to make it less attractive as a candidate for memory storage in the brain. Another is that it has often been confused with non-associative increases in synaptic strength such as post-tetanic potentiation (PTP), which are already understood quite well, particularly at the neuromuscular junction (NMJ). As a result, the true nature of STP has only begun to become apparent in the last 3 or 4 years.

#### *5.1.2 STP is distinct from PTP*

As I described briefly in Chapter Three, STP has sometimes been mistakenly identified as a long-lasting component of PTP that decays over a period of several minutes (McNaughton, 1982; Racine & Milgram, 1983). This is understandable since both PTP and STP can be produced by a tetanus and hippocampal STP has a similar duration to PTP at the NMJ. However, the two processes are different because, unlike PTP, induction of STP is associative (Sastry *et al.*, 1986) and depends on the activity of NMDA receptors (Larson & Lynch, 1988; Anwyl *et al.*, 1989). In hippocampal slices at 30°C, true PTP lasts for less than a minute while STP usually lasts for several minutes (Malenka, 1991).

Partly as a result of this confusion, there has been a lot of inconsistency in the names used to refer to the various brief forms of enhancement seen at hippocampal synapses.

Some researchers have used the terms PTP and STP almost interchangeably. Thus paired-pulse facilitation (PPF), PTP and other similar presynaptic phenomena have been described collectively as types of STP (Deadwyler *et al.*, 1975; McNaughton, 1982; Racine & Milgram, 1983). On the other hand, true STP has been mistaken for PTP (Sastry *et al.*, 1986; Anwyl *et al.*, 1988). The first report of STP induced by pairing postsynaptic depolarisation with simultaneous low-frequency presynaptic activity (a process often known simply as *pairing*) described the effect as an ‘associative PTP’ despite the fact that the potentiated synapses were not subjected to a tetanus at all (Sastry *et al.*, 1986).

### 5.1.3 STP shows a range of durations

As I described in Chapter One, experimental procedures designed to investigate the properties of LTP induction and expression often produce a decremental form of potentiation, which has also been called STP. This is seen, for example, when NMDA is applied to hippocampal slices or calcium ions ( $\text{Ca}^{2+}$ ) are allowed to enter a postsynaptic neurone through voltage-dependent  $\text{Ca}^{2+}$  channels. However, these enhancements usually last about 30 minutes, while the types of STP produced by tetanus or pairing often decay in 5 minutes or less. Is it right to call them by the same name?

For the time being at least, I would suggest that it is. Different research groups use a variety of experimental conditions and these are likely to result in a variety of STP durations, even if the same basic potentiation mechanisms are involved. One factor that is likely to be particularly important is the test stimulation rate. There does seem to be a tendency for STP to be briefer when higher rates of stimulation are used. Sastry *et al.* (1986), for example, stimulate at 0.2Hz and see STP lasting only 2-3 minutes; Malenka (1991) uses only half this rate and reports potentiation lasting about 15 minutes; Behnisch & Reymann (1993) stimulated extremely slowly—once every 10 minutes—and see a decremending form of potentiation that lasts over an hour.

Different stimulation rates alone do not explain all of the variability in STP duration and, of course, the different methods of induction used may well be activating different underlying mechanisms. But there is not yet any direct evidence to suggest that this is the case and, until there is, it is reasonable to assume that STPs with different durations are merely quantitatively, rather than qualitatively, different.

#### *5.1.4 Induction of STP and LTP are closely related*

What is the relationship between STP and LTP? Some research has suggested that their induction mechanisms are different. Larson *et al.* (1986) found that STP and LTP are affected differently by changes to the induction procedure. McGuinness *et al.* (1991b) found that their inductions were affected differently by changes to the extracellular  $\text{Ca}^{2+}$  concentration. However, as I have mentioned above, it now seems that STP induction involves many of the same processes as LTP induction. In particular, both types of potentiation are triggered by a rise in postsynaptic  $\text{Ca}^{2+}$  concentration caused by the opening of NMDA-receptor ion channels.

The study by Sastry *et al.* (1986) showed that a brief pairing produces STP while longer ones produce LTP. More recent work has shown that this is because the two types of potentiation are triggered by different amounts of postsynaptic  $\text{Ca}^{2+}$ . Strongly inhibiting all rises in postsynaptic  $\text{Ca}^{2+}$  concentration prevents both STP and LTP (Lynch *et al.*, 1983) but more subtle manipulations can tease the two apart. Work by Malenka (1991) has shown that a relatively small  $\text{Ca}^{2+}$  influx will produce STP and a larger one, LTP. This agrees with a more recent report by another group (Hanse & Gustafsson, 1992), who showed that the duration of potentiation in the dentate gyrus depends on the amount of postsynaptic activity during the induction period and does not depend directly on the amount of presynaptic activity. The duration of the rise in postsynaptic  $\text{Ca}^{2+}$  concentration seems to be as important as its size. Malenka *et al.*, (1992) showed that a tetanus that normally produces LTP will result in STP if

the rise in postsynaptic  $\text{Ca}^{2+}$  is artificially limited to a duration of less than 2-2.5s and will produce only PTP if it less than 1.5-2s long.

Other work in Malenka's laboratory (Colino *et al.*, 1992) showed that during pairing, the presynaptic stimuli must be no more than a few tens of seconds apart to reliably induce STP. This might be because the increase in postsynaptic  $\text{Ca}^{2+}$  levels during pairing stimulates a  $\text{Ca}^{2+}$ -dependent process that decays over a period of seconds and must summate to reach some threshold in order to trigger STP. Presumably there is another, higher threshold for LTP induction. The level of this threshold seems to be affected by the recent activity of the synapse. A tetanus that produces LTP in synapses that have been inactive during the preceding hour or so will often produce only STP in synapses that have been stimulated during the same period (Huang *et al.*, 1992a). However, as I described in Chapter One, this may be due to a negative feedback loop in which  $\text{Ca}^{2+}$  influx stimulates the production of nitric oxide (NO), which in turn inhibits the action of NMDA receptors.

### *5.1.5 How is STP expressed?*

Although the induction of STP and LTP are very similar, there are grounds for thinking that their expression mechanisms are quite distinct. As I described in Chapter One, blockers of protein kinase activity or putative retrograde messenger production inhibit LTP induction but appear to leave STP intact.

One way to test this more directly is to see whether or not saturating LTP inhibits expression of STP. Unfortunately, this question remains unresolved. Some groups report that it does (Gustafsson *et al.*, 1989; Asztely *et al.*, 1991) and others that it does not (Kauer *et al.*, 1988a; Kullmann *et al.*, 1992).

Little is known about whether STP is presynaptically or postsynaptically expressed. It has sometimes been equated with the early stages of LTP, which is sometimes (wrongly, it now seems) considered to be purely presynaptic (e.g. Stevens, 1989).

What little direct evidence there is suggests that STP is mainly postsynaptic. It is reported not to affect paired-pulse facilitation (PPF) (McNaughton, 1982; Anwyl *et al.*, 1989; Colino *et al.*, 1992) and a kind of 'STP' can occur in a purely postsynaptic preparation in which brief puffs of a solution containing glutamate and glycine are applied to an isolated neurone (Zilberter *et al.*, 1990). A quantal analysis specifically addressing the changes underlying STP expression has not been reported before.

The aim of my experiments was to investigate STP induced by two different methods: tetanic stimulation and pairing. I used quantal analysis methods to find out whether changes were presynaptic or postsynaptic and then compared the results with those for LTP in Chapter Four. In this way, I hoped to discover more about the nature of STP and its relation to LTP. These results have been published as a paper (Hannay *et al.*, 1993).

## 5.2 Methods

### 5.2.1 *STP induced by tetanus*

I made voltage recordings from pyramidal neurones in area CA1 of rat hippocampal slices. The recording and stimulating arrangements as well as the incubation conditions were identical to those described for the LTP study in Chapter Four. All recordings in this chapter were carried out in artificial cerebrospinal fluid (ACSF) containing 4mM Ca<sup>2+</sup> and 4mM Mg<sup>2+</sup>. I used a metal bipole resting on the slice to apply test stimuli to Schaffer collateral axons and recorded the small (usually <1mV) EPSPs that this produced in the postsynaptic cell. After 500 trials, I stopped stimulation for one minute and then applied a series of 5 tetani, each composed of 20 stimuli at 100Hz and separated by 15 seconds. During the tetani, the test EPSP was paired with a second, much larger EPSP that I stimulated with a separate bipole. This usually caused the neurone to fire action potentials during each tetanus. Following the

last tetanus, there was another one-minute pause before I resumed test stimulation at 1Hz.

### *5.2.2 STP induced by pairing*

In a separate set of experiments, I tried to induce STP in 34 separate EPSPs (recorded from 19 different neurones) by pairing low-frequency presynaptic stimulation with postsynaptic depolarisation. The incubation conditions were identical to those for tetanus-induced STP (see above) except that in some experiments I added 100 $\mu$ M of the NMDA receptor antagonist, 2-amino-5-phosphonovalerate (AP5) to the ACSF.

The pairing procedure was as follows. After 500 test stimuli, I allowed 1Hz stimulation of the test EPSP to continue uninterrupted and depolarised the postsynaptic cell by injecting current through the recording electrode. In each case, I applied a steady current of +3nA superimposed on the negative holding current for that particular cell (typically around -0.5nA, giving a net positive current of about 2.5nA). This always took the cell through the threshold for firing action potentials. The peak (i.e. least negative) membrane potential reached during depolarisation, averaged across all 32 EPSPs, was  $-41\pm 15$ mV. After a further 40 trials I switched off this depolarising current and returned the cell to its original holding current.

## 5.3 Results

### *5.3.1 Tetanus-induced and pairing-induced STP have similar amplitudes and durations*

I gave tetani to a total of 159 separate EPSPs. As described in Chapter Four, I discarded 134 of these, usually because they showed no enhancement. A further 13 showed LTP and are described in Chapter Four. In this chapter, I will describe the remaining 12 that showed STP.

The EPSP amplitude during the 50 trials before tetanus ranged from 218 to 897 $\mu$ V and averaged 572 $\pm$ 185 $\mu$ V for all 12 EPSPs. STP was defined as an increase in EPSP amplitude during the first 50 trials after tetanus to at least 150% of this baseline value. The largest increase was to 364% of the baseline and the average was 230 $\pm$ 74%. The amount of potentiation did not correlate with the initial EPSP amplitude.

Table 5.1 shows EPSP rise time and latency, resting potential, and the SD of both the noise and the EPSP for each of the 12 examples of tetanus-induced STP. EPSP waveforms were digitised at 5kHz so rise times and latencies were measured to an accuracy of  $\pm$ 0.2ms. The average rise time during the last 50 trials before tetanus was 3.7 $\pm$ 1.0ms and this increased slightly to 3.9 $\pm$ 0.9ms during the 50 trials immediately afterwards. On the whole, individual EPSPs did not show much change in rise time but there were exceptions (S5 & S8). The average latency before tetanus was 3.5 $\pm$ 0.9ms and 3.5 $\pm$ 0.8ms afterwards. Two EPSPs (S5 & S10) showed a change in latency of more than 0.4ms.

In only two cases (S6 & S9) did the resting potential change by more than 2mV. The average resting potential immediately before the tetanus was 74 $\pm$ 9mV and afterwards it was almost unchanged at 74 $\pm$ 8mV. This suggests that potentiation was not caused by changes in the input resistance. The amount of potentiation did not correlate with the resting potential of the neurone.

The SD of the noise during the 500 trials before tetanus was 109 $\pm$ 29 $\mu$ V and this increased slightly to 122 $\pm$ 31 $\mu$ V during the 500 trials after the tetanus. The SD of the EPSP increased more substantially, from 311 $\pm$ 77 $\mu$ V before tetanus to 398 $\pm$ 106 $\mu$ V afterwards. The SD of the noise did not correlate with either the SD of the EPSP or the estimate of quantal size,  $Q$ .

The mean half-width of the EPSP amplitude time course was 51 $\pm$ 20s and the total duration of STP ranged from less than 2 minutes to about 5 minutes. On this basis

EPSP number	Tet. or Pair	10-90%		Latency		Rest. Pot.		Noise SD		EPSP SD	
		before/after	before/after	before/after	before/after	before/after	before/after	before/after	before/after		
S1	Tet.	2.4	2.8	2.4	2.2	-85	-85	85	96	445	362
S2	Tet.	4.0	4.2	3.6	3.6	-72	-73	67	81	253	415
S3	Tet.	3.6	3.0	2.8	3.2	-89	-88	161	165	422	608
S4	Tet.	4.6	5.2	4.0	3.8	-72	-74	83	105	274	481
S5	Tet.	3.0	4.4	6.0	4.8	-77	-78	79	116	350	512
S6	Tet.	4.8	4.6	3.2	3.4	-60	-63	115	130	328	339
S7	Tet.	3.2	3.6	2.4	2.6	-66	-67	108	90	388	507
S8	Tet.	3.8	5.0	3.2	3.4	-60	-60	129	121	261	326
S9	Tet.	2.0	2.4	3.2	3.2	-72	-75	129	185	190	239
S10	Tet.	3.6	3.2	4.2	5.0	-84	-82	82	98	213	278
S11	Tet.	5.6	5.2	3.4	3.4	-69	-71	150	161	277	405
S12	Tet.	3.2	3.2	3.0	3.4	-76	-76	118	118	330	308
Average	—	3.7	3.9	3.5	3.5	-74	-74	109	122	398	311
±SD	—	±1.0	±0.9	±0.9	±0.8	±9	±8	±29	±31	±106	±77

**Table 5.1** *Properties of all 12 EPSPs that showed tetanus-induced STP* 10-90% rise times and latencies for the mean waveforms of 50 trials before and after tetanus are given in ms. Sweeps were digitised at 5kHz so these measurements are accurate to  $\pm 0.2$ ms. The **resting potential** of the neurones immediately before and immediately after tetanus are given in mV. In some cases the recorded potential has been corrected for estimated changes in the tip potential of the electrode so the absolute values are approximate only. However, assuming that no significant change in tip potential accompanied the tetanus, the relative values before and after should be accurate. The SD of the baseline **noise** and the **EPSP** during the 500 trials before and after tetanus are given in  $\mu$ V.

alone, STP was always easy to distinguish from LTP. Figure 5.1a shows the mean time course of all 12 examples of tetanus-induced STP.

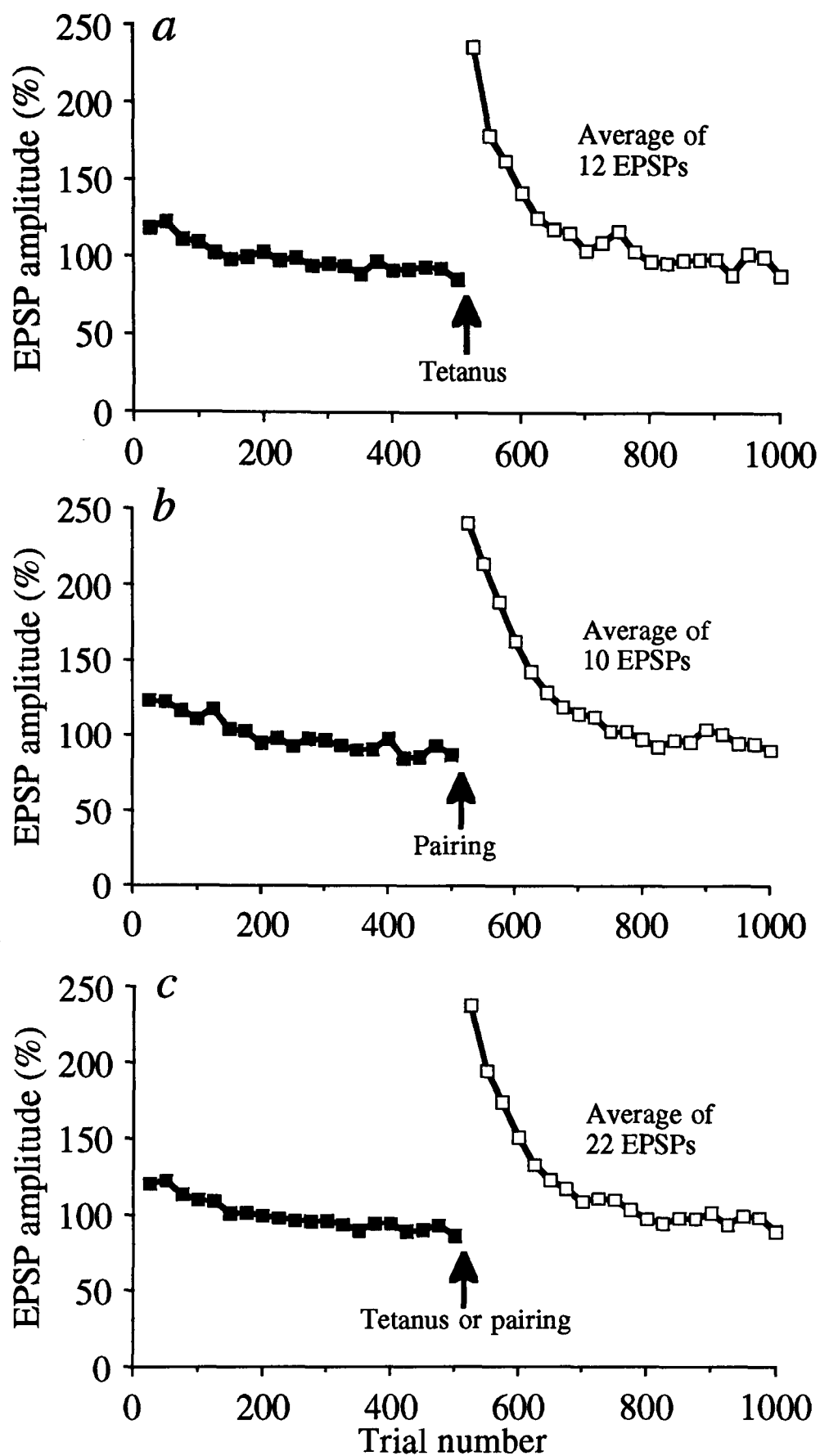
The pairing procedure never resulted in LTP but proved to be a much more reliable way of producing STP. Ten of the 22 EPSPs that I recorded in the absence of AP5 showed substantial STP. Once again, I defined this as an immediate increase in EPSP amplitude to at least 150% of the baseline value.

The average initial amplitude of these 10 EPSPs during the 50 pre-pairing trials ranged from 310 to 1042 $\mu$ V and averaged 657 $\pm$ 240 $\mu$ V. The average increase in amplitude for all 10 cells was to 255 $\pm$ 99% of the baseline value. The amount of potentiation did not correlate with the initial EPSP amplitude.

Table 5.2 shows information about EPSP rise time and latency, resting potential, and the SD of both the noise and the EPSP for each of the 10 examples of pairing-induced STP. The 10-90% rise time during the 50 trials just before pairing was 3.3 $\pm$ 1.0ms and this reduced slightly to 3.2 $\pm$ 0.9ms during the first 50 trials immediately after pairing. Two EPSPs (S20 & S22) showed changes in rise time of more than 0.4ms. The average latency before pairing was 3.2 $\pm$ 0.5ms and this was unchanged at 3.5 $\pm$ 0.5ms afterwards. No EPSPs showed changes in latency of more than 0.4ms.

The large postsynaptic depolarisation applied during pairing could have had significant effects on the input resistance of the neurone. The average resting potential immediately before pairing was -74 $\pm$ 5mV and that immediately after pairing was -76 $\pm$ 6mV. Only 3 EPSPs (S15, S21 & S22) showed changes of more than 2mV. This suggests that, on the whole, changes in input resistance were not large. However, the possibility of relatively small changes in input resistance contributing to the potentiation cannot be ruled out. I will return to this point in the next section.

The average SD of the noise during the 500 trials before pairing was 149 $\pm$ 59 $\mu$ V and this increased to 162 $\pm$ 57 $\mu$ V during the 500 trials after pairing. The SD of the EPSPs



**Figure 5.1** *STP induced by tetanus and pairing methods* (a) The average time course of 12 EPSPs that showed STP after tetanic stimulation (given at the arrow). Each point represents 25 trials evoked at 1Hz. The whole period of 1,000 trials covers about 15 minutes. Amplitudes are expressed as a percentage of the mean amplitude during the first 500 trials. On average, the initial enhancement took about 4 minutes to decline back to the baseline. (b) The average time course of 10 EPSPs that showed STP after pairing low-frequency presynaptic stimulation with postsynaptic depolarisation. Each point represents 25 trials evoked at 1Hz. The potentiation seen after pairing is very similar in size and duration to that seen after tetanic stimulation. (c) The average time course of all 22 EPSPs that showed either tetanus-induced or pairing-induced STP.

EPSP number	Tet. or Pair	10-90%		Latency		Rest. Pot.		Noise SD		EPSP SD	
		before/after	before/after	before/after	before/after	before/after	before/after	before/after	before/after		
S13	Pair	1.8	1.6	2.6	2.8	-68	-67	171	201	395	760
S14	Pair	3.0	3.4	3.0	3.4	-67	-67	92	139	265	402
S15	Pair	4.6	4.4	2.6	2.6	-74	-78	86	93	321	263
S16	Pair	2.6	2.6	3.8	3.6	-68	-70	129	152	334	365
S17	Pair	3.8	3.4	4.0	4.0	-72	-80	138	119	232	273
S18	Pair	4.6	4.2	2.8	2.8	-78	-78	190	197	293	363
S19	Pair	1.8	1.8	2.4	2.4	-72	-74	96	107	286	427
S20	Pair	3.8	4.4	3.4	3.6	-77	-78	241	238	369	595
S21	Pair	3.4	3.2	3.6	3.4	-75	-79	254	268	363	443
S22	Pair	3.8	3.0	3.4	3.4	-84	-89	96	110	266	1200
Average	—	3.3	3.2	3.2	3.2	-74	-76	149	162	312	509
±SD	—	±1.0	±0.9	±0.5	±0.5	±5	±6	±59	±57	±50	±270

**Table 5.2 Properties of all 10 EPSPs that showed pairing-induced STP** 10-90% rise times and latencies for the mean waveforms of 50 trials before and after tetanus are given in ms. Sweeps were digitised at 5kHz so these measurements are accurate to  $\pm 0.2$ ms. The resting potential of the neurones immediately before and immediately after pairing are given in mV. In some cases the recorded potential has been corrected for estimated changes in the tip potential of the electrode so the absolute values are approximate only. However, assuming that no significant change in tip potential accompanied the tetanus, the relative values before and after should be accurate. The SD of the baseline noise and the EPSP during the 500 trials before and after tetanus are given in  $\mu$ V.

increased much more, rising from  $312 \pm 50 \mu\text{V}$  before pairing to  $509 \pm 270 \mu\text{V}$  afterwards. The SD of the noise did not correlate with either the SD of the EPSPs or the estimates of quantal size,  $Q$ .

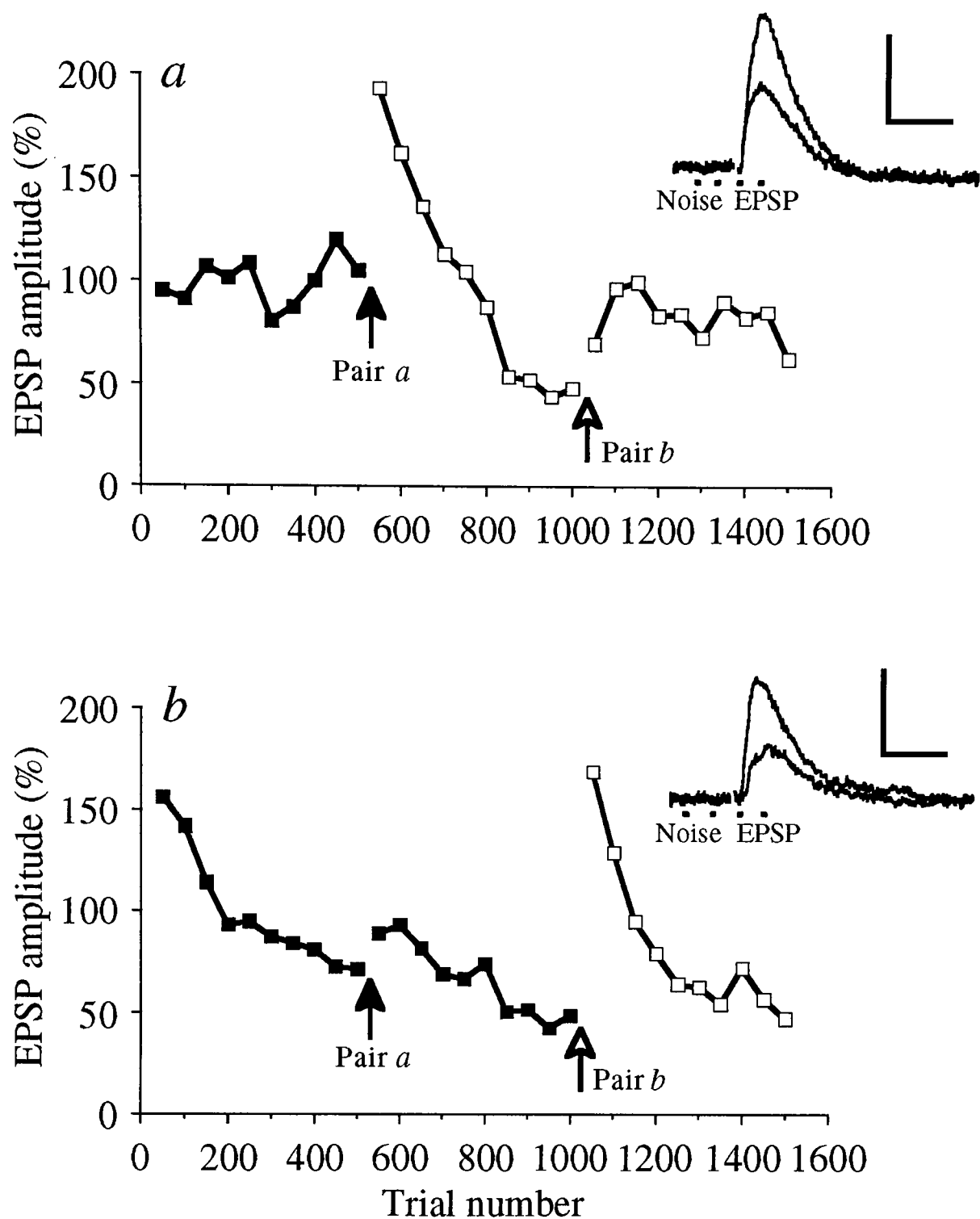
The mean half-width of the EPSP amplitude time courses was  $78 \pm 24 \text{s}$ . This was a bit longer than the average of tetanus-induced STP, although, like tetanus-induced STP, the total duration usually ranged from just under 2 minutes to about 5 minutes. One exceptional EPSP showed an initial enhancement to 510% and took almost 12 minutes to decline back to the baseline.

Figure 5.1*b* shows the mean time course of these 10 EPSPs. It is very similar to the time course of STP induced by tetanus. The rate of the decline in EPSP amplitude seems to be a bit slower immediately after pairing than immediately after tetanus. This could reflect a genuine difference in the behaviour of the EPSPs but, as I will discuss later, there might also have been a small increase in the input resistance of the cell just after pairing. Figure 5.1*c* shows the mean time course of all 22 examples of STP induced by either method. The average enhancement is to  $238 \pm 90\%$  of the baseline value.

### *5.3.2 STP induced by pairing is synapse-specific and requires NMDA receptor activity*

Figure 5.2 shows that pairing-induced STP only occurs when presynaptic activity coincides with postsynaptic depolarisation. I recorded both of these EPSPs at the same time in the same cell using a single intracellular electrode but two separate stimulating bipoles. I stimulated the inputs alternately, each at 1Hz, so that stimuli to input *a* and input *b* alternated at 0.5s intervals.

At the point marked by the filled arrow, I turned off stimulator *b* and depolarised the cell for 40 trials of stimulator *a*. After this I switched off the depolarising current and switched stimulator *b* on again. Only EPSP *a*, which had been paired with the



**Figure 5.2 Pairing-induced STP is synapse-specific** (a) The time course of an EPSP that showed STP when I paired low-frequency presynaptic stimulation with postsynaptic depolarisation at trial 500 (filled arrow). Each point represents 50 trials expressed as a percentage of the mean during the first 500 trials. During the postsynaptic depolarisation at trial 1,000 (unfilled arrow) I temporarily stopped stimulation of this EPSP and there was no STP. The small increase in EPSP amplitude seen might be due to a small increase in input resistance. The inset shows the average EPSP waveform of 50 trials immediately before and after pairing. The stimulus artefact has been removed. Horizontal bars mark the zones used for measuring the amplitudes of the noise and the EPSP during each sweep. Scale bars: 1,000 $\mu$ V, 20ms. (b) A second EPSP recorded in the same cell at the same time using a separate stimulating bipole. In this case, I did not pair the postsynaptic depolarisation at trial 500 (filled arrow) with presynaptic activity and there was only a small enhancement, possibly caused by a rise in input resistance. However, when I paired the depolarisation at trial 1,000 (unfilled arrow) with presynaptic activity, this did result in STP. The inset shows the average EPSP waveform of 50 trials immediately before and after pairing. Scale bars: 1,000 $\mu$ V, 20ms.

postsynaptic depolarisation, showed STP. To demonstrate that EPSP *b* could show STP, I carried out the reverse procedure, switching off stimulator *a* and depolarising the cell once again. This time STP was induced only in EPSP *b*. This shows that pairing-induced STP, like LTP, is associative and synapse-specific.

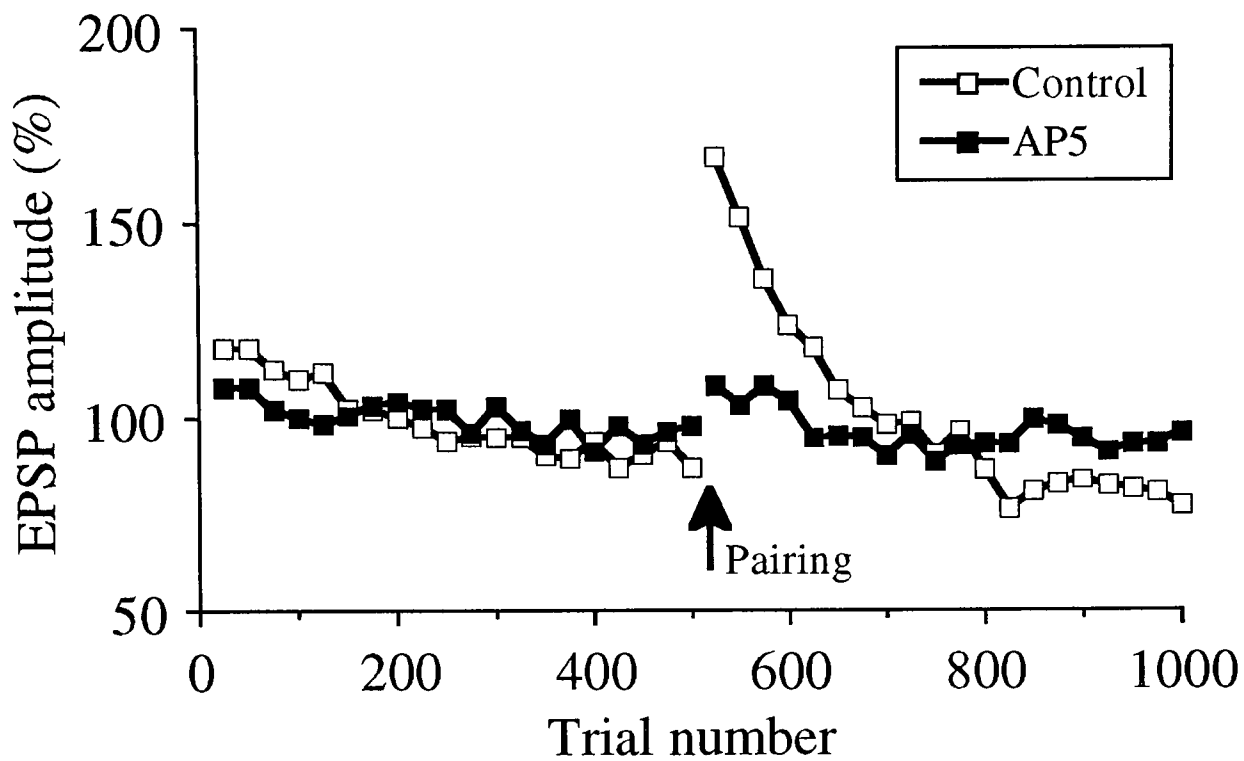
Both EPSPs showed small enhancements even when their activity was not paired with postsynaptic depolarisation. This was most likely caused by small increases in input resistance. The fact that the majority of the potentiation is specific to the paired input, however, indicates that most of the potentiation was not caused by changes in non-specific factors such as input resistance.

Figure 5.3 shows that AP5, an antagonist of the NMDA-type glutamate receptor, blocks STP induced by the pairing method. The open squares show the mean amplitude of all 22 EPSPs that I subjected to pairing, 10 of which showed STP. Filled squares show the mean amplitude for the 12 other EPSPs that I incubated in ACSF containing 100 $\mu$ M AP5. None of these showed STP. However, they did, on average, show a small enhancement. Once again, this was probably the result of small increases in input resistance after pairing.

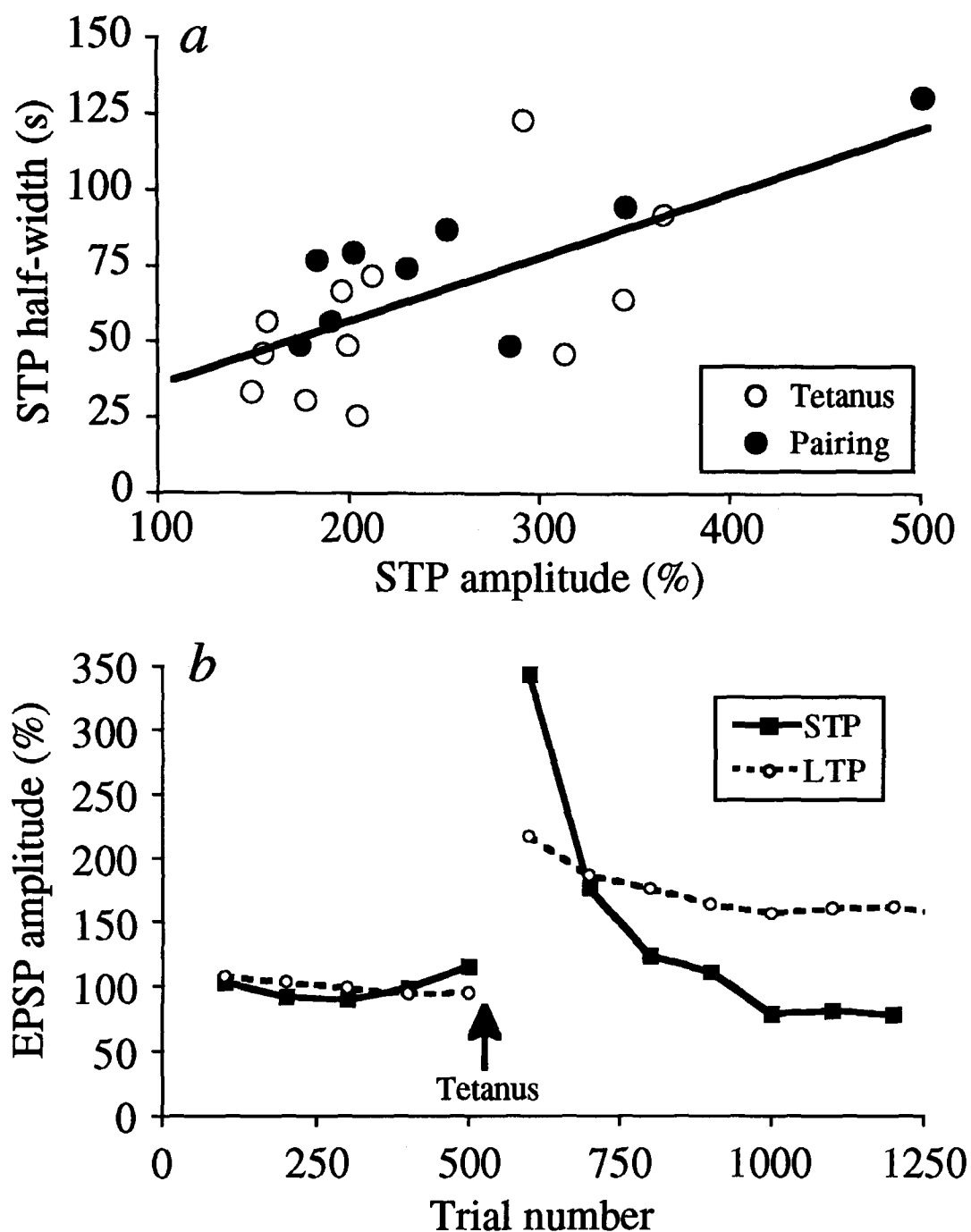
### *5.3.3 Large STP lasts longer than small STP but is distinct from LTP*

Figure 5.4*a* shows the correlation between the half-width of the STP time course (a measure of its duration) and its amplitude immediately after induction by tetanus (open circles) or pairing (filled circles). Half-widths ranged from 26 to 131s and STP with a higher peak amplitude tends to last longer.

This could suggest that LTP is a simple extension of STP and that its longer duration is simply a result of a larger initial amplitude. But this is not so. Figure 5.4*b* shows the time course of the example of tetanus-induced STP that showed the largest enhancement (open squares) superimposed on the average time course of all 13 examples of tetanus-induced LTP (filled circles) described in Chapter Four. The STP



**Figure 5.3 *Pairing-induced STP requires NMDA receptor activity*** The average time course of all 22 EPSPs that I subjected to the pairing method (open squares), of which 10 showed STP. Each point represents 50 trials expressed as a percentage of the mean during the first 500 trials. The mean amplitude during the 50 trials immediately after pairing was  $167 \pm 91\%$ . I recorded a further 12 EPSPs in ACSF containing  $100 \mu\text{M}$  of the NMDA receptor antagonist, AP5. I subjected these to the same pairing procedure but none of them showed STP (closed squares). In this case, the average amplitude during the first 50 trials after pairing was  $109 \pm 23\%$ . This small increase might be due to a rise in input resistance.

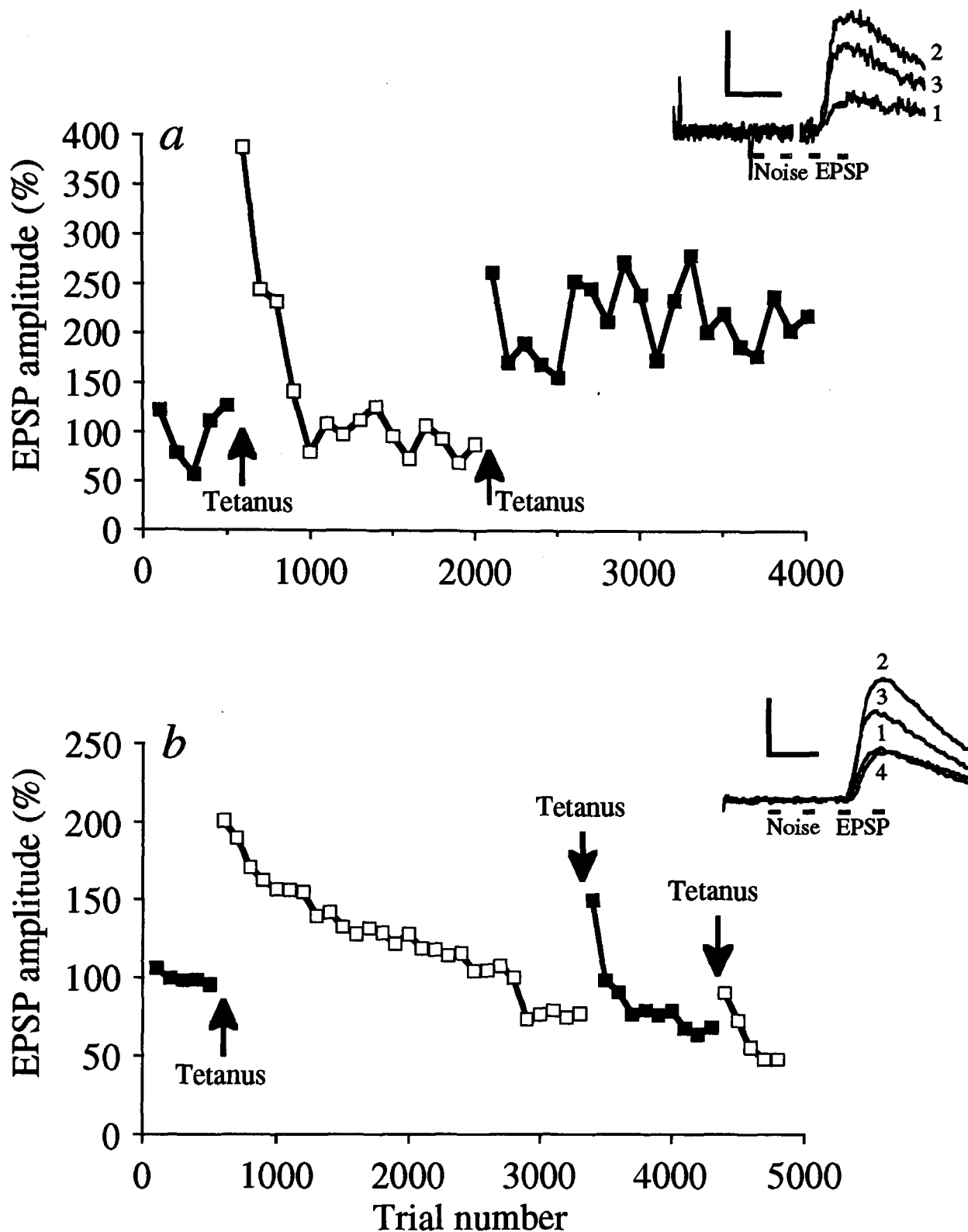


**Figure 5.4 Large STP lasts longer than small STP but is distinct from LTP** (a) There is a significant correlation between STP amplitude and the half-width of its time course. The STP amplitude is the mean amplitude during the first 50 trials after STP induction, expressed as a percentage of the mean during the 50 trials before induction. The half-width is the time taken for the EPSP amplitude to fall from this peak value to a point half-way between the peak and the baseline. I measured this by hand directly from graphs of individual EPSP time courses. For both the tetanus (open circles) and pairing (closed circles) methods, EPSPs that show a greater initial potentiation tended to have a longer half-width (correlation coefficient for all points,  $r=0.65$ ,  $p<0.001$ ). (b) Even STP that shows very large initial enhancement does not last as long as LTP. The example of tetanus-induced STP that showed the greatest potentiation (open squares) was initially much larger than the average for the 13 examples of LTP described in Chapter Four (filled circles) but falls back to baseline much more rapidly. Each point represents 100 trials expressed as a percentage of the mean amplitude during the 500 pre-tetanus trials.

example shows a far greater initial amplitude than the average for LTP and yet has a much shorter duration.

The same point is more convincingly illustrated in Figure 5.5a, which shows the mean time course of an EPSP that showed first STP and then LTP in response to two successive tetani. The initial amplitude of the STP is larger than that of LTP but its duration is much shorter (about 6 minutes). Other examples of STP showed quite a modest increase in EPSP amplitude, so neither is it true to say that STP always shows larger initial enhancements than LTP. Therefore, the amount of potentiation immediately after induction does not seem to be a reliable indicator of whether a particular EPSP will go on to show STP or LTP.

During my LTP studies, I also saw brief enhancements resembling STP when I gave tetanic stimulation to synapses that had already expressed LTP and returned to baseline. Figure 5.5b shows an example. In this case, LTP lasted for just under 40 minutes. Once this had decayed, I applied another tetanus. This produced a small, brief enhancement. A third tetanus resulted in an even smaller, even more transient change. I saw similar behaviour in both of two other EPSPs for which I tried this. The potentiation always got smaller and shorter with successive tetani. These brief enhancements may be comparable to STP. Alternatively they could be a form of 'stunted' LTP and indicate that these synapses, perhaps fatigued by prolonged 1Hz stimulation, could no longer express increases in synaptic strength as effectively as before. Because of this uncertainty, I did not include any of these examples in my analysis of STP. I also excluded the EPSP illustrated in Figure 5.5a, which formed part of the LTP study in Chapter Four.



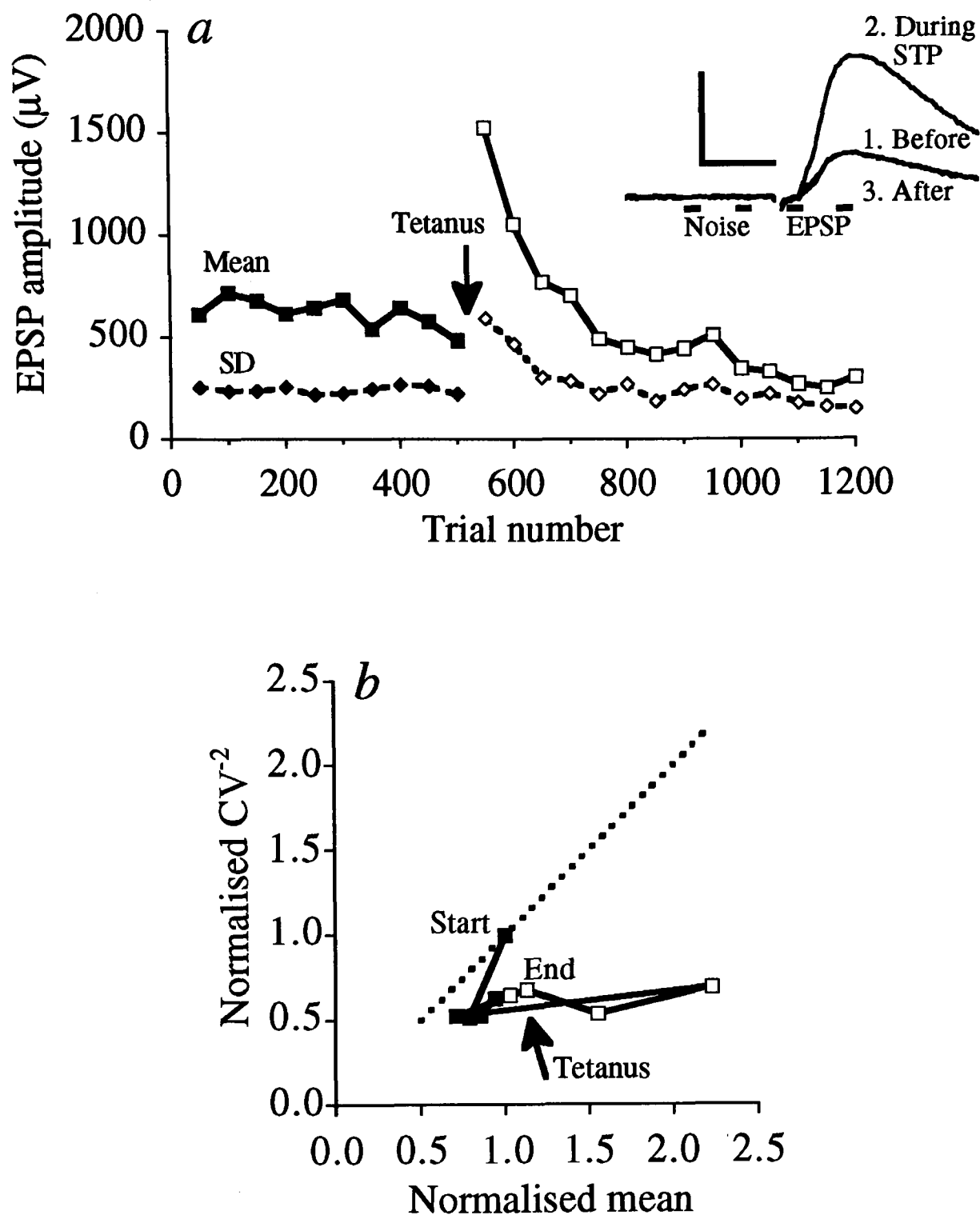
**Figure 5.5** *EPSPs that showed STP and LTP* (a) An EPSP that showed STP followed by LTP. A tetanus given after 500 trials produced STP. A similar tetanus given after a total of 2,000 trials resulted in LTP. The initial amplitude of the STP is higher than for LTP but its duration is much shorter, falling back to baseline in about 6 minutes. Each point represents 100 trials stimulated at 1Hz. The whole recording period shown lasted just over an hour. The inset shows average EPSP waveforms for 50 trials before the first tetanus (1), 50 trials immediately after the first tetanus (2) and 50 trials immediately after the second tetanus (3). Horizontal bars mark the zones used for measuring the amplitudes of the noise and the EPSP during each sweep. Scale bars: 200 $\mu$ V, 10ms. (b) An EPSP that showed LTP after a tetanus at trial 500. After the EPSP amplitude had returned to baseline, I applied two further tetani, one after 3,400 trials and the other after 4,400 trials. In both cases, there was a brief enhancement that lasted for 2-5 minutes. However, the amounts of potentiation were quite small. The size and duration of the enhancements decrease with successive tetani. The inset shows average EPSP waveforms for 50 trials before the first tetanus (1), 50 trials immediately after the first tetanus (2), 50 trials immediately after the second tetanus (3) and 50 trials immediately after the third tetanus (4). Scale bars: 500 $\mu$ V, 10ms.

### 5.3.4 Examples of postsynaptic STP

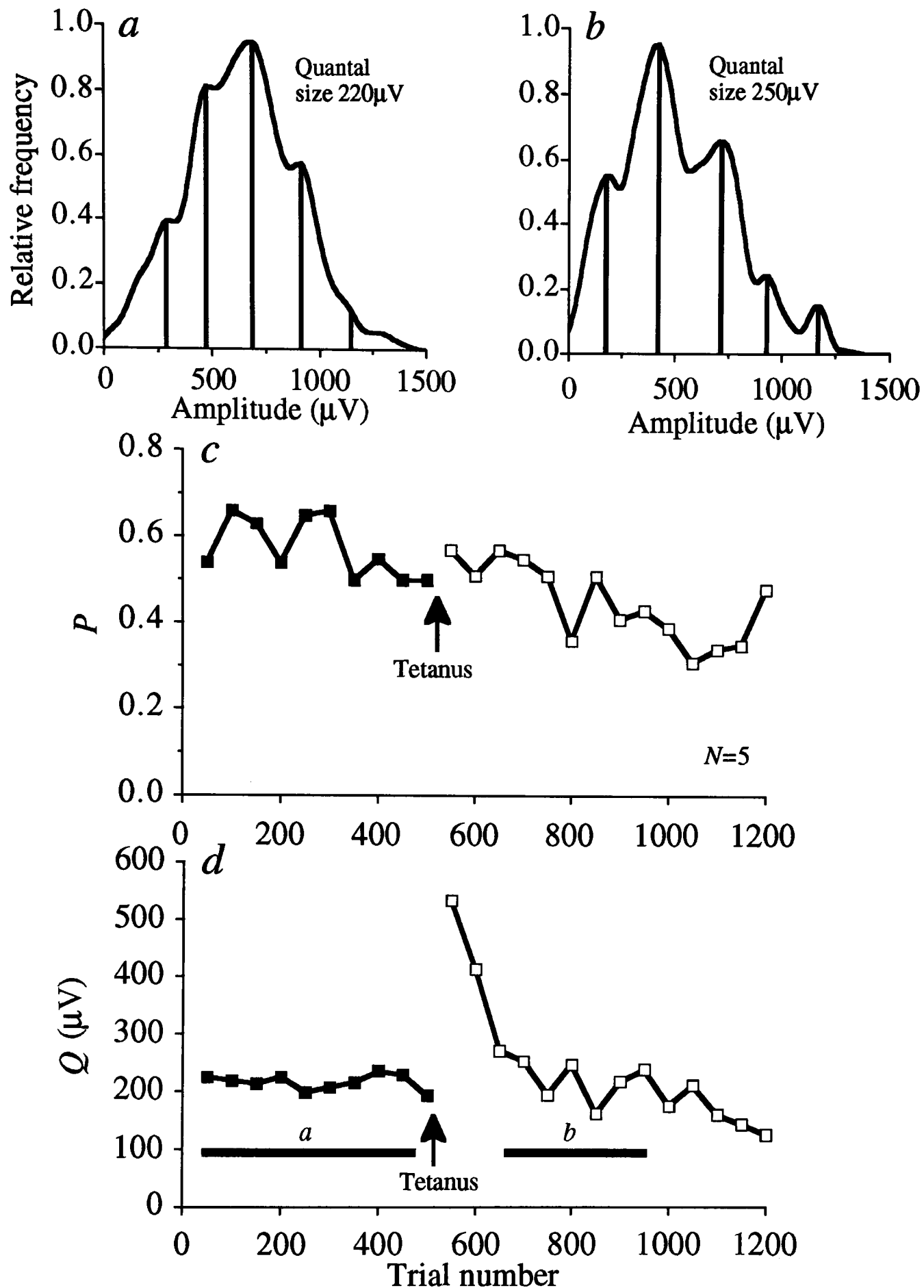
Figure 5.6 shows data from an EPSP (S2) that showed STP after a tetanus. The initial rise in amplitude was roughly three-fold and this decayed back to baseline in about 4 minutes (Figure 5.6a). The SD also increased substantially so the  $1/CV^2$  graph is almost horizontal (Figure 5.6b). This type of analysis makes certain assumptions about the nature of synaptic transmission that I have discussed in general terms in Chapter Two and in relation to my data in Chapter Four. Within these assumptions, this type of trajectory suggests that the change is mainly or wholly in quantal size and there is unlikely to have been any substantial change in the number of release sites or their release probabilities.

Figure 5.7 shows a quantitative quantal analysis of this EPSP. A histogram composed of 460 pre-tetanus trials (selected from the 500 available) had peaks with a mean spacing of about  $220\mu\text{V}$  (Figure 5.7a). Assuming that transmission obeys simple binomial release statistics, this value together with the mean and SD of the histogram can be used to calculate the number of release sites ( $N$ ). In this case,  $N=5$  to the nearest whole number. Making the further assumption that  $N$  is the same for all the recorded data, 'constant  $N$  analysis' (see Chapter Two) shows that the release probability ( $P$ ) declines gradually throughout the period but changes little following the tetanus (Figure 5.7c). Most of the potentiation was due to an increase in quantal size ( $Q$ ) (Figure 5.7d). A histogram of 300 post-tetanus trials shows a quantal size of  $250\mu\text{V}$  (Figure 5.7b), which agrees well with the value of  $Q$  estimated by constant  $N$  analysis for the same period of data (bar  $b$  in Figure 5.7d).

Using histograms to confirm the results of constant  $N$  analysis is less satisfactory in the case of STP than it is for the LTP examples described in Chapter Four. Peaky histograms composed of a sufficient number of trials were practically impossible to find during the time that STP was being expressed. This is not surprising since the



**Figure 5.6** An EPSP that showed a predominantly postsynaptic form of tetanus-induced STP (a) The mean and SD of the EPSP amplitude for 1,200 trials (about 19 minutes) of recording (S2). Each point represents 50 trials. Closed squares show pre-tetanus data and open squares, post-tetanus data. Both the mean and the SD increased about three-fold. The inset shows the average EPSP waveforms of the last 50 trials before the tetanus (1), the first 50 trials during STP (2) and 50 trials just after the EPSP amplitude had returned to baseline (3). (b) A  $1/CV^2$  graph incorporating the 250 trials immediately before STP induction and the 250 trials immediately afterwards. Each point represents 50 trials. Closed squares show data from before the tetanus while open squares show data from after the tetanus. The trajectory is almost horizontal and lies well below the diagonal (dotted line).



**Figure 5.7** *Quantal analysis of the EPSP shown in Figure 5.6* (a) A histogram plotted from 460 pre-tetanus trials (selected from the 500 available). The mean peak spacing is  $220\mu\text{V}$ . (b) A histogram plotted from 300 post-tetanus trials (selected from the 700 available) shows a quantal size of  $250\mu\text{V}$ . (c) Assuming simple binomial release statistics, the value of  $N=5$  (to the nearest integer) for the region of data used to construct the pre-tetanus histogram in *a*. Using this value of  $N$  for all of the recorded data, constant  $N$  analysis shows that  $P$  declined gradually during the recording period but there was no significant increase after the tetanus. (d) Similar calculations for  $Q$  show a much more substantial change that can account for most or all of the change in EPSP amplitude. Bars mark the regions of data used to plot the histograms in *a* and *b*. The estimated value of  $Q$  for the region of data used to construct the post-tetanus histogram in *b* agrees reasonably well with the quantal size measured from this histogram.

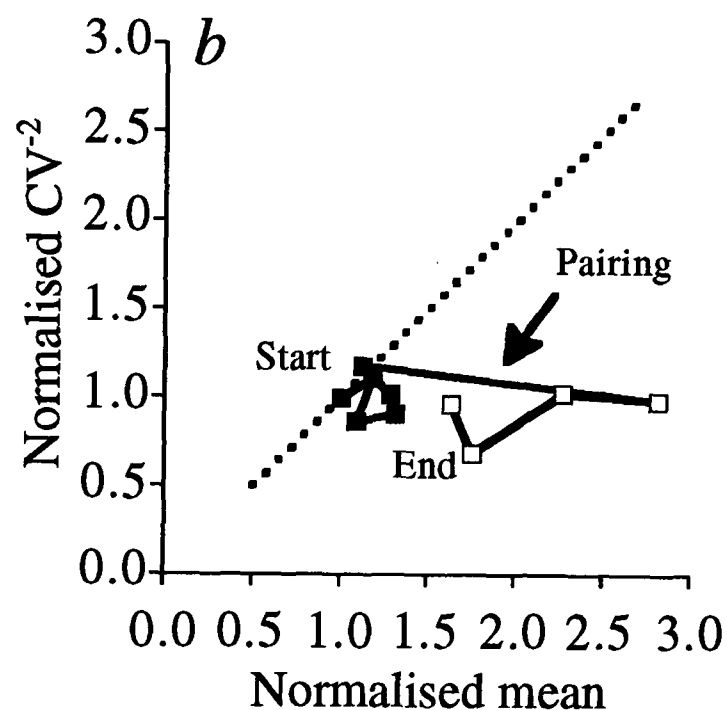
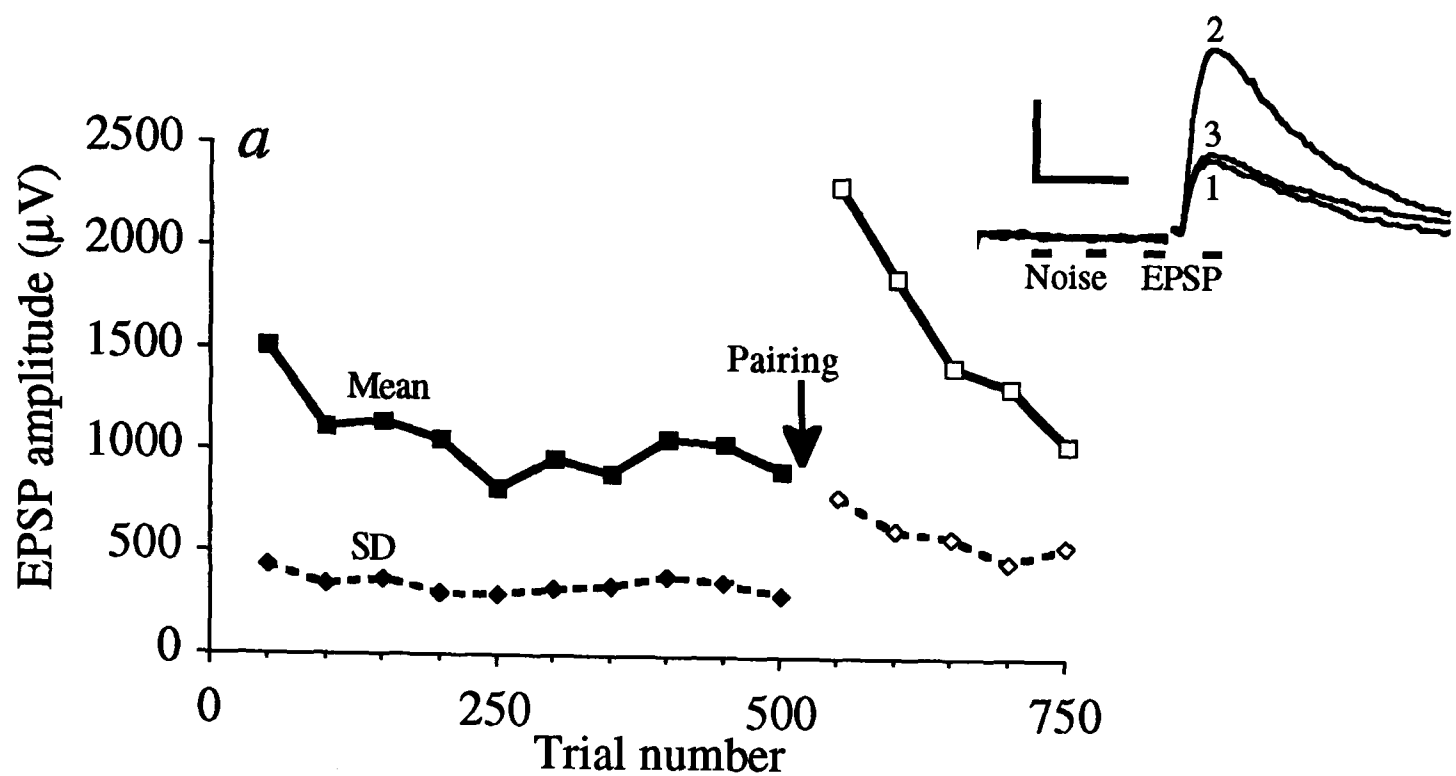
EPSP mean is changing rapidly. The best I could do was to look for histograms in regions of data recorded after STP had subsided and use these to confirm that the results of constant  $N$  analysis are reasonably accurate for those periods. This shows that the assumptions implicit in this method—in particular, the assumption that  $N$  is constant—hold true for these later times. This makes it unlikely that there has been a substantial and long-lasting increase in  $N$  but the possibility of a brief increase in  $N$  during STP cannot be ruled out .

In defence of using constant  $N$  analysis in this way, it is important to note that although the absence of histograms from the period of potentiation make the assumption of constant  $N$  harder to justify, it also makes this form of analysis indispensable. Without using this kind of analysis method, there would be no way of carrying out a quantal analysis of STP.

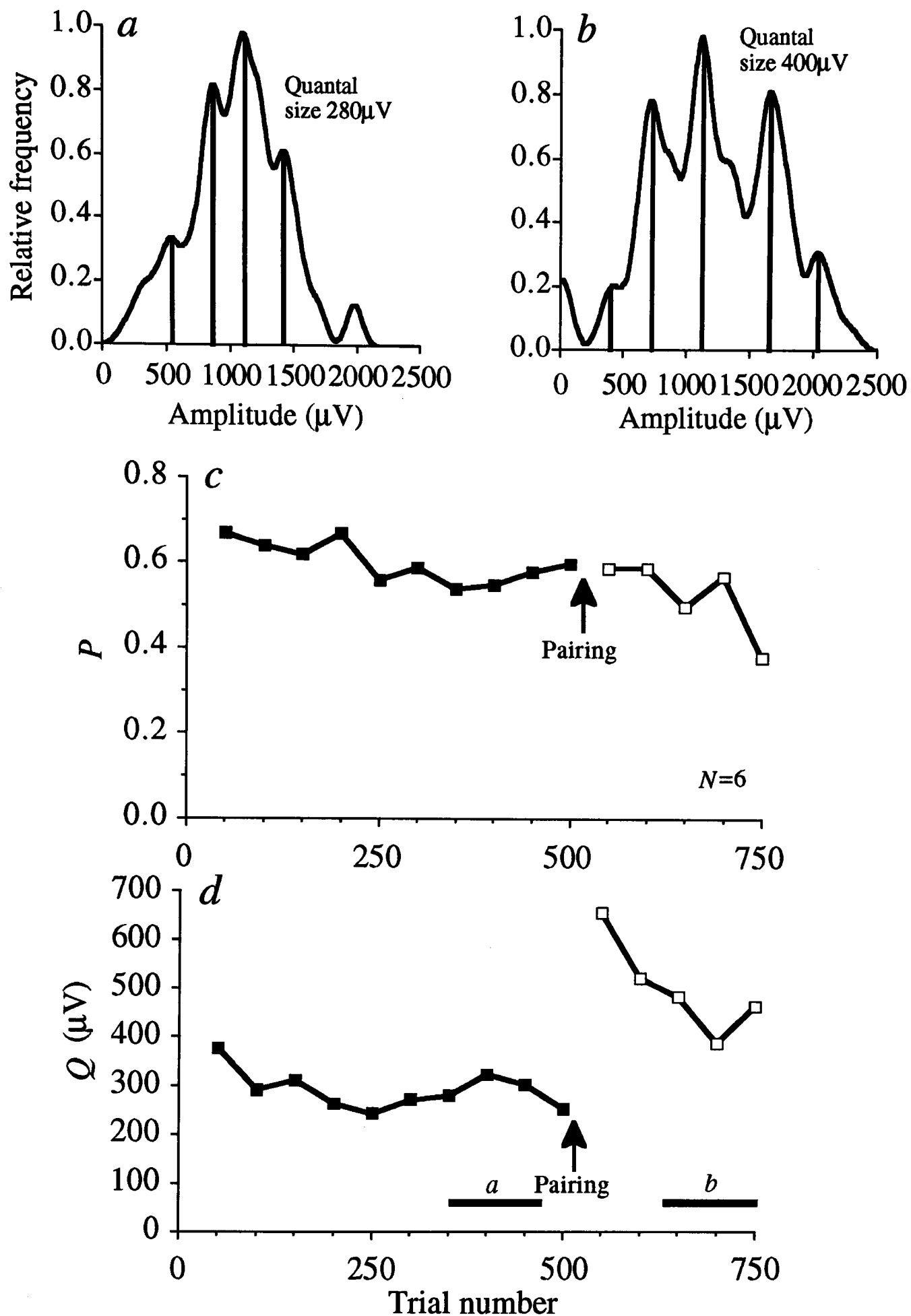
With these reservations, the three analysis methods I have described are all consistent with the idea that this EPSP showed STP that is largely or wholly caused by a increase in quantal size. This is usually interpreted as a postsynaptic change (see Chapter One).

Figure 5.8 shows an example of STP induced by the pairing method (S13). The potentiated response reaches about 2.5 times that of the unpotentiated one (Figure 5.8a) and declined quite rapidly until the impalement was lost at trial 750. Once again, the  $1/CV^2$  graph has an almost horizontal trajectory (Figure 5.8b), which suggests a change predominantly in quantal size.

A histogram from the period before pairing has a quantal size of  $280\mu V$  (Figure 5.9a). Assuming simple binomial statistics, the mean and SD for this period gives  $N=6$ . Using this value of  $N$  for all of the data, constant  $N$  analysis calculates that, once again,  $P$  changes little during the experiment and shows no rise in response to the pairing procedure (Figure 5.9c). In contrast,  $Q$  increases substantially and accounts for most or all of the potentiation (Figure 5.9d). A histogram for the period after



**Figure 5.8** An EPSP that showed a predominantly postsynaptic form of pairing-induced STP (a) The mean and SD of the EPSP amplitude for 750 trials (about 12 minutes) of recording (S13), after which the impalement was lost. Each point represents 50 trials. The mean and SD both increased by about 2.5 times. The inset shows average EPSP waveforms for 50 trials immediately before (1) and after (2) pairing and the last 50 trials recorded (3). Scale bars: 1,000 $\mu$ V and 10ms. (c) A  $1/CV^2$  graph incorporating the 300 trials immediately before STP induction and the 200 trials immediately afterwards. Each point represents 50 trials. The trajectory is almost horizontal and lies well below the diagonal (dotted line).



**Figure 5.9** *Quantal analysis of the EPSP shown in Figure 5.8* (a) A histogram plotted from 120 trials recorded before pairing. The quantal size, is  $280\mu\text{V}$ . (b) A histogram plotted from 120 trials after pairing has a quantal size of  $400\mu\text{V}$ . (c) Assuming simple binomial release statistics,  $N=6$  (to the nearest integer) for the region of data used to construct the histogram in a. Constant  $N$  analysis shows that  $P$  declined gradually during the recording period but there was no significant increase after the pairing. (d) Similar calculations for  $Q$  show a much more substantial increase that can account for most or all of the change in EPSP amplitude. Bars mark the regions of data used to plot the histograms in a and b. The estimated value of  $Q$  for the region of data used to construct the histogram in b agrees well with the quantal size measured from this histogram.

pairing (Figure 5.9*b*) has a quantal size of  $400\mu\text{V}$ , which agrees well with the constant  $N$  analysis results for the same period (bar  $b$  in Figure 5.9*d*).

Once again, the three analysis methods agree reasonably well and attribute most of the change to an increase in quantal size, which is normally interpreted as a postsynaptic change (see Chapter One).

### 5.3.5 *An example of presynaptic STP*

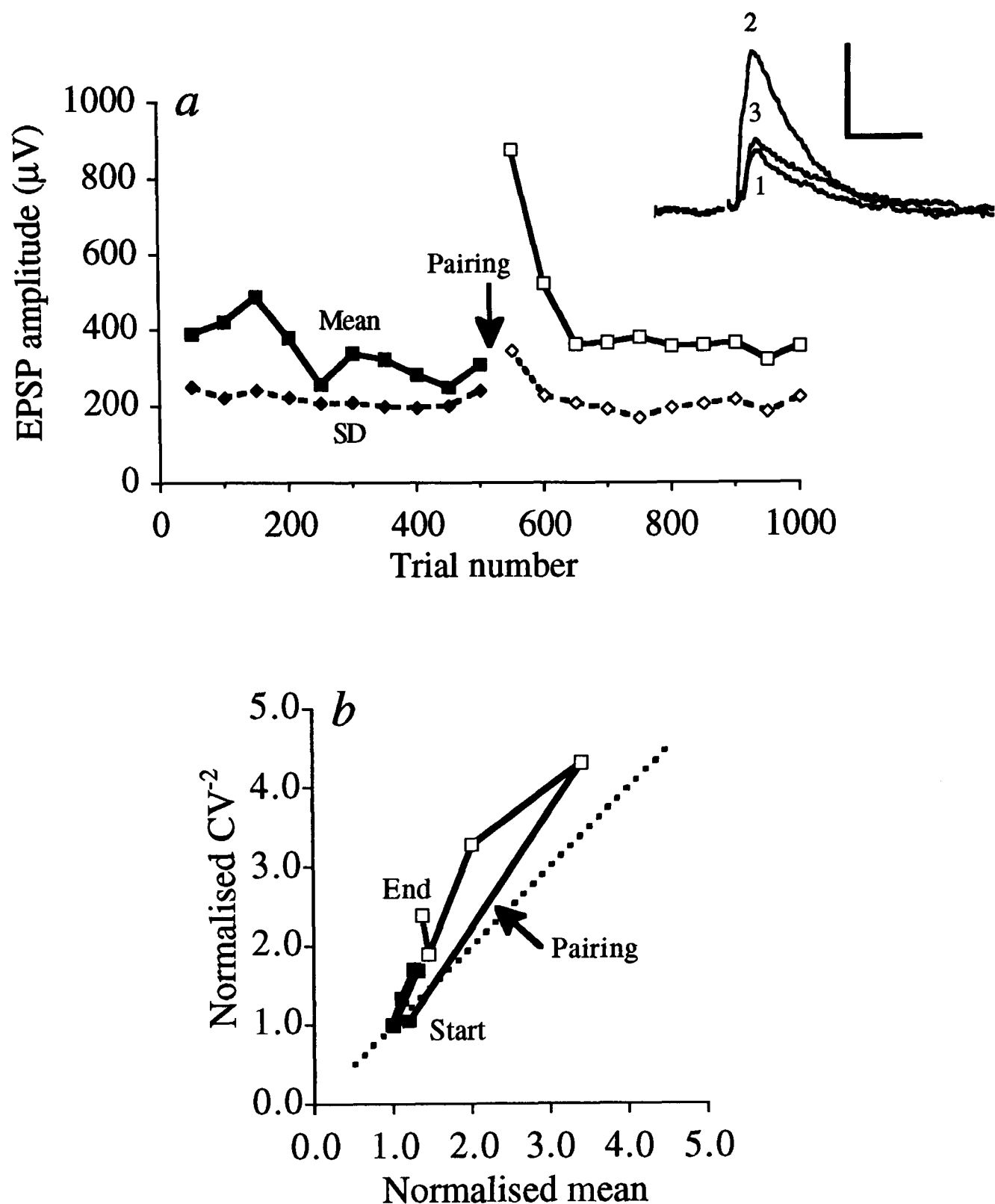
Figure 5.10 shows another example of STP induced by the pairing method (S17). The initial increase is almost three-fold (Figure 5.10*a*). This time the increase in SD is more modest and, as a result, the  $1/CV^2$  graph has a steep trajectory that lies above the diagonal (Figure 5.10*b*). This suggests an increase in release probability, possibly accompanied by an increase in the number of release sites. A large increase in quantal size is unlikely.

A histogram plotted from data recorded before the pairing has a quantal size of  $180\mu\text{V}$  (Figure 5.11*a*). This gives a value of  $N=7$ . Constant  $N$  analysis calculates that there was a large increase in  $P$  during the period of STP (Figure 5.11*c*) but little change in  $Q$  (Figure 5.11*d*). A histogram plotted from data recorded after the pairing has a quantal size of  $140\mu\text{V}$  (Figure 5.11*b*), which agrees reasonably well with the estimate made by constant  $N$  analysis for the same period (bar  $b$  in Figure 5.11*d*).

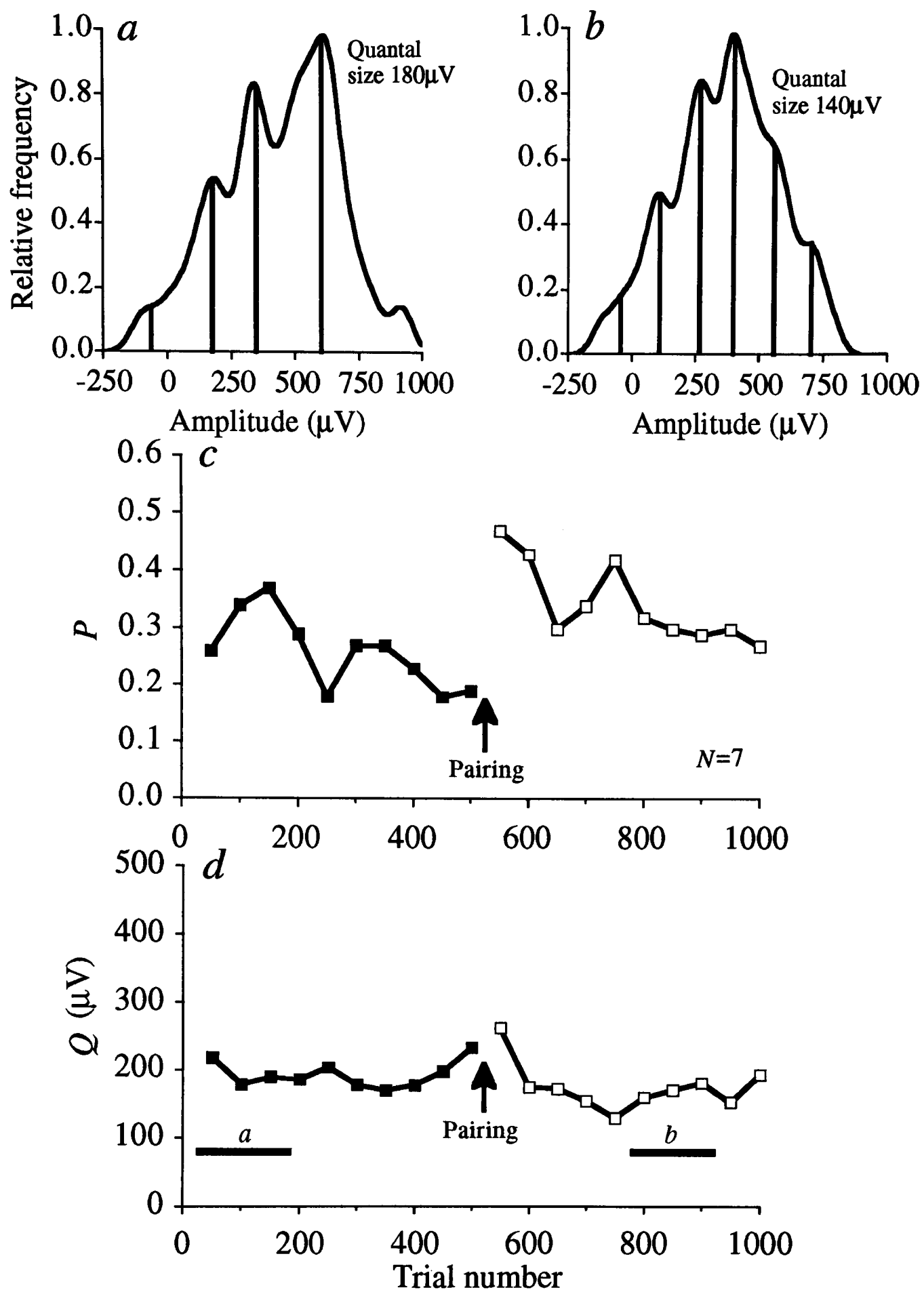
Once again, the analysis methods all agree quite well but this time the increase was predominantly in release probability rather than quantal size. This is normally interpreted as a presynaptic change (see Chapter One).

### 5.3.6 *Quantal analysis results for STP and LTP are very similar*

I analysed the other 19 EPSPs in the same way. Tables 5.3 and 5.4 give the amplitude, duration half-width,  $1/CV^2$  graph gradient, and values of  $N$ ,  $P$  and  $Q$  for



**Figure 5.10** *An EPSP that showed a predominantly presynaptic form of pairing-induced STP* (a) The mean and SD of the EPSP amplitude for 1,000 trials (about 16 minutes) of recording (S17). Each point represents 50 trials. The mean increased almost three-fold and the SD went up by a smaller amount. The inset shows average EPSP waveforms for 50 trials immediately before and after pairing. Scale bars: 500 $\mu\text{V}$ , 20ms. (c) A  $1/\text{CV}^2$  graph incorporating the 300 trials immediately before STP induction and the 200 trials immediately afterwards. Each point represents 50 trials. The trajectory is steep and above the diagonal (dotted line).



**Figure 5.11** *Quantal analysis of the EPSP shown in Figure 5.10* (a) A histogram plotted from 160 pre-tetanus trials. The quantal size, is  $180\mu\text{V}$ . (b) Another histogram, this time plotted from 140 post-tetanus trials, has a quantal size of  $140\mu\text{V}$ . (c) Assuming simple binomial release statistics, the value of  $N$  for the region of data used to construct the histogram in *a* is (to the nearest integer)  $N=7$ . Using this, constant  $N$  analysis showed that there was a large increase in  $P$  after the pairing. This can account for most or all of the change in the EPSP amplitude. (d) Similar calculations for  $Q$  show a gradual decline but no substantial change after the pairing. Bars mark the regions of data used to plot the histograms in *a* and *b*. The estimated value of  $Q$  for the region of data used to construct the histogram in *b* agrees reasonably well with the quantal size measured from this histogram.

EPSP number	Amplitude		Duration HW (s)	1/CV <sup>2</sup> grad.	N	P		Q	
	before/after					before/after		before/after	
S1	496	989	49	0.47	8	0.29	0.41	223	298
S2	487	1525	46	0.18	5	0.50	0.68	193	450
S3	897	3269	92	1.57	6	0.53	0.86	283	636
S4	745	2560	64	-0.09	8	0.70	0.71	133	455
S5	678	1970	23	2.77	10	0.34	0.73	203	269
S6	535	948	31	0.42	6	0.58	0.50	167	353
S7	835	1311	57	1.00	7	0.53	0.58	230	321
S8	453	674	33	0.47	5	0.37	0.42	246	324
S9	218	461	72	1.30	4	0.38	0.53	148	218
S10	396	776	67	-0.06	4	0.56	0.56	181	363
S11	626	1276	26	-0.24	4	0.58	0.63	268	515
S12	500	774	46	1.89	5	0.41	0.59	242	265
Average	572	1378	51	0.81	6.0	0.48	0.60	210	372
±SD	±185	±804	±20	±0.88	±1.8	±0.12	±0.13	±45	±115

**Table 5.3 Results of quantal analysis for all 12 EPSPs that showed tetanus-induced STP** Mean amplitudes for the 50-trial periods before and after tetanus are given in  $\mu\text{V}$ . Duration half-widths are the approximate time it took the EPSP amplitude to fall from its peak to a value half-way between the peak and the baseline.  $1/\text{CV}^2$  graph gradients are for graphs binned at 50 trials, and normalised to the last 50 trials before tetanus. The gradient is the change in  $1/\text{CV}^2$  that accompanied STP induction divided by the change in mean over the same period. A value of zero indicates a horizontal trajectory, 1 indicates a trajectory along the diagonal and a value  $>1$  indicates a trajectory steeper than the diagonal.  $N$ ,  $P$  and  $Q$  (in  $\mu\text{V}$ ) are binomial parameters calculated using constant  $N$  analysis. In the cases of  $P$  and  $Q$ , mean values for the 50-trials immediately before and after tetanus are shown.

EPSP number	Amplitude		Duration HW (s)	1/CV <sup>2</sup> grad.	N	P		Q	
	before/after					before/after		before/after	
S13	920	2312	87	-0.05	6	0.62	0.59	248	649
S14	503	911	—	1.38	4	0.41	0.65	301	353
S15	417	960	74	0.08	6	0.52	0.55	133	293
S16	666	1265	57	0.57	7	0.46	0.58	208	309
S17	310	879	49	1.67	7	0.20	0.53	230	237
S18	726	1262	49	0.43	5	0.69	0.74	219	343
S19	685	1383	80	0.17	8	0.59	0.64	145	272
S20	927	1699	77	0.40	7	0.53	0.60	251	404
S21	374	1290	95	1.36	6	0.21	0.53	303	406
S22	1042	5311	131	0.36	8	0.76	0.89	172	744
Average	657	1727	78	0.64	6.4	0.50	0.63	221	401
±SD	±240	±1261	±24	±0.58	±1.2	±0.18	±0.11	±56	±158

**Table 5.4 Results of quantal analysis for all 10 EPSPs that showed pairing-induced STP** Mean amplitudes for the 50-trial periods before and after pairing are given in  $\mu\text{V}$ . Duration half-widths are the approximate time it took the EPSP amplitude to fall from its peak to a value half-way between the peak and the baseline. In the case of S14, insufficient post-pairing trials were collected to determine this value. 1/CV<sup>2</sup> graph gradients are for graphs binned at 50 trials, and normalised to the last 50 trials before pairing. The gradient is the change in 1/CV<sup>2</sup> that accompanied STP induction divided by the change in mean over the same period. A value of zero indicates a horizontal trajectory, 1 indicates a trajectory along the diagonal and a value >1 indicates a trajectory steeper than the diagonal. Values for N, P and Q (in  $\mu\text{V}$ ) are binomial parameters calculated using constant N analysis. In the cases of P and Q, mean values for the 50-trials immediately before and after pairing are shown.

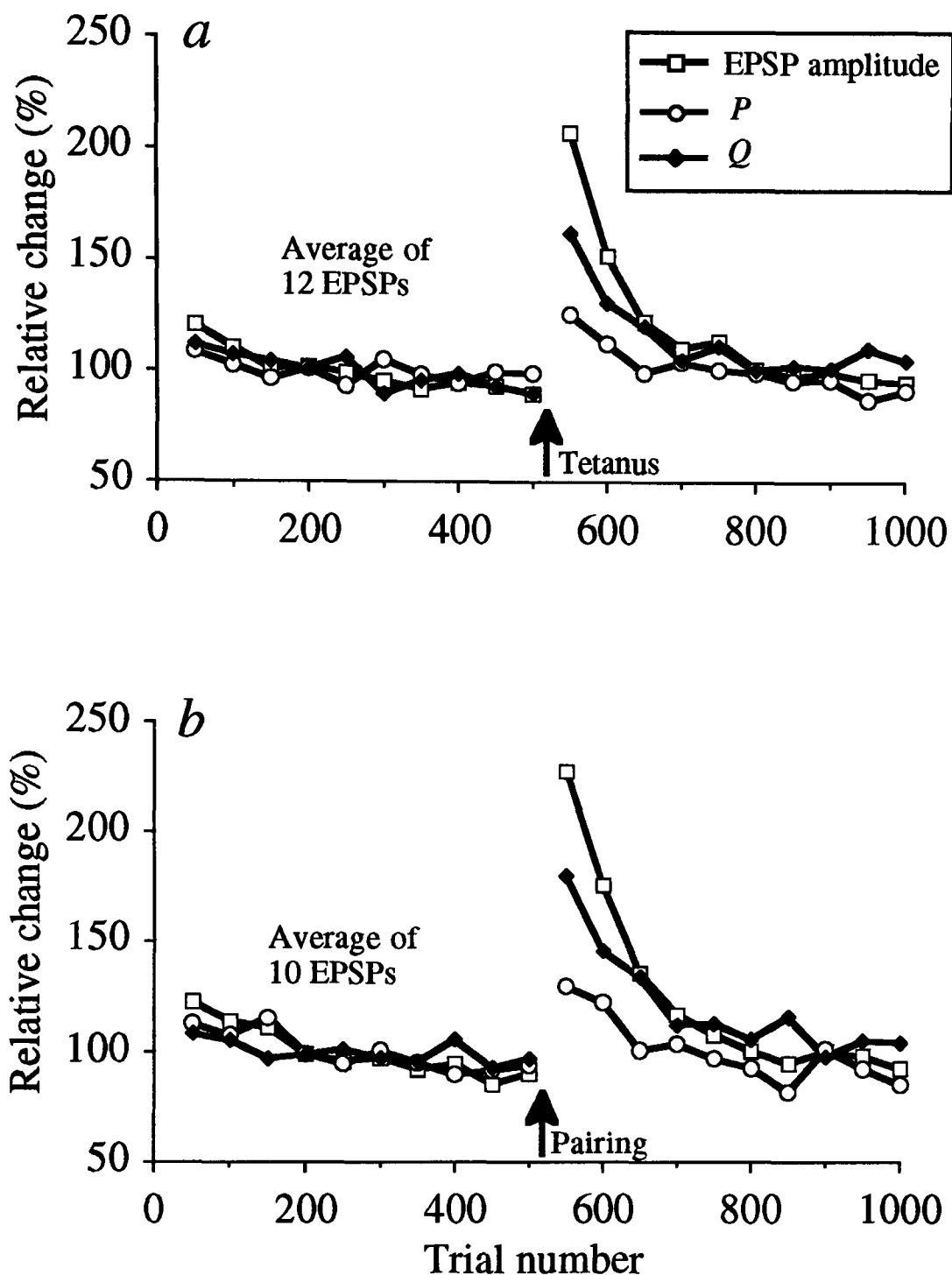
the EPSPs that showed tetanus-induced and pairing-induced STP. Values of  $N$  for all 22 EPSPs were in the range 4-10 and averaged  $6.2 \pm 1.6$ . Mean  $P$  values during the 50 trials before STP induction ranged from 0.20 to 0.76 and averaged  $0.49 \pm 0.15$ . Initial values of  $Q$  ranged from 133 to  $316 \mu\text{V}$  and averaged  $215 \pm 50 \mu\text{V}$ .

The quantal analysis results showed that the changes underlying STP can vary greatly between individual EPSPs. Some EPSPs show predominantly presynaptic or postsynaptic changes while others show a more equal mixture of both. These results are almost identical to the ones I obtained for LTP in Chapter Four.

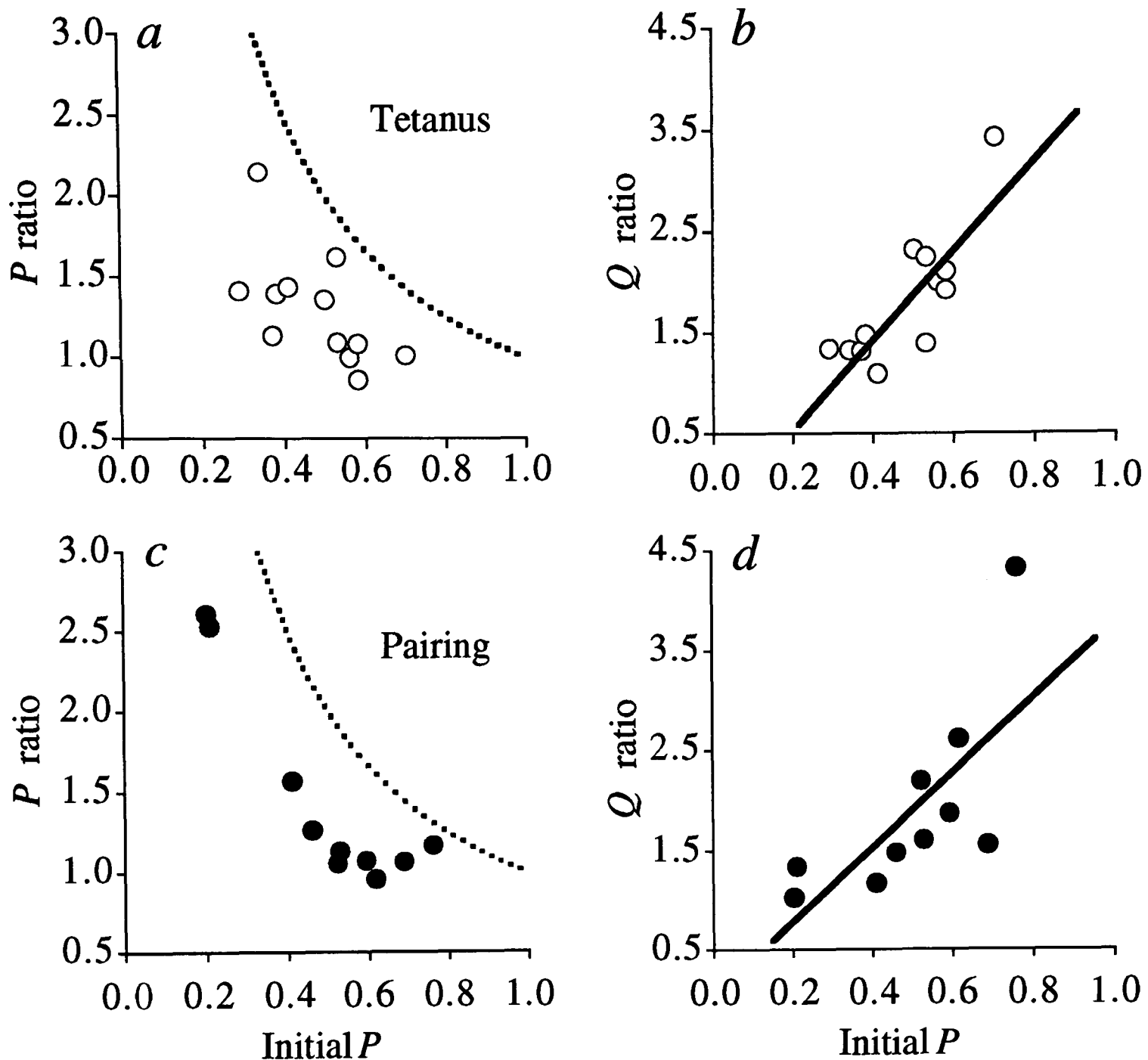
### *5.3.7 Changes in $P$ and $Q$ correlate with initial $P$*

The average changes in mean,  $P$  and  $Q$  for all of the EPSPs that showed STP after either tetanic stimulation or the pairing method are plotted in Figure 5.12. Although there was a lot of variation between the behaviour of individual EPSPs, the averaged results of both groups look remarkably similar. Apart from the briefer time course, they also closely match the results for LTP (see Chapter Four). Both induction methods resulted in STP that involved changes in  $P$  and  $Q$ . On average, the change in  $Q$  was larger and slightly longer-lasting than the change in  $P$ .

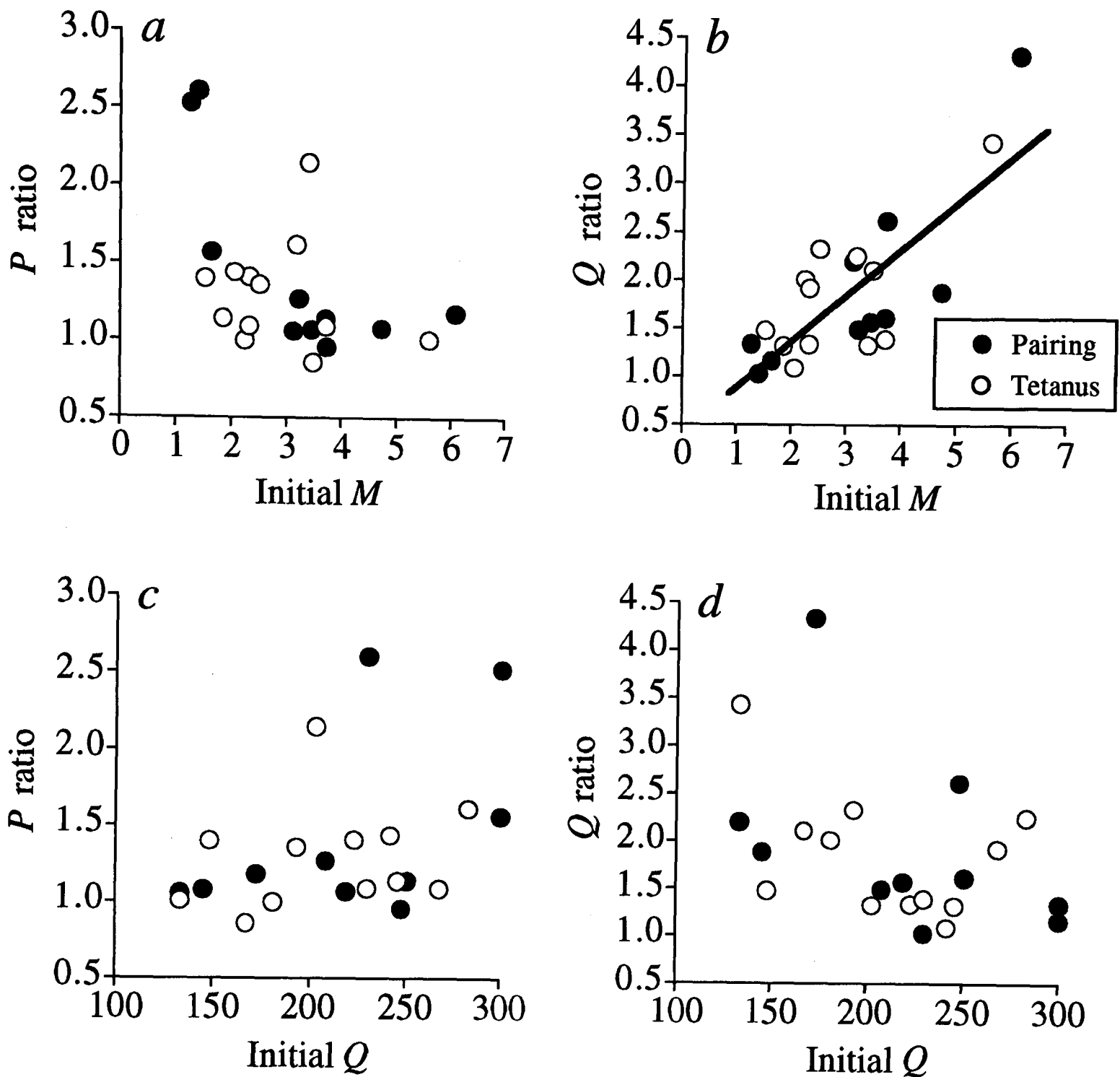
The ' $P$  ratio' and ' $Q$  ratio' are measures of the increases in  $P$  and  $Q$  respectively. Figure 5.13 shows these plotted against the initial (pre-STP) values of  $P$ . For the tetanus induction method, synapses at which  $P$  was initially low showed a large change in  $P$  (Figure 5.13a) but little change in  $Q$  (Figure 5.13b), while synapses at which  $P$  was already high showed little change in  $P$  but a large change in  $Q$ . Data from the pairing induction method show similar correlations (Figure 5.13c&d).  $P$  and  $Q$  ratios also correlate with initial values of the quantal content,  $M$  (equal to  $NP$ ) (Figure 5.14a&b) but not with initial values of  $Q$  (Figure 5.14c&d). Neither the size nor the duration of STP correlate with initial release probability or quantal content (Figure 5.15a&b).



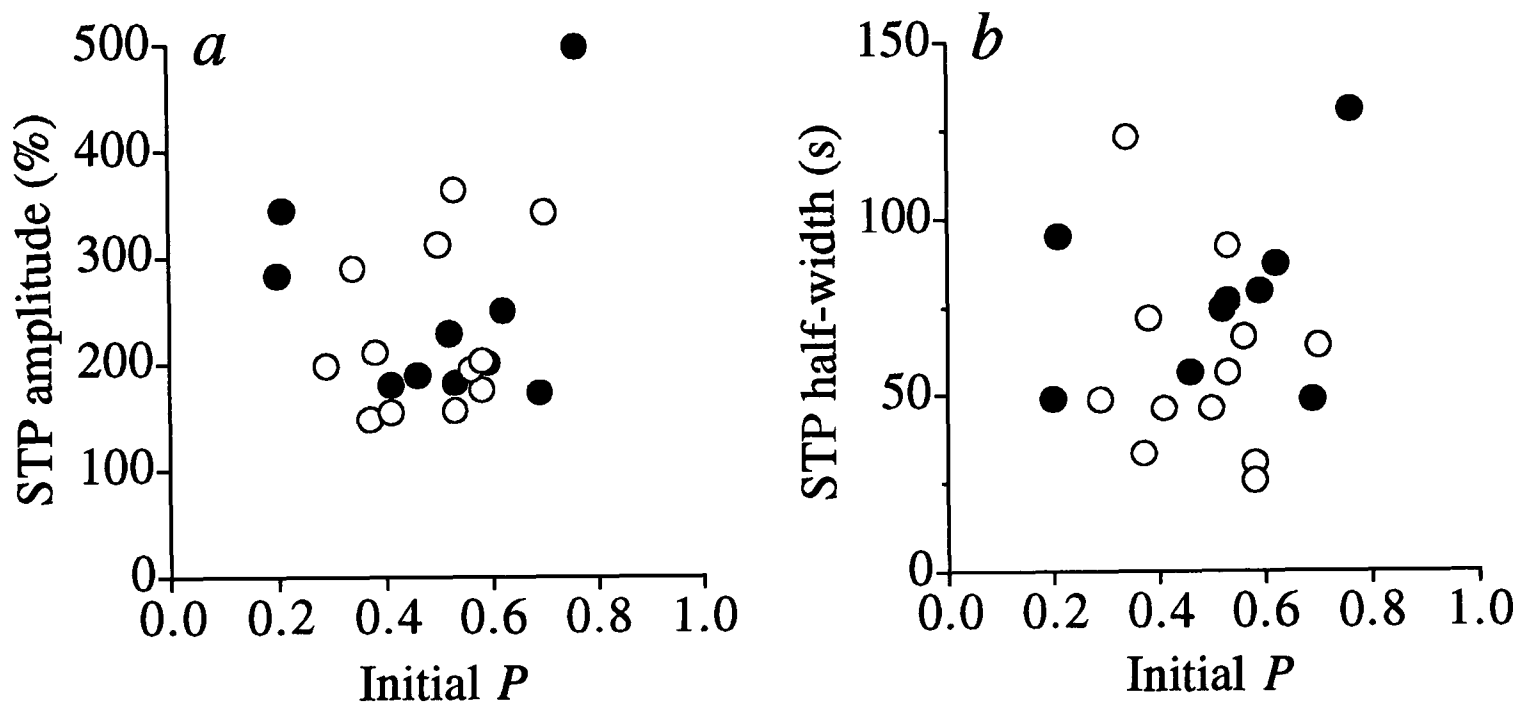
**Figure 5.12** *The average time courses of changes in mean EPSP amplitude, P and Q for tetanus-induced and pairing-induced STP* (a) The average change in mean, P and Q for the 12 examples of tetanus-induced STP. Each point represents 50 trials expressed as a percentage of the mean value during the 500 pre-tetanus trials. The increases in EPSP amplitude (open squares) are accompanied by changes in both Q (closed diamonds) and P (open circles). The change in Q was, on average, about twice as great as the change in P and seemed, if anything, to be slightly longer-lasting. (b) A similar plot for all 10 examples of pairing-induced STP. The results are almost identical to those for tetanus-induced STP. It is important to note that although the two groups showed very similar averaged results, the individual EPSPs within each group showed extremely diverse behaviour.



**Figure 5.13** *Increases in P and Q correlate with initial P* (a) For the 12 EPSPs that showed tetanus-induced STP, the increase in  $P$  is negatively correlated with its pre-tetanus value. Initial  $P$  is the mean value of  $P$  for the 50 trials immediately before the tetanus. The  $P$  ratio is the mean value of  $P$  for the 50 trials just after the tetanus divided by initial  $P$ . An upper limit is imposed on the  $P$  ratio by the fact that  $P$  can never be greater than one (dotted line). Linear regression gave a correlation coefficient,  $r$ , of  $-0.61$ . (b) The increase in  $Q$  is positively correlated with the pre-tetanus value of  $P$ . The  $Q$  ratio is the mean value of  $Q$  during the 50 trials immediately after the tetanus divided by the mean value of  $Q$  during the 50 trials just before. ( $r=0.82$ ,  $p<0.001$ ). (c) The change in  $P$  accompanying pairing-induced STP shows a similar correlation with initial  $P$  ( $r=-0.89$ ) (d) The same is also true for the change in  $Q$  ( $r=0.71$ ,  $p<0.021$ ).

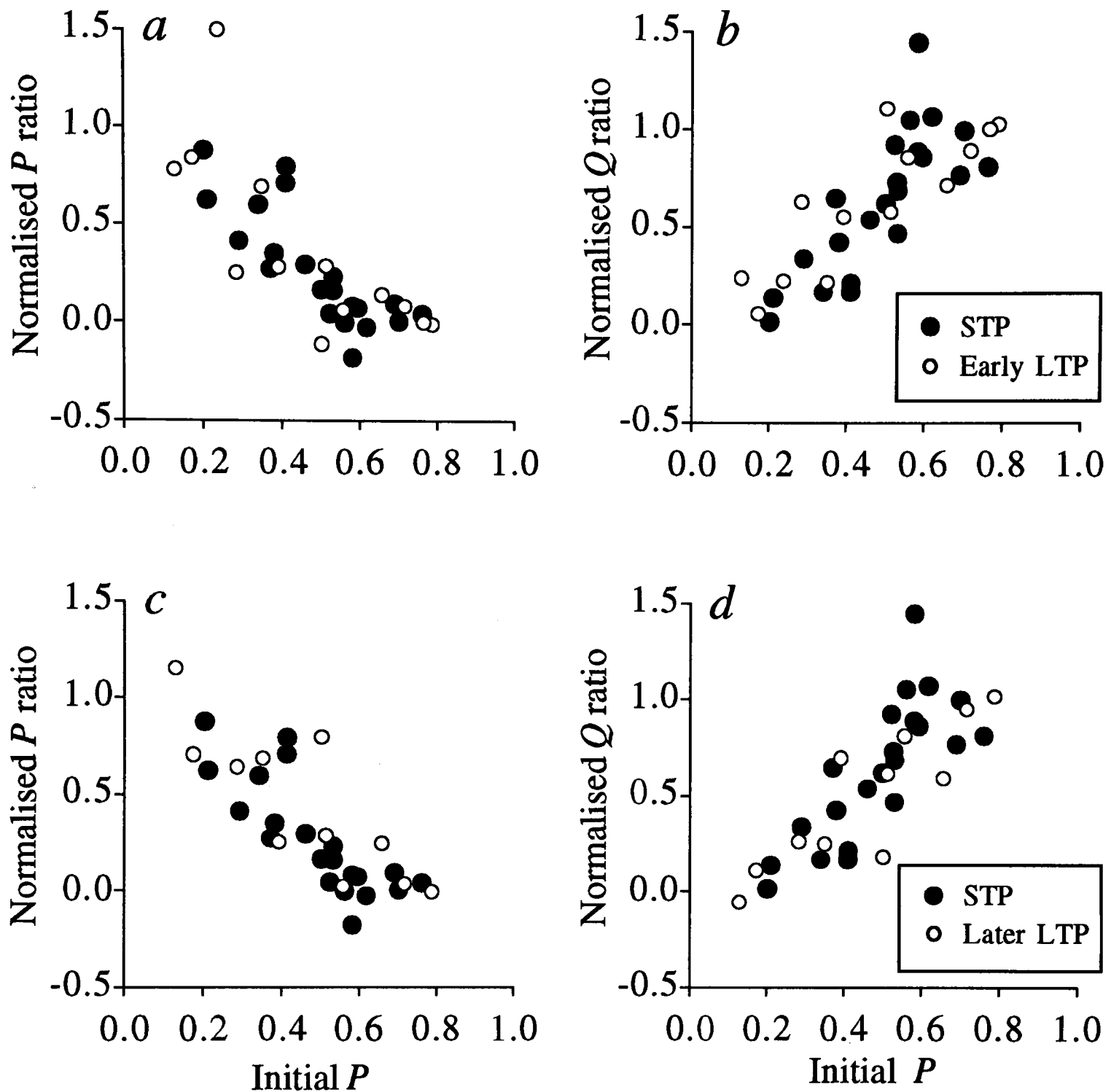


**Figure 5.14 P and Q ratios correlate with initial M but not initial Q** (a) When P ratios are plotted against initial M rather than initial P, there is a similar but weaker correlation ( $r=0.49$ ). Filled circles show data from pairing-induced STP and open circles show data from tetanus-induced STP. (b) The Q ratios show a much stronger correlation with initial M ( $r=0.77$ ,  $p<0.001$ ). (c&d) Neither P ratios nor Q ratios correlate strongly with initial Q ( $r=0.42$ ,  $p<0.06$  and  $r=0.44$ ,  $p<0.04$ , respectively).



**Figure 5.15** *Neither STP amplitude nor STP duration correlate with initial  $P$*  (a) STP amplitude is the EPSP amplitude during the first 50 trials after STP induction expressed as a percentage of the mean EPSP amplitude during the 50 preceding trials. There is no significant correlation with initial  $P$  ( $r=0.19$ ,  $p<0.41$ ). Closed circles show data from pairing-induced STP and open circles, data from tetanus-induced STP. (b) The half-width of the EPSP amplitude time course, a measure of its duration, also shows no correlation with initial  $P$  ( $r=0.11$ ,  $p<0.65$ ).

The absolute changes in  $P$  and  $Q$  that accompanied STP are difficult to compare directly with those that accompanied LTP. This is because STP showed more variability in the degree of potentiation (from 150 to almost 500% of the baseline value) than LTP (130-240% and usually around 200%). To allow for this, I normalised the increases in  $P$  and  $Q$  by the amount that the EPSP mean had increased. This gives a measure of the relative contributions of  $P$  and  $Q$  to the increase in EPSP amplitude, regardless of the absolute size of the increase. When these normalised ratios are plotted against initial  $P$ , the correlations improve and are very similar to those for the early stages (about the first 8 minutes) of LTP (Figure 5.16a&b). This suggests that LTP and STP share very similar expression mechanisms. An alternative explanation might be that only STP shows these correlations and that STP contaminates the early stages of LTP. However, Figure 5.16c&d shows that data from the later stages of LTP (about 8-16 minutes after induction), when STP would have returned to baseline, show similar correlations. Of course, this does not rule out the possibility of other processes at even later times.



**Figure 5.16** *The correlations of normalised P and Q ratios with initial P are similar for STP and LTP* (a) The normalised P ratio was calculated as  $(P \text{ ratio} - 1) / (\text{mean ratio} - 1)$ , where the mean ratio is the mean EPSP amplitude during the 50 trials just after STP induction divided by the mean during the 50 trials before induction. The normalised P ratio shows a negative correlation with initial P (filled circles) ( $r=0.81$ ). Normalised P ratios for the first 500 trials (about 8 minutes) of LTP show a very similar correlation (open circles). (b) Normalised Q ratios can be calculated in a similar way and these show positive correlations with initial P for both STP (filled circles) ( $r=0.78$ ) and LTP (open circles). (c & d) The correlations for STP also match those for later stages of LTP (trials 500-1,000, equivalent to about 8-16 minutes after LTP induction), by which time any accompanying STP would have declined back to baseline.

## 5.4 Discussion

### 5.4.1 *The duration of STP*

In my experiments, STP induced by either tetanus or pairing usually lasted for between 2 and 5 minutes. These values could be underestimates of the true duration because STP often seemed to be superimposed on a gradual depression of EPSP amplitude. Nevertheless, these durations lie at the lower end of the range reported by other researchers. One reason for this might be that I gave test stimuli at 1Hz rather than at frequencies of 0.2-0.05Hz, which most other researchers in this field use. As I mentioned in the introduction to this chapter, there does seem to be a tendency for low test stimulation rates to result in STP with longer durations. This point is also relevant to my studies of LTP and is discussed in more detail in Chapter Four.

### 5.4.2 *STP and LTP induction share the same properties*

One striking feature of these results is that STP seems to be remarkably similar to LTP. The induction of pairing-induced STP depends on NMDA receptor activity and its expression is synapse-specific. This confirms the results of several other studies (Sastry *et al.*, 1986; Larson & Lynch, 1988; Anwyl *et al.*, 1989; Malenka, 1991). The low success rate of the tetanus induction method meant that I could not investigate this in the same way, but it is outwardly so similar to pairing-induced STP that it is not unreasonable to suppose that it shares the same properties.

My results disagree with findings by McGuinness *et al.* (1991b), who could induce LTP but not STP in hippocampal slices bathed in ACSF containing 4mM Ca<sup>2+</sup>. They concluded that STP and LTP must therefore involve different processes. All of the slices in my experiments were bathed in ACSF containing 4mM Ca<sup>2+</sup> and yet many showed STP. My results also show a great deal of similarity between STP and LTP.

### *5.4.3 A common rule governs the changes that underlie the expression of STP and LTP*

My quantal analysis results show that increases in either release probability or quantal size can underlie STP, with many EPSPs showing both types of change. These are very similar to the results I obtained for LTP (see Chapter Four). In both cases, an identical rule seems to govern the synaptic locus of the change: synapses that have a low release probability express STP or LTP by showing an increase in that probability, while those where the release probability is already high show an increase in quantal size. It is also interesting to note that induction by pairing can produce an increase in release probability (Figures 5.10 & 5.11). Pairing involves manipulating only the postsynaptic cell so these results provide further good evidence for the existence of a retrograde synaptic message.

### *5.4.4 What makes STP shorter than LTP?*

The fact that the induction and expression mechanisms of STP and LTP are so similar makes their different time courses all the more difficult to explain. Work by Malenka and others (see the Introduction to this chapter and Malenka & Nicoll, 1993 for reviews) suggests that  $\text{Ca}^{2+}$  or some  $\text{Ca}^{2+}$ -dependent process has to reach a threshold level in the postsynaptic neurone to induce STP or LTP. It also seems that the threshold for LTP is higher than for STP. Perhaps the higher level of  $\text{Ca}^{2+}$  needed to induce LTP activates the same biochemical processes that are responsible for STP but does so in such a way that they become constitutively active. I will discuss possible mechanisms in more detail in Chapter Seven.

In my experiments, STP was produced much more reliably by pairing than by tetanic stimulation. However, there was no corresponding improvement in the success rate for LTP. How can this be if STP and LTP rely on such similar induction mechanisms? Taken on their own, my results suggests that LTP induction requires

high-frequency presynaptic activity, while STP does not. But this is not the case: in some preparations, LTP can be induced reliably by pairing low-frequency presynaptic activity with very strong postsynaptic depolarisation (e.g. Malinow & Tsien, 1990; Malinow, 1991; Manabe *et al.*, 1993).

There are alternative explanations. Since NMDA receptors are voltage-dependent, the amount of  $\text{Ca}^{2+}$  entering the postsynaptic neurone during induction depends critically on its level of depolarisation. It may be that the pairing procedure produced a more reproducible, relatively mild depolarisation throughout the dendritic tree of the neurone that was enough to induce STP (but not LTP) at many synapses. Tetanic stimulation, on the other hand, might have resulted in an uneven depolarisation that is deep enough in some places to elicit LTP but too weak in others to produce any potentiation at all. If this is the case then LTP might be produced by using deeper depolarisation during pairing or, perhaps, by pairing for a larger number of trials.

Another difference is that tetanic stimulation might involve greater activation of metabotropic glutamate receptors. These are normally thought to enhance LTP by increasing NMDA receptor activity (see Chapter One) and might explain why a large proportion of those synapses that potentiated in response to a tetanus showed LTP rather than STP.

#### *5.4.5 What determines the amount of potentiation?*

The idea that  $\text{Ca}^{2+}$  levels determine the duration of potentiation still leaves unexplained the question of the different amounts of potentiation seen. My finding that large STP tends to last longer than small STP agrees with similar results reported by Malenka (1991). But, as I have shown, this rule cannot be extended to explain the longer duration of LTP; examples of STP often show a much greater initial enhancement than any of the EPSPs that expressed LTP. Many observers of the field would probably guess that the initial degree of potentiation also depends on the amount of  $\text{Ca}^{2+}$  that flows into the postsynaptic cell during induction. But the degree

and the duration of potentiation seem, to some extent, to be independent of each other. This makes it difficult to see how both amplitude and duration could be controlled by the same signal and suggests that other messengers might play a role. These could conceivably include second messengers such as inositol trisphosphate (IP<sub>3</sub>) and cyclic adenosine monophosphate (cAMP) or putative retrograde messengers such as nitric oxide (NO) (see Chapter One). Needless to say, other unknown substances might also be involved.

#### *5.4.6 Both presynaptic and postsynaptic changes are rapid*

My STP results also reinforce the point made in Chapter Four that both presynaptic and postsynaptic changes occur very quickly after induction. The changes were always complete by the time I resumed test stimulation. This is particularly striking for the pairing-induced examples, for which the pause between the end of pairing and the recommencement of recording was always less than 5s. I will return to this point in Chapter Seven.

# Chapter Six

## Area CA3

### 6.1 Introduction

This chapter describes the results of a preliminary study of synaptic transmission in area CA3 of hippocampal slices. My intention was to carry out a quantal analysis of long-term potentiation (LTP) at mossy fibre synapses.

#### 6.1.1 *Pyramidal neurones and mossy fibre synapses in area CA3*

Pyramidal neurones in area CA3 of the hippocampus are similar to their counterparts in area CA1 but have larger cell bodies and thicker apical dendrites. The hippocampus of a Sprague-Dawley rat contains about 300,000 CA3 pyramidal cells (Boss *et al.*, 1987; Amaral *et al.*, 1990) and each one receives excitatory glutamatergic inputs from a variety of sources. Most come from other CA3 pyramidal neurones. These can be either in the same hemisphere of the brain (*associational* connections) or the opposite one (*commissural* connections). Many inputs also come from the perforant path, which not only makes synapses onto dentate gyrus granule neurones (see Chapter One) but also has substantial connections with pyramidal neurones in areas CA1 and CA3.

The most widely studied connections on CA3 pyramidal neurones, however, are the giant 'mossy fibre' synapses made by axons of dentate gyrus granule neurones. There are much fewer of these than the other types of synapses I have mentioned. For example, Amaral *et al.* (1990) estimate that CA3 pyramidal neurones in rats have, on average, about 12,000 excitatory synapses, which include inputs from around 6,000 other CA3 pyramidal neurones, but only 46 dentate gyrus granule neurones. One reason that mossy fibre synapses have attracted so much interest is that they are much

larger than most vertebrate synapses and can easily be seen with an optical microscope. The giant presynaptic terminals are 3-4µm across and make contact with unusual branched postsynaptic spines ('thorny excrescences') on the CA3 pyramidal cells (Ramón y Cajal, 1911; Blackstad & Kjaerheim, 1961; Hamlyn, 1961, 1962; Chicurel & Harris, 1992). They are physically and electrotonically close to the cell body (Johnston & Brown, 1983), which makes them particularly attractive for electrophysiological experiments because voltages and currents created at the synapse are relatively undistorted by the time they reach the recording site. In contrast, synapses of commissural or associational axons tend to make contact with dendrites further from the cell body (Hjorth-Simonsen, 1973) and the resulting EPSPs and EPSCs have longer rise and decay times (Miles & Wong, 1986; Williams & Johnston, 1991).

As I described in Chapter One, area CA3 was among the first brain regions to be subjected to a quantal analysis (Yamamoto, 1982). These early studies did not produce histograms with clear peaks but more recent experiments by Jonas *et al.* (1993) have. These show beyond reasonable doubt that, in favourable cases at least, transmission at mossy fibre synapses is quantal and that quantal variance is low. Jonas *et al.* estimated that the mean quantal content is in the range 2-6 and that each quantum involves the opening of 14-65 non-NMDA receptors.

### *6.1.2 Changes in the strength of mossy fibre synapses*

Mossy fibre EPSPs recorded from hippocampal slices show paired-pulse facilitation (PPF), post-tetanic potentiation (PTP) and long-term potentiation (LTP) (Alger & Tyler, 1976; Yamamoto *et al.*, 1980). PPF and PTP seem to be caused by residual calcium (Ca<sup>2+</sup>) in the presynaptic terminal but LTP is not (Regehr & Tank, 1991).

They also show *frequency facilitation* (sometimes known as frequency potentiation) (Alger & Tyler, 1976). This is the tendency for the size of the postsynaptic response to increase as the rate of stimulation goes up and is a natural consequence of the

residual  $\text{Ca}^{2+}$  phenomenon that causes PPF (see Chapter Three). Faster stimulation rates mean that successive pulses are closer together and so there is an increase in the level of intracellular  $\text{Ca}^{2+}$  to which the presynaptic terminal returns just before each stimulus. This causes more transmitter release and hence a bigger postsynaptic response. Frequency facilitation has been described in areas CA1 and CA3 of intact animals (Andersen, 1960a, b) and hippocampal slices (Alger & Tyler, 1976; Creager *et al.*, 1980). In area CA1 of hippocampal slices the effect is enhanced by increasing the magnesium ( $\text{Mg}^{2+}$ ) concentration or decreasing the  $\text{Ca}^{2+}$  concentration (Landfield *et al.*, 1986), which is consistent with the view that it is caused by residual  $\text{Ca}^{2+}$  in presynaptic terminals.

Area CA3 contains NMDA receptors but they are less abundant than in area CA1 and the lowest concentration is in the region of the proximal apical dendrites, where mossy fibres make contact (Monaghan *et al.*, 1983; Monaghan & Cotman, 1985). Spontaneous miniature EPSCs recorded from CA3 pyramidal neurones have components mediated by both NMDA receptors and non-NMDA receptors, so the two types probably exist together at individual synaptic connections (McBain & Dingledine, 1992). However, mossy fibre responses are reported to have very small NMDA-receptor-mediated components (Andreasen *et al.*, 1989; Katsuki *et al.*, 1991; Jonas *et al.*, 1993).

Consistent with this view is the finding that induction of LTP at mossy fibre synapses does not require NMDA receptor activity but LTP at associational or commissural synapses does (Harris & Cotman, 1986; Zalutsky & Nicoll, 1990). As I described in Chapter One, induction of LTP at synapses in the dentate gyrus and area CA1 does involve NMDA receptors and it is the way in which these receptors respond to both neurotransmitter and membrane potential that gives LTP at these synapses its Hebbian properties.

There is some disagreement over whether or not mossy fibre LTP is Hebbian. Zalutsky & Nicoll (1990, 1992) show that it does not require a rise in postsynaptic

Ca<sup>2+</sup> or postsynaptic depolarisation and conclude that it is not Hebbian. Katsuki *et al.* (1991) have reported very similar results. Ito & Sugiyama (1991) found that they could induce LTP at mossy fibre synapses even when synaptic transmission was temporarily inhibited with a combination of antagonists that blocked both NMDA-receptors and non-NMDA-receptors. This also supports the view that postsynaptic depolarisation is not necessary for LTP induction.

But Johnston and his colleagues disagree. They find that LTP at mossy fibre synapses does depend on postsynaptic depolarisation and a rise in postsynaptic Ca<sup>2+</sup> concentration (Williams & Johnston, 1989; Jaffe & Johnston, 1990, reviewed in Johnston *et al.*, 1992) and is therefore Hebbian. They suggest that Ca<sup>2+</sup> enters the postsynaptic cell during induction, coming through voltage-dependent channels. This is consistent with their finding that neuromodulators known to affect mossy fibre LTP seem to alter the activity of these channels (Fisher & Johnston, 1990). They also find that presynaptic activity seems to be necessary, perhaps indicating a role for metabotropic glutamate receptors. This last point is consistent with other results reported by Ito's group, who showed that mossy fibre LTP seems to require the activity of a pertussis-toxin-sensitive G-protein (Ito *et al.*, 1988) and metabotropic glutamate receptors (Ito & Sugiyama, 1991). They did not, however, distinguish between presynaptic and postsynaptic sites of action.

Yamamoto *et al.* (1980), found that mossy fibre LTP induces heterosynaptic potentiation in other mossy fibre inputs but not in other types of synapses on the same neurone. Bradler & Barrionuevo (1989) have found the reverse. In their hands, LTP at mossy fibre synapses seems to produce a heterosynaptic increase in the strength of commissural and fimbrial connections on the same cell. LTP at commissural synapses, on the other hand, results in heterosynaptic depression at mossy fibre synapses. These heterosynaptic changes suggest that a postsynaptic signal is produced during mossy fibre LTP induction. However, it does not necessarily mean that induction is postsynaptic because Bradler & Barrionuevo (1990) found that

heterosynaptic depression could occur even when homosynaptic mossy fibre LTP failed to develop, so the two may be triggered by different signals.

Mossy fibre presynaptic terminals also contain peptide neurotransmitters (Gall *et al.*, 1981; Chavkin *et al.*, 1983). These seem to modulate mossy fibre LTP but there is some disagreement over whether they enhance (Ishihara *et al.*, 1990; Derrick *et al.*, 1991) or inhibit it (Weisskopf *et al.*, 1993).

### 6.1.3 *The site of mossy fibre LTP expression*

Mossy fibre LTP is accompanied by a reduction in PPF (Zalutsky & Nicoll, 1990; Staubli *et al.*, 1990), which suggests that expression is at least partly presynaptic. As I described in Chapter One, the first quantal analyses of hippocampal LTP were carried out by Voronin in region CA3 and these indicated a presynaptic locus of expression (reviewed in Voronin, 1983). The only other group to report a quantal analysis of mossy fibre LTP have produced inconclusive results. On the basis of an increase in  $1/CV^2$  and a reduction in the number of failures, Hirata *et al.* (1991) concluded that it has a substantial presynaptic component. But a later quantal analysis by the same group, this time based on histograms (Yamamoto *et al.*, 1992), showed an increase only in quantal size.

I studied synaptic plasticity at a subset of synapses in area CA3 that had fast rise times and showed pronounced frequency facilitation and, in one case, NMDA-receptor-independent LTP. These properties suggest that they might have been mossy fibre synapses. I used  $1/CV^2$  graphs to determine whether these changes in synaptic strength were presynaptic or postsynaptic.

## 6.2 Methods

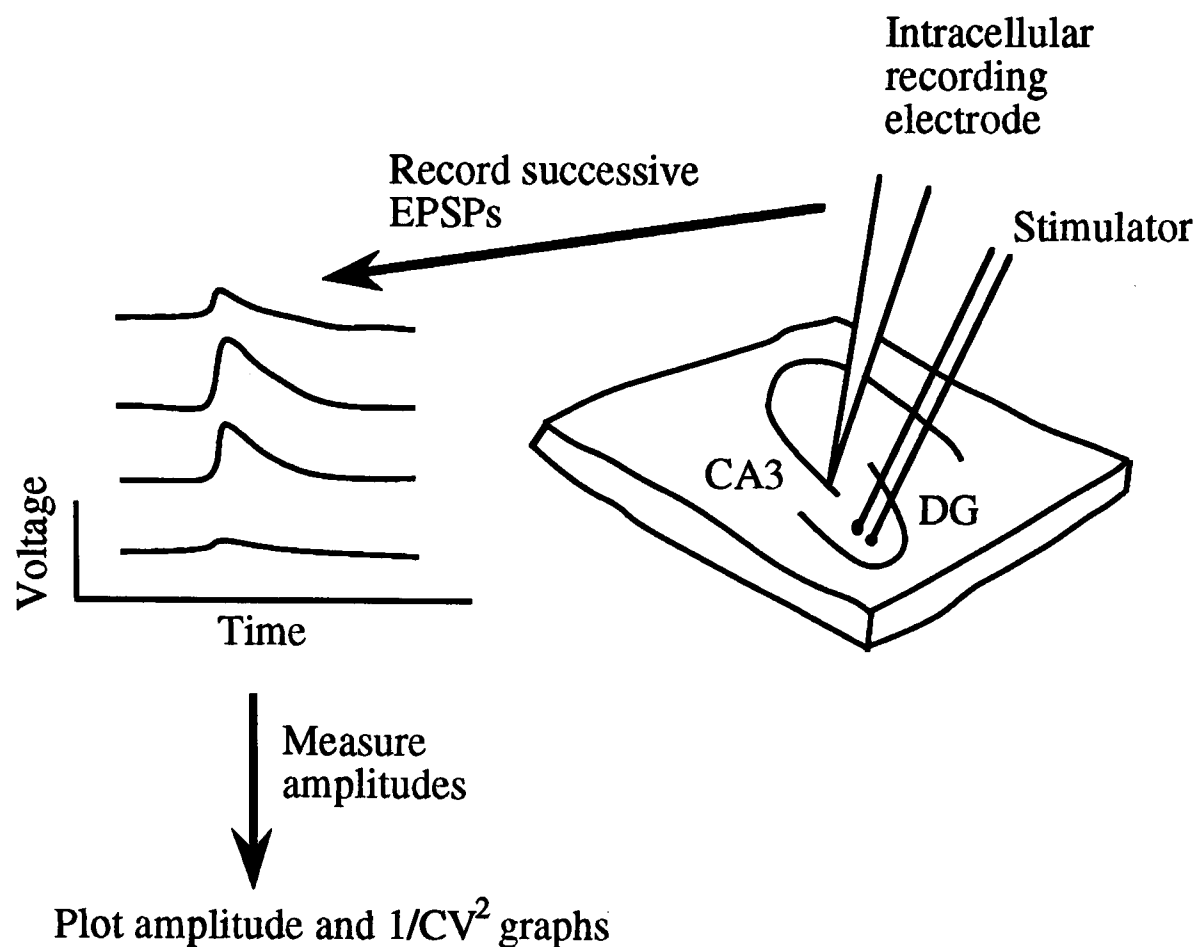
The slice preparation method varied slightly for these experiments. For about half of them, I prepared the slices exactly as described in Chapter Two. For the other half I

sliced from the bottom of the brain towards the top (rather than from the side to the mid-line) at an angle of about 30° to the horizontal (inclined towards the back of the brain). This is thought to preserve connections between the dentate gyrus and area CA3 more effectively (Guy Major, personal communication) but I found no noticeable differences in the results I got from these two types of slices. When I trimmed the slices, I left the dentate gyrus and area CA3 (as well as area CA1) intact so the resulting slices were larger than the ones I used to record from area CA1.

The artificial cerebrospinal fluid (ACSF) was different in two respects from that described in Chapter One. First, I added 100µM of the NMDA receptor antagonist, 2-amino-5-phosphonovalerate (AP5) for all experiments and, second, I did not add any glycine. NMDA receptor activity would therefore have been inhibited. This should not have affected mossy fibre LTP but might have had the beneficial effect of slowing the rate of neuronal death in the slices (Choi & Rothman, 1990).

In a few cases I used the same tetanus procedure described in Chapter Four. However, for most of these experiments I gave two tetani, each of 100 stimuli at 100Hz, separated by a 30s interval. As described in Chapter Two, there were always one-minute pauses between the end of test stimulation and the first tetanus, and between the last tetanus and resuming test stimulation. During the tetanus, I stimulated only the small test EPSP (i.e. without using a second bipole) on the assumption that LTP at mossy fibre inputs is not co-operative.

Figure 6.1 shows a diagram of the recording arrangement. The CA3 cell body layer was usually only faintly visible so I used the position of the dentate gyrus to help locate it. I placed the electrode in a region of CA3 close to the dentate gyrus to improve the chances of eliciting a mossy fibre input and put the stimulating bipole in the hilus region, between the electrode and the granule cell layer of the dentate gyrus. Test stimulation was at 0.2, 1, 3 or 5Hz. The rates used are given in the text and figures where appropriate.



**Figure 6.1** *The arrangement for stimulating and recording from synapses in area CA3* I made recordings from the cell body layer of area CA3 and stimulated axons with a metal bipole placed between this and the granule cell layer of the dentate gyrus (DG).

## 6.3 Results

### 6.3.1 *Single-component EPSPs were hard to find and many EPSPs had long rise times*

Compared to my experiments in CA1, I found it harder to get clean, apparently single-component EPSPs. Many were contaminated with extra components that seemed to be polysynaptic. This may be due to the large number of excitatory local connections in area CA3 and the fact that I blocked inhibitory transmission with picrotoxin.

The first 8 EPSPs that I recorded had amplitudes between 498 and 2,414 $\mu$ V, with an average of 1,482 $\pm$ 786 $\mu$ V. Their 10-90% rise times ranged from 3.6 to 6.2ms and averaged 4.6 $\pm$ 1.0ms. Two of them obviously had more than one component with

different latencies but the other six appeared to have smooth rising and decaying phases. Latencies were in the range 3.0-6.0ms and averaged  $4.0 \pm 0.9$ ms. In each case I gave test stimulation at 1Hz. None of these EPSPs showed any noticeable potentiation after tetanic stimulation.

### *6.3.2 Some EPSPs showed marked frequency facilitation*

The ninth EPSP that I recorded had very different properties. It was large (about  $3,000\mu\text{V}$ ) and had a short 10-90% rise time (1.6ms). It showed very noticeable frequency facilitation. The mean EPSP amplitude at a steady 0.2Hz was about  $3,100\mu\text{V}$  and this rose to  $7,500\mu\text{V}$  at a stimulation rate of 1Hz. Returning the stimulation rate to 0.2Hz again brought the EPSP amplitude back down to about  $2,500\mu\text{V}$ . The characteristics of short rise time and large amplitude suggested that it might be a mossy fibre EPSP. After this I began to look for other similar EPSPs. I selected these on the basis of pronounced frequency facilitation.

### *6.3.3 EPSPs that showed frequency facilitation were rare*

EPSPs that showed noticeable frequency facilitation were rare; I found only 9 others among roughly 400-500 EPSPs that I examined. Most other EPSPs had slow rise times, comparable to those of the 8 EPSPs described above. They also showed no significant response to a change in the stimulation rate between 0.2 and 5Hz. I gave tetanic stimulation to 14 of them but none of these showed any significant increase in amplitude.

For 4 of the 9 EPSPs that showed frequency facilitation, I could not complete a full cycle of first increasing and then decreasing the stimulation rate. For this reason, it was impossible to be sure how much of the change in amplitude was caused by an underlying drift (see Chapter Three) and how much was caused by the change in stimulation frequency. I did not analyse any of these EPSPs further. The rest of this chapter describes the properties of the other 6 EPSPs. All of these were stimulated at

both 0.2 Hz and 1Hz. In addition, two of them were also stimulated at 3Hz and two others were stimulated at 5Hz.

#### *6.3.4 Six EPSPs that showed frequency facilitation*

Mean amplitudes of these 6 EPSPs at the various stimulation rates used are given in Table 6.1. The range at 0.2Hz was between 762 and 3,140 $\mu$ V, and the average for all of them was 1,600 $\pm$ 797. This rose to 3,449 $\pm$ 1,863 $\mu$ V at 1Hz and returned to 1,401 $\pm$ 652 $\mu$ V when I lowered the stimulation rate back to 0.2Hz. The average amplitudes at 3Hz and 5Hz were even higher than at 1Hz, although these figures are likely to be less accurate because they are each based on only two EPSPs.

Table 6.2 shows the 10-90% rise time and latency for all six EPSPs at the various stimulation frequencies used. Rise times during the baseline period at 0.2Hz were between 1.6 and 4.0ms, with an average of 2.6 $\pm$ 0.8ms. This decreased very slightly to 2.5 $\pm$ 0.8ms when I increased the stimulation rate to 1Hz, and remained at 2.5 $\pm$ 0.8ms when I lowered the stimulation rate back to 0.2Hz. The only EPSP to show a substantial change in rise time was C4. The EPSPs I stimulated at 3 or 5Hz did not show any large differences in their rise times at these higher frequencies as compared to their rise times at 0.2Hz.

Latencies ranged from 2.6 to 5.2ms and averaged 3.4 $\pm$ 0.9ms during the baseline period at 0.2Hz. There was a small tendency for this to increase as the stimulation rate rose. At 1Hz the average latency was 3.5 $\pm$ 0.8ms. This fell to 3.3 $\pm$ 0.9ms when I lowered the stimulation rate back to 0.2Hz.

Table 6.3 shows the resting potential and the SD of the noise and EPSP for each of the six examples. Inhibitory synapses were blocked during these experiments and under these conditions, recordings in CA3 tends to be contaminated with a lot of spontaneous synaptic activity and bouts of epileptiform activity. Largely because of

EPSP No.	Amplitude ( $\mu\text{V}$ )				
	0.2Hz	1Hz	3Hz	5Hz	0.2Hz
C1	3140	7468	—	—	2467
C2	1165	2626	—	—	1261
C3	2027	3213	—	5879	1555
C4	1007	2280	—	6448	798
C5	762	1861	3150	—	493
C6	1497	3248	4441	—	1832
Average	1600	3449	3796	6164	1401
$\pm\text{SD}$	$\pm 797$	$\pm 1863$	$\pm 646$	$\pm 285$	$\pm 652$

**Table 6.1** *Amplitudes of all 6 EPSPs at different stimulation rates* The amplitudes given are in  $\mu\text{V}$  and represent the mean for all trials collected at the indicated stimulation rate. A dash (‘—’) indicates that the EPSP was not stimulated at that rate. All 6 EPSPs showed significant increases in amplitude as the stimulation rate was raised from 0.2Hz to 1, 3 or 5Hz. Amplitudes then fell back to close to their original values when the stimulation rate was returned to 0.2Hz again (right-hand column).

EPSP No.	10-90% rise time (ms)					Latency (ms)				
	0.2Hz	1Hz	3Hz	5Hz	0.2Hz	0.2Hz	1Hz	3Hz	5Hz	0.2Hz
C1	1.6	1.6	—	—	1.4	5.2	5.2	—	—	5.2
C2	1.8	1.8	—	—	1.8	2.8	2.8	—	—	2.6
C3	2.8	2.8	—	3.0	3.0	2.6	3.0	—	3.4	2.6
C4	2.2	2.2	—	2.8	2.6	2.6	3.0	—	3.0	2.6
C5	2.4	2.4	2.4	—	2.4	3.4	3.4	3.6	—	3.4
C6	4.0	3.6	3.6	—	4.0	3.6	3.4	3.6	—	3.4
Average	2.8	2.5	3.0	2.9	2.5	3.4	3.5	3.6	3.2	3.3
±SD	±1.2	±0.8	±0.6	±0.1	±0.8	±0.9	±0.8	±0.0	±0.2	±0.9

**Table 6.2** *Rise times and latencies for all 6 EPSPs at different stimulation rates* The rise times and latencies are given in ms. They are for the average EPSP waveform of all trials collected at the indicated stimulation rate. These were digitised at 5kHz so the figures are accurate to  $\pm 0.2$ ms.

EPSP No.	Rest. Pot.	Noise SD ( $\mu\text{V}$ )					EPSP SD ( $\mu\text{V}$ )				
		0.2Hz	1Hz	3Hz	5Hz	0.2Hz	0.2Hz	1Hz	3Hz	5Hz	0.2Hz
C1	-70	—	—	—	—	—	—	—	—	—	—
C2	-72	174	125	—	—	148	1278	1603	—	—	989
C3	-75	353	269	—	341	354	617	1037	—	1475	577
C4	-79	269	233	—	219	273	590	787	—	1376	843
C5	-79	172	135	141	—	122	598	954	1556	—	707
C6	-76	114	167	124	—	123	885	1431	1652	—	1016
Average	-75	216	186	133	280	218	794	1162	1604	1426	826
$\pm\text{SD}$	$\pm 3$	$\pm 84$	$\pm 56$	$\pm 9$	$\pm 61$	$\pm 100$	$\pm 266$	$\pm 305$	$\pm 48$	$\pm 50$	$\pm 167$

**Table 6.3 Resting potential, noise SD and EPSP SD for all 6 EPSPs**  
**Resting potentials** are given in mV. In some cases they have been corrected for estimated changes in tip potential so figures are only approximate. Values for **noise SD** and **EPSP SD** are given in  $\mu\text{V}$ . They are calculated from all of the trials collected at the indicated stimulation rate. In the case of C1, I recorded only the average EPSP waveform at each stimulation rate so these figures are not available.

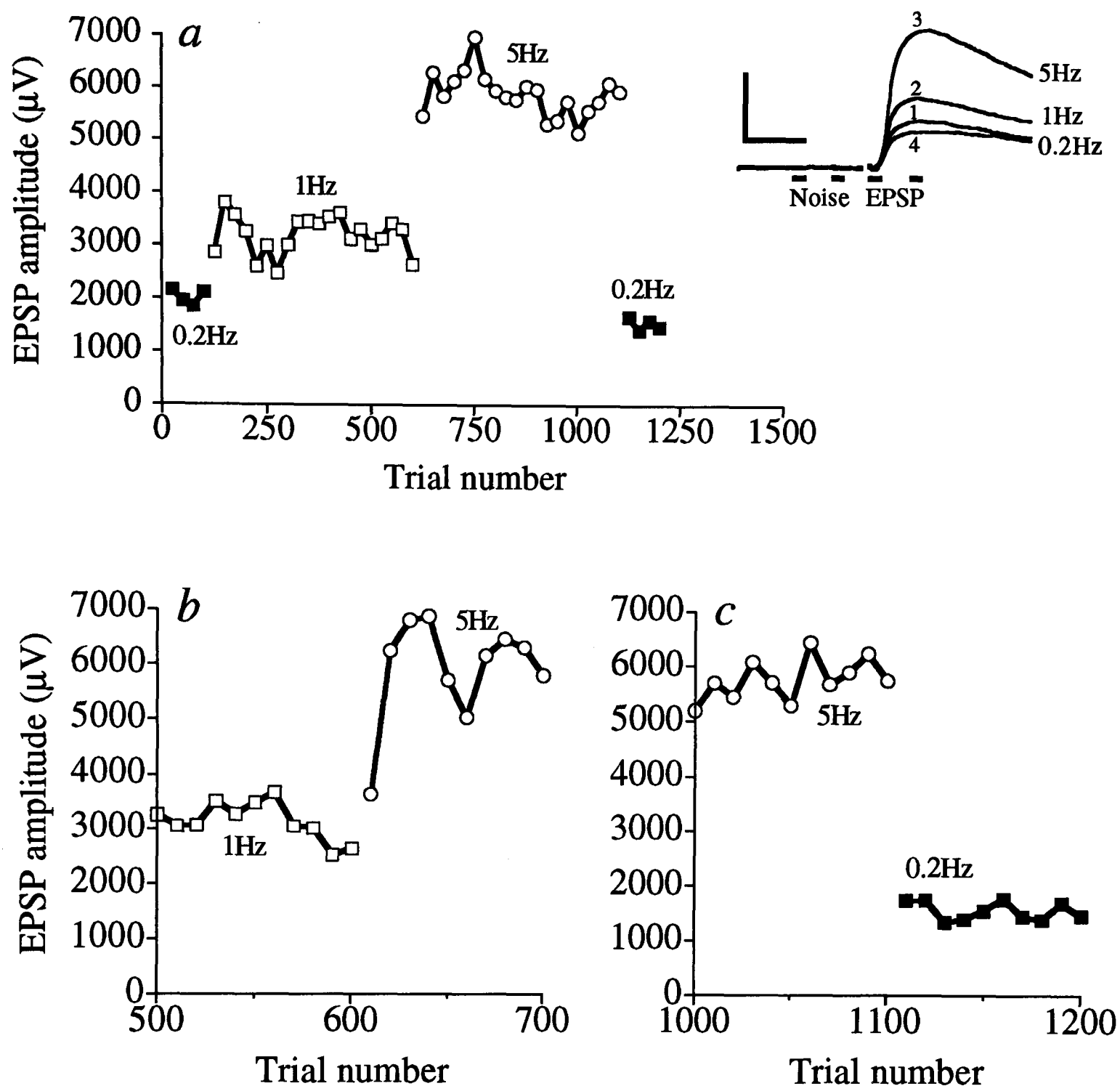
this, I found noise levels here to be higher than for most of my recordings from area CA1. At 0.2Hz the standard deviation of the noise ranged from 114 $\mu$ V to 353 $\mu$ V and averaged 216 $\pm$ 84 $\mu$ V. This seemed, if anything, to reduce when the stimulation rate went up, falling to 186 $\pm$ 56 $\mu$ V at 1Hz. It rose again to 218 $\pm$ 100 $\mu$ V when I resumed 0.2Hz stimulation again. In contrast, the SD of the EPSPs tended to rise with increasing stimulation rate. From an average of 794 $\pm$ 266 $\mu$ V at 0.2Hz, it rose to 1162 $\pm$ 305 $\mu$ V at 1Hz and fell back again to 826 $\pm$ 167 when I resumed 0.2Hz stimulation.

Figure 6.2a shows an EPSP (C3) that I stimulated at three different rates, 0.2Hz, 1Hz and 5Hz. The mean EPSP amplitude increased from 2,027 to 5,879 $\mu$ V. When I lowered the stimulation rate back to 0.2Hz, the EPSP amplitude fell to 1,555 $\mu$ V. Figure 6.2b shows the transition from 1Hz to 5Hz at higher resolution (each point represents the mean of 10 trials). The onset of frequency facilitation takes 5-6s to develop. The fall in amplitude when I lowered the stimulation rate to 0.2Hz appeared to be instantaneous at this resolution (Figure 6.2c).

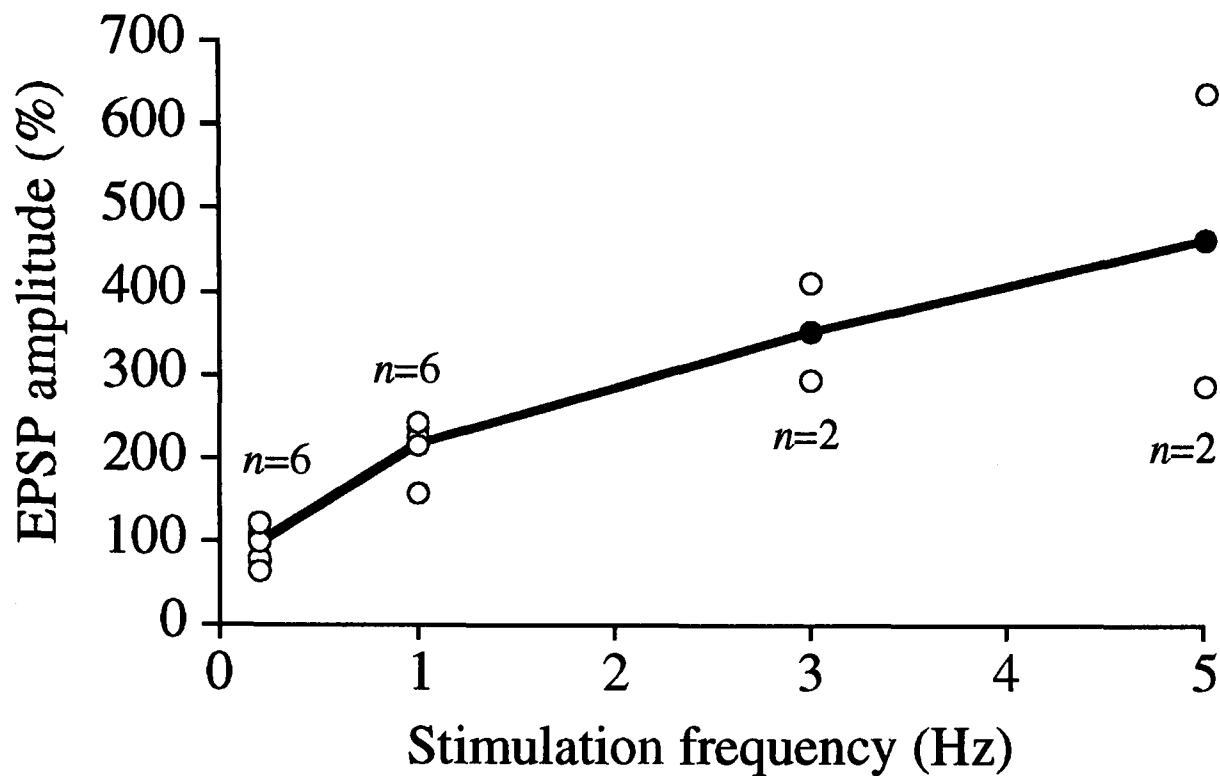
Figure 6.3 shows a summary of the frequency facilitation shown by all 6 EPSPs. The open circles show the amplitudes of individual EPSPs at different frequencies. The line and filled circles show the average for all EPSPs. On average, 1Hz stimulation caused the EPSP amplitudes to increase to 218 $\pm$ 28% of their amplitudes at 0.2Hz. At 3Hz this went up to 355 $\pm$ 58% and at 5Hz it was 465 $\pm$ 175%. The average amplitude when I returned the stimulation frequency to 0.2Hz was 88 $\pm$ 20%. The values for 3 and 5Hz are the least reliable because they are each based on results from only 2 EPSPs. The other values are calculated from all 6 EPSPs.

### *6.3.5 Frequency facilitation appears to involve a presynaptic change*

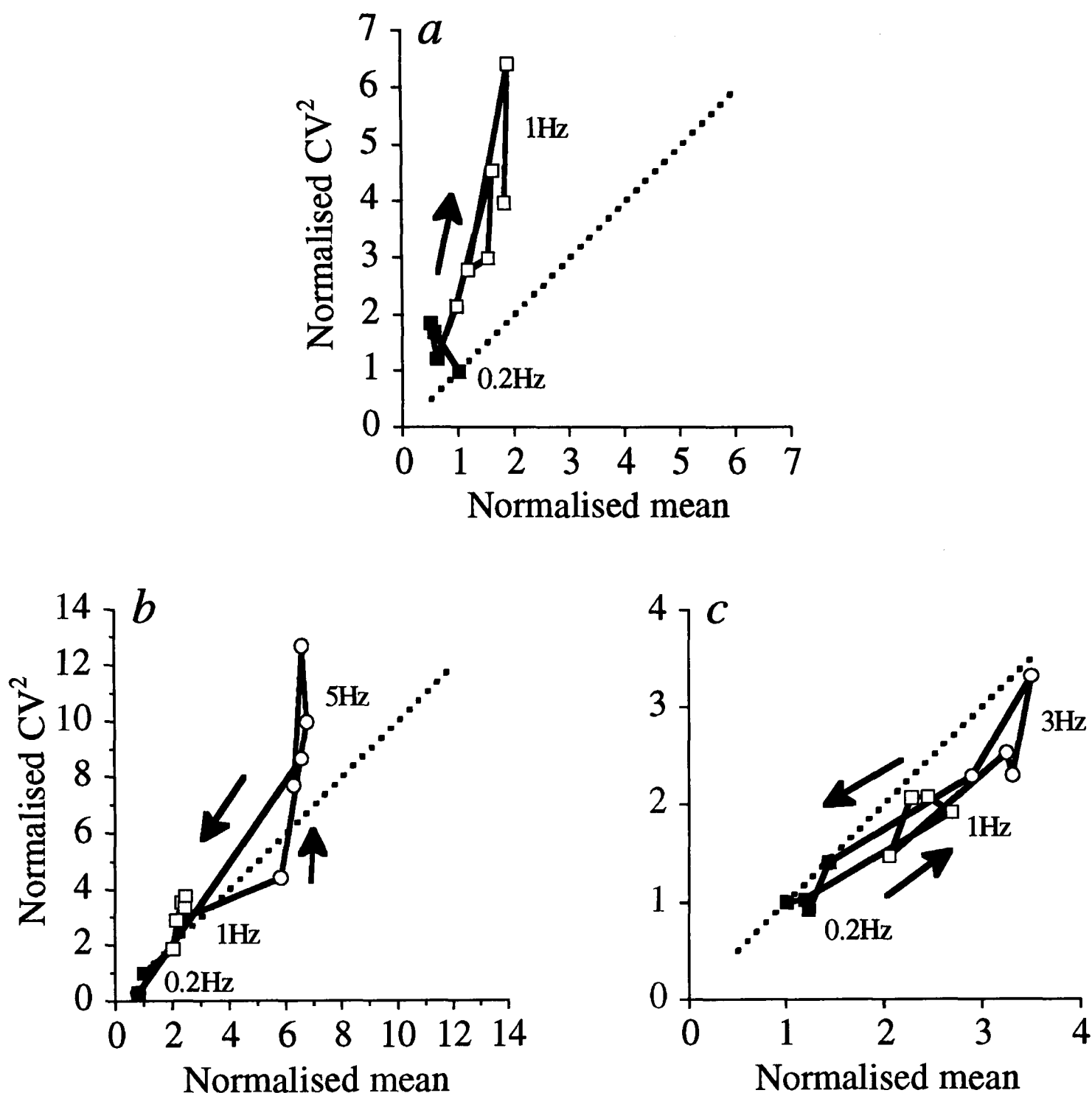
Figure 6.4a shows a  $1/CV^2$  graph for an EPSP (C2) that I stimulated at 0.2Hz and 1Hz only. The graph shows data from the baseline period of 100 trials at 0.2Hz and



**Figure 6.2 An EPSP that showed frequency facilitation** (a) At a stimulation rate of 0.2Hz, this EPSP (C3) had a mean amplitude of 2,027 $\mu$ V (closed squares). This rose to 3,213 $\mu$ V at 1Hz (open squares) and 5,879 $\mu$ V at 5Hz (open circles). Lowering the stimulation rate back to 0.2Hz (closed squares) caused the EPSP amplitude to fall to 1,555 $\mu$ V, a little below its starting amplitude. Each point represents the mean of 25 trials. The whole recording period shown took just over 25 minutes. The inset shows the EPSP waveforms of the last 50 trials at 0.2Hz (1), the last 50 trials at 1Hz (2), the last 50 trials at 5Hz (3) and the first 50 trials after the stimulation had been returned to 0.2Hz again (4). The 10-90% rise time was 3.0ms and did not change significantly when the stimulation rate and amplitude went up or down. Bars under the EPSPs show the time zones used to measure the amplitude of the noise and EPSP on each trial. Scale bar: 3,000 $\mu$ V, 10ms. (b) The onset of frequency facilitation as the stimulation frequency was increased from 1Hz (open squares) to 5Hz (open circles) shown at higher resolution. Each point represents the mean of 10 trials. Frequency facilitation takes 5-6s to develop. (c) The drop in EPSP amplitude when the stimulation rate was decreased from 5Hz (open circles) to 0.2Hz (filled squares) shown at the same higher resolution. At this resolution it appears to be abrupt.



**Figure 6.3** *The increase in EPSP amplitude with stimulation rate for all 6 EPSPs* Open circles show the mean amplitude of individual EPSPs at different frequencies. All values are expressed as a percentage of the mean amplitude during the initial period of stimulation at 0.2Hz for that particular EPSP. The open circles at 0.2Hz show the mean EPSP amplitudes when the stimulation rate was returned to 0.2Hz after stimulating at the various higher rates. The filled circles and line show the average amplitude for all EPSPs. On average, stimulating at 1Hz produced an EPSP that had an amplitude  $218 \pm 28\%$  of that at 0.2Hz. At 3Hz this increased to  $355 \pm 58\%$  and at 5Hz it was  $465 \pm 175\%$ , although these last two figures may not be particularly accurate because they are each averages of only 2 EPSPs.



**Figure 6.4**  $1/CV^2$  graphs of three EPSPs that showed frequency facilitation (a) A  $1/CV^2$  graph of one of the EPSPs (C2) for which the stimulation rate was increased from 0.2Hz (filled squares) to 1Hz (open squares). The trajectory is much steeper than the diagonal (dotted line) and curves upwards. Each point represents 25 trials. The period shown covers all 100 trials at 0.2Hz and the first 150 trials at 1Hz. (b) A  $1/CV^2$  graph of all 1,200 trials recorded from another EPSP (C4). As the stimulation rate increased (upward-pointing arrow) from 0.2Hz (closed squares) to 1Hz (open squares) and then to 5Hz (open circles), the trajectory tends to curve upwards and most points lie above the diagonal (dotted line). When the stimulation rate was reduced back to 0.2Hz (downward-pointing arrow), both the mean and  $1/CV^2$  fell back to values close to their starting points. Each point represents 100 trials. (c) A  $1/CV^2$  graph of all 1,500 trials recorded from another EPSP (C6). As the stimulation rate increased (upward-pointing arrow) from 0.2Hz (closed squares) to 1Hz (open squares) and then to 3Hz (open circles), the mean and  $1/CV^2$  increase by similar amounts so the trajectory lies close to the diagonal. When I reduced the stimulation rate to 0.2Hz again (downward-pointing arrow), both the mean and  $1/CV^2$  returned to values similar to those at the start.

the first 150 trials at 1Hz. The trajectory is very steep and curves upwards. Within the assumptions implicit in this type of analysis (see Chapter Two), this suggests a presynaptic increase in release probability.

The  $1/CV^2$  graph in Figure 6.4b shows all 1,200 trials recorded from another EPSP (C4) at three different frequencies, 0.2, 1 and 5Hz. Once again, the trajectory is steep and curves upwards, which suggests a presynaptic change in release probability, possibly with an accompanying increase in the number of release sites. The trajectory briefly dips below the diagonal (dotted line in Figure 6.4b) when the stimulation rate increases from 1 to 5Hz. This could indicate a brief postsynaptic change but there is another possibility. When the mean is changing very rapidly, as it is here (from about 2mV to about 7mV in less than 100 trials), a substantial part of the variance for each point on the  $1/CV^2$  graph is due to this changing mean. This adds to the variance caused by random synaptic transmission to produce a spurious increase in CV (and hence a decrease in  $1/CV^2$ ), which makes the trajectory more horizontal than it would otherwise be.

Figure 6.4c shows a  $1/CV^2$  graph for an EPSP (C6) that I stimulated at 0.2, 1 and 3Hz. The trajectory lies just underneath the diagonal (dotted line in Figure 6.4c). If simple binomial statistics are assumed to apply, this would suggest a change in the number of release sites, or alternatively some indeterminate combination of changes in release probability, number of release sites and quantal size. Another possibility is that the behaviour of this EPSP is well-described by Poisson statistics (i.e. release probability  $\ll$  number of release sites). If this is the case then a trajectory along the diagonal would indicate a change in quantal content, which is usually interpreted as being presynaptic.

Each EPSP showed different  $1/CV^2$  plot trajectories but they were usually steep. Some were below the diagonal but none were horizontal or nearly horizontal. This

suggests that the predominant change is presynaptic, although it is impossible to rule out the possibility that some proportion of the change was postsynaptic.

A preliminary search for peaky histograms suggested that they exist but they appeared to be harder to find than in data recorded from area CA1. This might be because of the higher levels of background noise.

### *6.3.6 An EPSP that showed LTP*

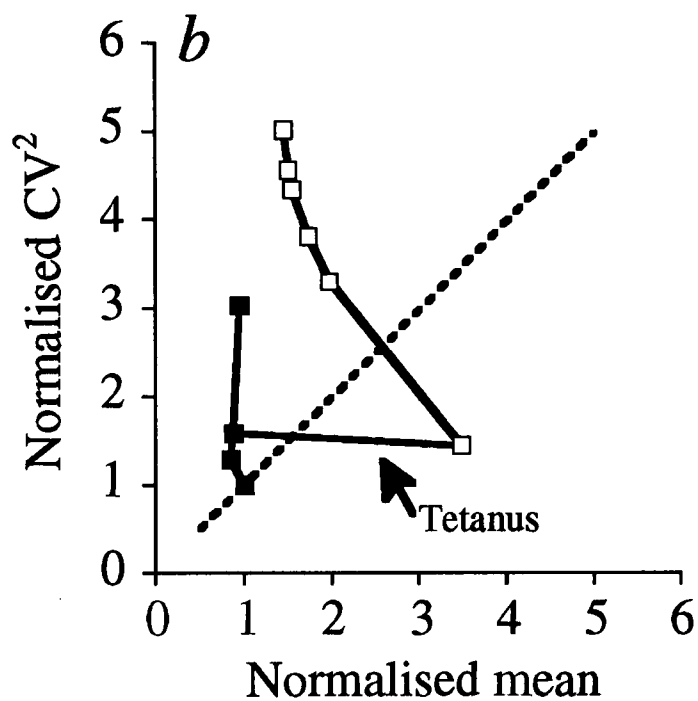
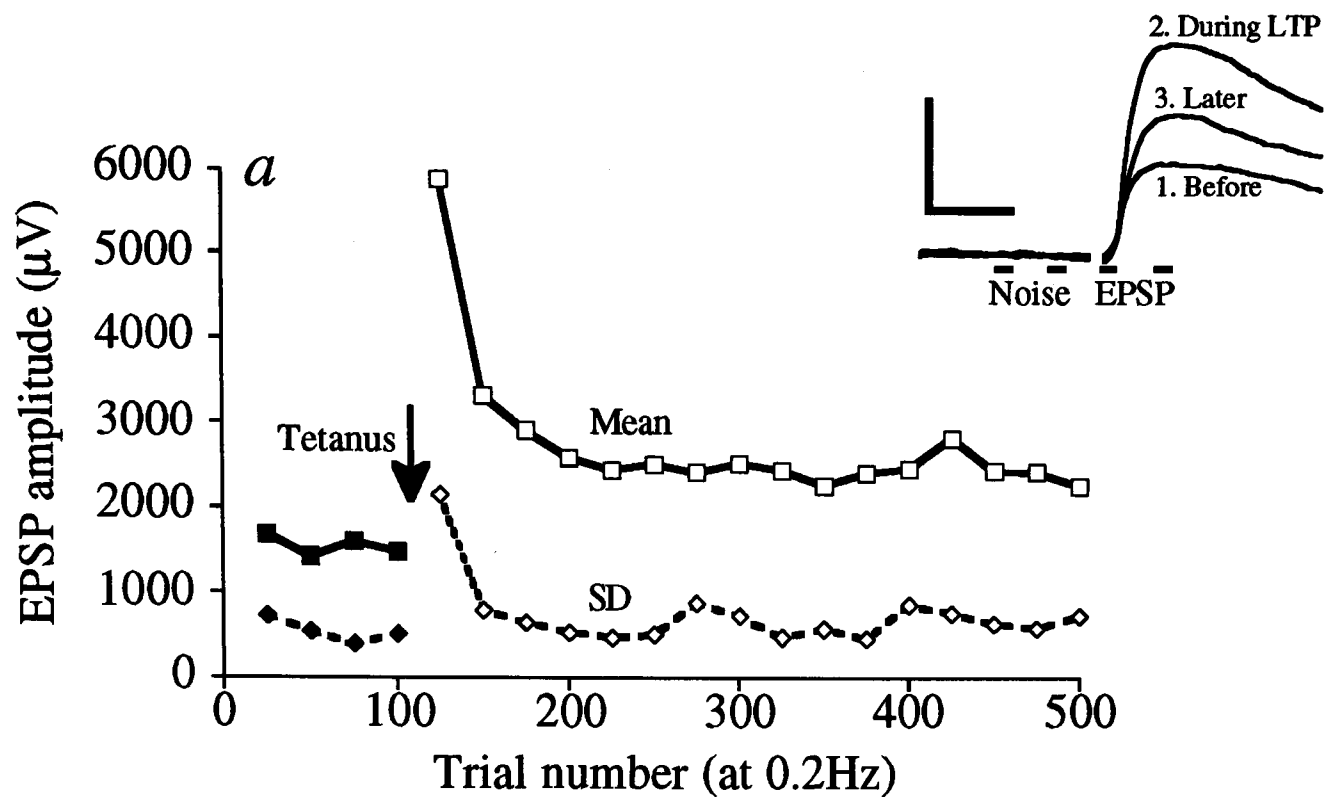
For 4 of these 6 EPSPs, I applied a tetanus<sup>1</sup> after I had lowered the stimulation rate back to 0.2Hz. Of these, 3 (C2, C5 & C6) showed only a very brief enhancement that decayed back to baseline in less than a minute after resuming test stimulation. This could have been PTP. In the other one (C3), the tetanus resulted in a longer-lasting enhancement that resembled LTP.

Figure 6.5a shows the time courses of the mean and SD for this EPSP. The mean was initially about 1,500 $\mu$ V but peaked at nearly 6,000 $\mu$ V immediately after the tetanus. After this, it declined rapidly to settle at about 2,500 $\mu$ V for the rest of the recording period (about 30 minutes). There was an abrupt rise in the SD after the tetanus but within 25 trials (about 2 minutes) it fell back to a level close to its original value.

This gives the  $1/CV^2$  graph an unusual trajectory. It is initially horizontal but this rapidly changes and all of the post-tetanus points after the first one lie above the diagonal. It is possible that at least some of this increase in SD is caused by the rapid change in mean during the period just after the tetanus. As I have discussed above, this might produce a spurious horizontal trajectory. However, this might not explain all of the increase in SD so it is impossible to rule out the existence of a very brief postsynaptic enhancement. Whatever the cause of the horizontal part of the graph, the

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<sup>1</sup>In these cases I used 2 tetani, each of 100 stimuli at 100Hz and separated by 30s.



**Figure 6.5 An EPSP that showed LTP** (a) The mean and SD of an EPSP that showed LTP in response to a tetanus. The initial potentiation is almost four-fold but this declines rapidly to leave a more persistent enhancement of about two-fold. Each point represents 25 trials and the stimulation rate throughout was 0.2Hz. The whole period shown covers about 40 minutes. The inset shows mean EPSP waveforms for all 100 trials before the tetanus (1), the first 100 trials after the tetanus (2) and the next 100 trials, once the potentiated EPSP had reached a stable amplitude (3). Bars underneath the EPSPs show the zones used to measure the amplitude of the noise and EPSP on each trial. Scale bar: 2mV, 10ms. (b) The  $1/CV^2$  graph for this EPSP, showing all 100 pre-tetanus trials (filled squares) and the next 150 trials immediately after the tetanus (open squares). The trajectory is horizontal at first but changes rapidly and all but the first point lie well above the horizontal (dotted line). Each point represents 25 trials.

later points suggest that the sustained component of LTP was due largely or wholly to a presynaptic increase in release probability.

## 6.4 Discussion

### 6.4.1 Frequency facilitation

I have shown data from 6 EPSPs recorded in CA3 pyramidal neurones that showed marked frequency facilitation. Increasing the stimulation rate in the range 0.2-5Hz caused mean EPSP amplitudes to rise and returning the stimulation rate back to 0.2Hz caused it to fall again. The mean during this second period of stimulation at 0.2Hz tended to be a bit lower than during the initial baseline period at the same stimulation rate. This difference might be due to an underlying synaptic depression during continuous synaptic stimulation, like the one that occurs at synapses in area CA1 (see Chapter Three).

The trajectories of the  $1/CV^2$  graphs are consistent with the idea that a presynaptic change underlies this frequency facilitation but do not rule the possibility of a postsynaptic component. The results are less certain than those for LTP (Chapter Four) and STP (Chapter Five) and are not quantitative because I did not use amplitude frequency histograms or constant  $N$  analysis. The fact that the trajectory of the  $1/CV^2$  graphs were sometimes on or below the diagonal suggests that there may have been a postsynaptic component. However, numerous studies have shown that frequency facilitation appears to be wholly presynaptic at the neuromuscular junction (NMJ) and synapses in the central nervous system (CNS) (see Chapter Three), including mossy fibre synapses (Yamamoto, 1982; Regehr & Tank, 1991). It therefore seems more likely that the frequency facilitation I saw is also presynaptic but that simple binomial statistics do not always apply. Trajectories along the diagonal suggest that Poisson statistics could give a better description. This might be expected: the large amounts of frequency facilitation that these synapses show

suggest that release probabilities are low (at least initially) and anatomical studies show that individual mossy fibre presynaptic boutons can contain 30 or more synaptic contacts, each of which may correspond to a single release site (Hamlyn, 1961, 1962; Chicurel & Harris, 1992).

#### *6.4.2 Long-term potentiation*

The  $1/CV^2$  graph of the only LTP example also suggests a largely presynaptic site of expression, although there may be a transient postsynaptic enhancement during the first 2 minutes or so. It is interesting to note that this LTP seems more robust than most of the examples I recorded in CA1. It showed no significant decline over a period of more than 30 minutes. There may be differences in the ways that LTP at these synapses is expressed but the lower test stimulation rate (0.2Hz compared to 1Hz) may also have had an effect.

The fact that I induced LTP in this EPSP with a small ( $< 2,000\mu V$ ) input also provides evidence, albeit weak, in favour of the view that LTP at these synapses is non-Hebbian. However, it would be unwise to conclude too much from this single example alone.

#### *6.4.3 Were these EPSPs produced by mossy fibre inputs?*

The short rise times of these EPSPs and the fact that one of them showed LTP in the presence of AP5 suggests that they might have been produced by mossy fibre synapses. Claiborne *et al.* (1993) have suggested six criteria for identifying mossy fibre inputs:

1. EPSCs should have a smooth rising phase and a rise time of  $< 3ms$  at  $31^\circ C$ .
2. EPSCs should have a smooth exponential decay.

3. Synaptic conductances and decay time constants should be voltage-independent.
4. EPSCs should have the same latency at different stimulation frequencies.
5. LTP induction should change the amplitude but not the shape of the EPSCs.
6. The latency of EPSCs should be  $<3.5\text{ms}$  at  $31^\circ\text{C}$ .

Unfortunately these criteria are based on excitatory postsynaptic currents (EPSCs) rather than voltages so some features do not apply directly to my data. Of the 6 EPSPs I studied, all but one had smooth rising phases and all had smooth decay phases<sup>2</sup>. In no case did the shape of the EPSP change substantially during frequency facilitation or LTP. But, as Claiborne *et al.* (1993) show, a smooth EPSP waveform does not necessarily mean that the associated EPSC is also smooth. The latencies and rise times of my EPSPs are short enough to satisfy these criteria, particularly since voltage changes inevitably lag synaptic currents. Latencies did, however, tend to increase by a small amount when I increased the stimulation frequency. These criteria, therefore, do not confirm that the EPSPs I have described came from mossy fibre synapses but they at least leave the possibility open.

Williams & Johnston (1991) have reported that mossy fibre EPSPs have average rise times of  $5.3\text{ms}$ , compared to  $7.5\text{ms}$  for associational and commissural EPSPs. The 6 EPSPs that I recorded had considerably faster rise times than this.

Figure 6.6 shows that, on average, EPSPs displaying frequency facilitation have shorter rise times than other, randomly selected EPSPs. The sample sizes are small and there is some overlap but the EPSPs do appear to fall into two groups. Since short

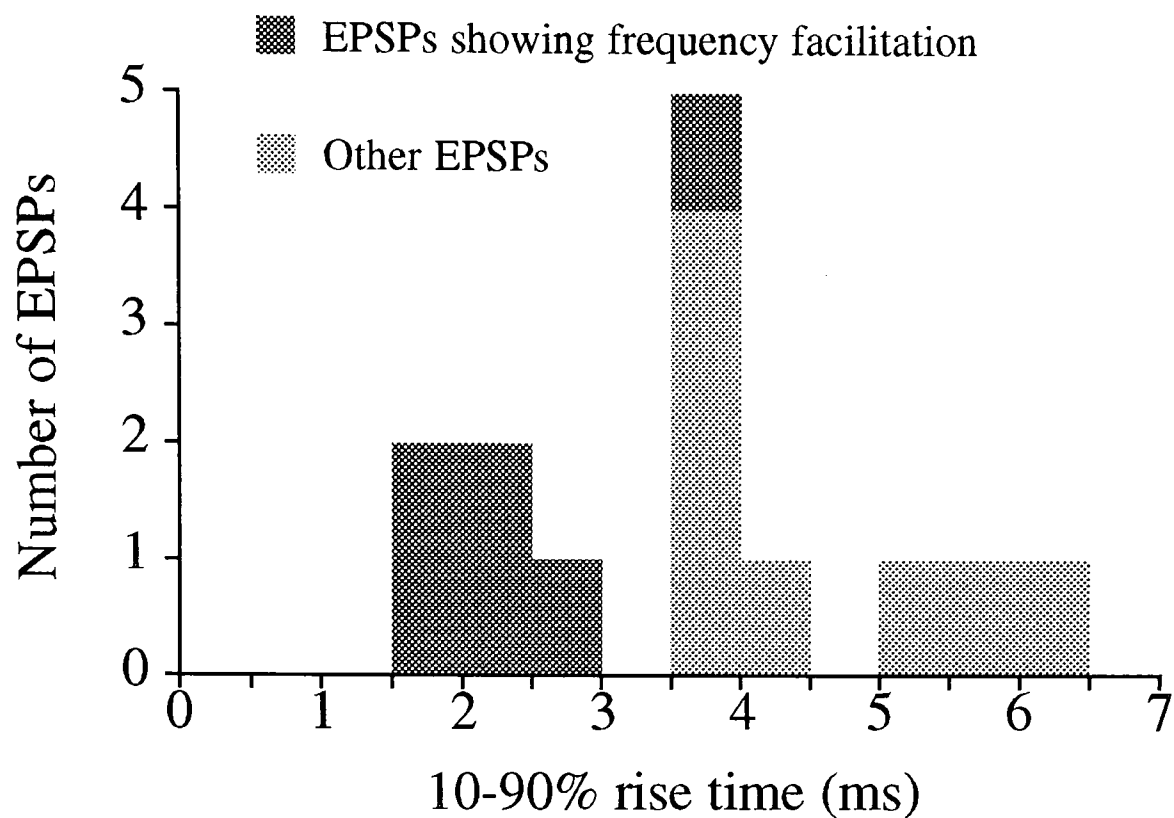
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<sup>2</sup>Although in some cases this was very slow, which might indicate contamination with longer-latency, slower-decaying polysynaptic inputs. The most extreme example of this is shown in the inset of Figure 6.5a.

rise times are thought to be indicative of mossy fibre inputs, it might be that the EPSPs I identified as showing pronounced frequency facilitation are also mossy fibre inputs. This does not necessarily mean that the method I used for selection identifies all mossy fibre inputs. It might select a small subset that, for example, have unusually low release probabilities. This could be one reason why I encountered them so rarely. However, it was my (admittedly subjective) impression that the EPSPs that showed frequency facilitation were a distinct population: it was always easy to distinguish them from other EPSPs because the effect was so pronounced.

If they do represent the whole mossy fibre population, then the question of their rarity remains unanswered. One possibility is that I cut the slices at such an angle that most mossy fibre axons left the plane of the slice before reaching area CA3. I cannot rule this out. Another possibility is that mossy fibre inputs are difficult to find even in slices that have been correctly cut. Given that the connection probability between dentate gyrus granule neurones and CA3 pyramidal neurones is very low (Amaral *et al.* (1990) estimate less than 1 in 20,000 *in vivo*), it is likely that a large number of mossy fibre axons need to be stimulated before one that is connected to the impaled neurone becomes active. This means that a large number of other CA3 pyramidal cells are likely to be stimulated at the same time. Mossy fibre EPSPs are large and it may take only a few to bring these cells to threshold (Jonas *et al.*, 1993) so many CA3 neurones might fire action potentials in response to each stimulus. Since CA3 neurones are highly interconnected (Amaral *et al.*, 1990, estimate a connection probability of about 1 in 80 *in vivo*) this is quite likely to produce an EPSP in the impaled cell via local associational connections. Therefore it is possible that a large proportion—perhaps the majority—of the EPSPs that I recorded were not produced by monosynaptic mossy fibre connections but polysynaptic mossy fibre-associational connections.

Claiborne *et al.* (1993) have suggested that this might be why some groups find that postsynaptic manipulations such as Ca<sup>2+</sup> buffers and voltage clamp do not affect



**Figure 6.6 Rise times of EPSPs that did and did not show frequency facilitation** When they were stimulated at 1Hz, the 6 EPSPs that showed frequency facilitation had rise times in the range 1.6-4.0ms and the average for all 6 was  $2.5 \pm 0.8$ ms. The first 8 EPSPs that I recorded, none of which showed signs of significant frequency facilitation, had rise times in the range 3.6-6.2ms and the average for all 8 was  $4.6 \pm 1.0$ . These two groups are significantly different (Student's t test gives  $p=0.002$ ). Thus EPSPs selected solely on the basis of their ability to show frequency facilitation have significantly shorter rise times than a group of randomly selected EPSPs that do not show frequency facilitation.

mossy fibre LTP (see Introduction, above). These researchers could have been studying polysynaptic associational inputs rather than monosynaptic mossy fibre connections. In this case, the potentiation might have happened not at synapses onto the cell from which they were recording but at intermediate mossy fibre synapses in the polysynaptic pathway. Note, however, that this does not explain how Ito & Sugiyama (1991) could apparently induce LTP in mossy fibre synapses at which the postsynaptic response was temporarily blocked because transmission at intermediate polysynaptic connections would also have been blocked.

My preliminary results are consistent with the idea that extracellular stimulation of dentate gyrus granule cell axons in hippocampal slices might not always reliably induce monosynaptic mossy fibre EPSPs in single CA3 pyramidal neurones. They broadly agree with observations made by Jonas *et al.* (1993), who estimate that they had to stimulate several thousand dentate gyrus granule neurones before they found one that made an apparently monosynaptic mossy-fibre contact with the CA3 neurone from which they were recording. This suggests that care must be taken to ensure that the responses being recorded from hippocampal slices are really coming from mossy fibre synapses.

Many papers describing experiments designed to investigate these connections do not specifically mention any selection of EPSPs, which suggests that some researchers may be assuming that any EPSP recorded in area CA3 and elicited by stimulating in the dentate gyrus or hilus are likely to arise from mossy fibre synapses. My results suggest that this may not necessarily be so. More work needs to be done to confirm whether or not EPSPs that show marked frequency potentiation are really a distinct population and, if so, whether or not they are mossy fibre inputs. If the impression given by my preliminary data is correct, it would suggest that obtaining and identifying clean mossy fibre EPSPs may not be easy. This would make investigations of LTP and other aspects of synaptic transmission at these synapses considerably more difficult than in area CA1.

# Chapter Seven

## Conclusions

### 7.1 Quantal Synaptic Transmission

#### *7.1.1 Transmission in area CA1 can be quantal*

There is still some disagreement as to whether or not synaptic transmission in the central nervous system (CNS) is truly quantal. The quantal hypothesis proposes that postsynaptic responses are made up of discrete, irreducible units. However, it does not predict that, for any particular excitatory postsynaptic potential (EPSP) or current (EPSC), these quanta will necessarily all be of the same size, nor that their sizes will be constant. Neither does it specify the likely size of quanta. This means that even if the theory is correct, not all histograms will have clear, evenly spaced peaks; there are any number of reasons that quantal transmission might produce histograms that are not peaky. These include low signal-to-noise ratios, large quantal variances and non-stationarity. For this reason, the hypothesis is practically impossible to disprove and it has rightly been left to the proponents of quantal transmission to prove their case instead.

Until recently this has been difficult to do, a fact that has been used as evidence against quantal transmission. But as recording and analysis methods have improved, it has become increasingly apparent that, under favourable conditions at least, transmission can be shown to be quantal (see Chapter Three). These findings could have important implications both for the way in which the brain functions *in vivo* and for the methods that we can use to investigate synaptic transmission.

The results of my investigations into long-term potentiation (LTP) and short-term potentiation (STP) in area CA1 of the hippocampus rely on the assumption that

transmission at these synapses is quantal but they also lend support to it. The close agreement between the different analysis methods would be extremely unlikely if transmission were not quantal. Indeed, they go further. The fact that quantal size estimates from histograms were close to the results of 'constant  $N$  analysis' suggests that a model using simple binomial statistics with the number of release sites held constant can describe the behaviour of these synapses with reasonable accuracy, even during abrupt changes in synaptic strength. This does not mean that simple binomial statistics provides a complete description, nor does it mean that the number of release sites never changes at all. Synaptic transmission is almost certainly more complicated. But the fortunate (perhaps surprising) finding that we can model it relatively simply allows us to investigate its properties without fully understanding the detail of the mechanisms involved.

### *7.1.2 Synapses in area CA1 have high release probabilities*

Most of the EPSPs I analysed, particularly the ones recorded in 4mM calcium ( $\text{Ca}^{2+}$ ) had high release probabilities. The average for both LTP and STP experiments was almost exactly 0.5. This would limit the amount of presynaptic enhancement these synapses can show and may explain why I saw very little paired-pulse facilitation (PPF) or post-tetanic potentiation (PTP) (see Chapter Three). These EPSPs did, however, show depression. During 1-4Hz stimulation, their average amplitude fell over a period of minutes, and depression at 4Hz was faster than at 1Hz.

Whether or not the group of EPSPs that I studied is a representative sample of CA1 synapses is not certain. As I discussed in Chapter Four, I may have inadvertently selected EPSPs with relatively high release probabilities so there may be a significant proportion of synapses that have lower release probabilities and show substantial PPF and PTP. However, the fact that compound EPSPs (which were not so highly selected) also failed to show PPF (see Chapter Three), tends to suggest that the effects of EPSP selection are not large.

### 7.1.3 Some CA3 synapses show frequency potentiation

In contrast, a small proportion of synapses onto pyramidal neurones in area CA3 (about 2% in my experiments) showed substantial frequency facilitation, which, on the basis of  $1/CV^2$  graph trajectories, appeared to be a presynaptic enhancement. Considering their fast rise times and relatively large amplitudes, these could be mossy fibre inputs from the dentate gyrus. The frequency facilitation effect was large and could play a significant role in the behaviour of these synapses *in vivo*. The relative scarcity of these EPSPs in my hippocampal slices is consistent with suggestions that uncontaminated mossy fibre EPSPs are difficult to elicit in this type of preparation. This would have implications for the interpretation of the literature that currently exists on these synapses and could hinder their future investigation.

## 7.2 Short-Term and Long-Term Potentiation

### 7.2.1 CA1 synapses show STP and LTP

As I described in Chapter Five, associative, NMDA-receptor-dependent forms of potentiation can be divided into two groups according to their different time courses. LTP lasts for tens of minutes, hours or even days, depending on the recording conditions. STP, on the other hand, typically declines back to baseline within a few minutes.

After giving a tetanus, I saw two forms of potentiation, one that lasted for 2-5 minutes (STP) and another that lasted for over 15 minutes (LTP). These durations are at the short end of ranges reported by other groups. However, this might be expected because I gave test stimuli at a higher rate (1Hz) than most other researchers use. Using the tetanus induction method, both STP and LTP were rare but this is likely to be because I used small EPSPs, which could have made it difficult to depolarise the postsynaptic cell sufficiently to induce potentiation. Indeed, a well-established feature

of LTP is cooperativity, implying that tetanising a weak input alone will not induce potentiation. For this reason, I stimulated a second, larger input during each tetanus. But, although this usually caused the neurone to fire action potentials, it was impossible to know what degree of depolarisation it produced in the region of the dendritic tree at which the test input made contact. The relatively high rate of test stimulation that I used might also have had an adverse effect on the success rate, since previous synaptic activity is known to inhibit LTP induction.

A pairing induction method was much more reliable at producing STP, probably because it produced a more reproducible depolarisation in the postsynaptic cell. However it never produced LTP. This could have been because it involved only low-frequency synaptic transmission. One effect of the larger amounts of neurotransmitter presumably released during a tetanus might be to activate metabotropic glutamate receptors, which appear to enhance LTP induction (see Chapter One).

### *7.2.2 STP and LTP involve both presynaptic and postsynaptic changes*

There has been a great deal of inconsistency in the results of different groups investigating the site of LTP expression. Some find that it is mainly presynaptic and others that it is mainly postsynaptic. Quantal analysis is a method well suited to answering this question but the results of these experiments have also been contradictory. Presynaptic and postsynaptic changes need not be mutually exclusive but only since I began this study have quantal analysis results shown that both types of change can happen together.

I used quantal analysis to investigate the changes underlying STP and the first 15 minutes or so of LTP at synapses in the CA1 area of rat hippocampal slices. Among the EPSPs that showed LTP, I found a wide range of behaviour. Some showed a predominantly presynaptic increase and in others it was largely postsynaptic. Most, however, showed some combination of the two.

The results for STP were remarkably similar to those for LTP. Once again, some EPSPs showed predominantly presynaptic or postsynaptic changes but most showed a combination of both. This suggests that, although they have very different durations, STP and LTP share not only common induction mechanisms, but common expression mechanisms too.

It is important to remember, however, that my results were obtained under a limited set of conditions and do not necessarily reflect the full range of cellular processes that could underlie LTP and STP. In particular, my analysis of LTP was restricted to the first 15 minutes or so after induction, and different mechanisms could be involved at later times.

### *7.2.3 The type of change depends on initial P*

For both STP and LTP, the amount by which the release probability and the quantal size changed was correlated with the initial release probability. EPSPs for which the release probability was low showed a substantial increase in this probability and little change in quantal size. On the other hand, those at which the release probability was already high showed little change in release probability but a much greater increase in quantal size. Thus whether potentiation is mainly presynaptic or postsynaptic seems to depend on the initial 'setting' of the synapse. This is in broad agreement with results published by two other groups (Kullmann & Nicoll, 1992; Liao *et al.*, 1992) after I had carried out this work.

It might therefore be possible to take a synapse with a low release probability and induce potentiation in two stages, first by increasing the release probability and then by increasing the quantal size. Sufficiently long or vigorous induction methods might produce both types of change together. This means that the correlation with initial release probability might not hold if the induction methods are particularly potent and produce very large potentiation. This could apply to the methods of extreme

depolarisation for relatively long periods used by some researchers recording with patch-clamp electrodes (see Chapter Four).

As I described in Chapter Four, the initial setting of synapses can be influenced by the incubation conditions used in the experiment. Synapses bathed in a lower calcium ( $\text{Ca}^{2+}$ ) concentration tend to have lower initial release probabilities and show predominantly presynaptic forms of potentiation. Since individual groups often use different  $\text{Ca}^{2+}$  concentrations, this may go some way to explaining why they seem to get different forms of potentiation.

#### *7.2.4 Possible mechanisms involved in LTP and STP induction*

Why should the site of the change that underlies STP or LTP depend on the initial release probability of the synapse? For a given induction method, the release probability presumably affects how much  $\text{Ca}^{2+}$  will enter the postsynaptic cell during induction. A synapse with a higher release probability will release neurotransmitter on a greater proportion of trials, so NMDA-receptor ion channels will open more frequently and allow more  $\text{Ca}^{2+}$  into the neurone. It is possible, then, that the smaller amount of  $\text{Ca}^{2+}$  that enters the postsynaptic cell when release probability is low triggers only the processes (e.g. retrograde messenger production) that lead to a presynaptic change. When the release probability is high, the larger amount of  $\text{Ca}^{2+}$  that enters the postsynaptic cell might trigger a postsynaptic change (e.g. receptor phosphorylation). Presumably this would be accompanied by messages that normally bring about presynaptic enhancement but, with release probability already high, the presynaptic cell might be unable to respond.

However, it may be wrong to attribute all of the variation seen in the size, duration and locus of potentiation to  $\text{Ca}^{2+}$  levels in the postsynaptic cell. STP, as well as LTP, can be either presynaptic or postsynaptic, or both. This suggests that, on these timescales at least, the locus of potentiation is largely independent of its ultimate duration. As I described in Chapter Five, the initial degree of potentiation is also, to

some extent, independent of its duration. It seems unlikely, then, that all of these aspects could be controlled by the same intracellular signal, and signals other than  $\text{Ca}^{2+}$  are almost certainly involved. The identity of these remains to be discovered but intracellular and intercellular messenger molecules such as cyclic adenosine monophosphate (cAMP), inositol trisphosphate ( $\text{IP}_3$ ) and nitric oxide (NO) are obvious candidates.

### *7.2.5 Possible mechanisms involved in LTP and STP expression*

The evidence that I described in Chapter One currently seems to favour a role for protein kinase C (PKC) in enhancing the amount of transmitter released from the presynaptic terminal and a role for calcium/calmodulin-dependent protein kinase II (CaMK II) in phosphorylating postsynaptic glutamate receptors, thus making them more sensitive to neurotransmitter. The possibility that different protein kinases might be involved in the expression of potentiation in the presynaptic and postsynaptic neurones suggests an interesting possibility. It might be that apparent inconsistencies in the literature regarding the importance or otherwise of these molecules in LTP come about because different laboratories use experimental conditions that favour predominantly presynaptic or predominantly postsynaptic LTP.

The fact that LTP and STP seem to share similar induction and expression properties argues strongly in favour of common mechanisms being involved. As I described in Chapter Five, a greater level of postsynaptic  $\text{Ca}^{2+}$  seems to be required for LTP induction than for STP induction. This suggests that the same process or processes could be activated but the higher level needed to induce LTP might activate them to a threshold beyond which they become 'self-perpetuating'.

In this regard, the ability of CaMK II to autophosphorylate and become  $\text{Ca}^{2+}$ -independent would seem to make it ideally suited (see Chapter One). The level of  $\text{Ca}^{2+}$  needed to induce STP might activate CaMK II insufficiently for autophosphorylation to outweigh the effects of phosphatase activity, which would

tend to dephosphorylate them. The higher level of  $\text{Ca}^{2+}$  needed to induce LTP, on the other hand, might activate CaMK II effectively enough for the rate of autophosphorylation to exceed the rate at which CaMK II molecules become dephosphorylated. In this way, a population of  $\text{Ca}^{2+}$ -independent CaMK II molecules could, in principle, be maintained for long periods. As I have discussed in Chapter One, PKA phosphorylates non-NMDA receptors and might be involved in mediating LTP expression despite the fact that it is activated by the second messenger cyclic-AMP (cAMP) rather than by  $\text{Ca}^{2+}$  directly. Interestingly, protein kinase A also shows the ability to phosphorylate its own regulatory subunits and become constitutively active. Whether or not PKC (or whatever else might be responsible for presynaptic enhancement) can show similar properties remains to be seen.

Even smaller influxes of  $\text{Ca}^{2+}$  into the postsynaptic cell than are required to produce STP seem to trigger long-term depression (LTD) (reviewed by Malenka & Nicoll, 1993). This is consistent with the results described in Chapter Three, in which a component of the depression seen during stimulation at constant frequency appears to be blocked by the NMDA receptor antagonist, AP5. One possibility is that the AP5-sensitive component of the depression is caused by the activation of phosphatases (Mulkey *et al.*, 1993) that antagonise the activities of protein kinase. The remaining, AP5-insensitive component could involve processes such as transmitter depletion from the presynaptic terminal or receptor desensitisation.

### *7.2.6 Presynaptic and postsynaptic changes are rapid*

As I pointed out in Chapters Four and Five, the presynaptic and postsynaptic changes that underlie STP and LTP seem to be effective within seconds. This agrees with similar findings by other groups (e.g. Malinow & Tsien, 1990; Kullmann & Nicoll, 1992), who also report abrupt changes on both sides of the synapse. In contrast, drugs that inhibit protein kinases leave the first few minutes of potentiation intact and only abolish later phases of LTP. The same is true of drugs that inhibit putative retrograde

messengers. Assuming that these drugs are fully effective in blocking their targets (which is not certain) this means that none of these molecules appear to be involved in the changes that underlie STP and the early stages of LTP, so new, as yet unidentified, candidates might have to be sought.

### 7.2.7 *The relevance of these results in vivo*

My work, like most investigations of synaptic transmission in the hippocampus, involved slices of tissue kept *in vitro*. This obviously raises the question of how relevant these findings are to synaptic transmission and plasticity in living animals.

The conditions in the recording chamber are designed to mimic conditions *in vivo* reasonably closely but there are many potentially important differences. The most obvious is that in making the slice, the structure of the hippocampus has been disrupted. Excitatory, inhibitory and modulatory inputs are severed and extracellular levels of important substances such as glutamate and oxygen could reach pathologically high or low levels.

The composition of the ACSF (see Chapter Two) is similar but not identical to that of the cerebrospinal fluid (CSF) in a living mammal (given in Rowland *et al.*, 1991). For the purposes of my experiments, I added picrotoxin to the ACSF to abolish inhibitory responses and, in some cases, I added AP5 too. I usually also increased the  $\text{Ca}^{2+}$  concentration from 2.5mM, which is close to the physiological level, to 4.0mM. This generally gave more stable impalements and allowed me to investigate the effect of different  $\text{Ca}^{2+}$  concentrations.

The other changes were not strictly necessary for the purposes of the experiment but were designed to counteract the consequences of some of the changes that were. Thus I raised the glucose concentration from its *in vivo* level of about 3mM to 10mM. This was to ensure an adequate energy supply to the cells. I also reduced the temperature slightly from 37°C to 34°C, at which the slices tend to live longer. The magnesium

(Mg<sup>2+</sup>) concentration was much higher than *in vivo* (2.5 or 4.0mM compared to about 0.3mM). This was to suppress neurotransmitter release and hence reduce the amount of uncontrolled nervous activity that otherwise happens in the presence of picrotoxin and high Ca<sup>2+</sup> concentrations. The levels of the other principle inorganic ions—in particular sodium (Na<sup>+</sup>), potassium (K<sup>+</sup>) and chloride (Cl<sup>-</sup>)—were close to *in vivo* levels and the overall osmolarity of the ACSF was close to that of CSF.

Although it is impossible to rule out, the glucose concentration seems unlikely to have had any significant effect on the results. The effect of temperature might be important but is likely to be quantitative rather than qualitative, although there are cases in which temperature can apparently have profound effects on the nature of LTP (Williams *et al.*, 1993b).

The changes in Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations might also have affected the type (and not just the size or duration) of the changes I saw. Indeed, my results show that changes in Ca<sup>2+</sup> concentration can do just this by influencing the initial release probability of synapses. In most of my experiments, I used a higher concentration of Ca<sup>2+</sup> than is found *in vivo* so it might be that my results overestimate the initial release probability. In doing this, they will tend to overestimate the average amount of postsynaptic change and underestimate the amount of presynaptic change. This effect might have been increased further by the possibility that I inadvertently selected synapses with high initial release probabilities (see above). However, it seems unlikely that all postsynaptic changes are artefacts of the high Ca<sup>2+</sup> concentration I have used. Two of the three EPSPs I recorded in a low Ca<sup>2+</sup> concentration did show some postsynaptic increase and the one in which it was largest had the highest initial release probability.

Intuitively, it seems sensible for a synapse to be able to change both its release probability and its quantal size. In principle, synapses that show presynaptic and postsynaptic changes can express a wider range of strengths than synapses at which

the change is limited to one side only. There may also be more subtle implications, which I will discuss later.

### 7.2.8 *The importance of STP and LTP in vivo*

Although the evidence is circumstantial, LTP or LTP-like processes do seem to play a role in certain types of memory (see Chapter One). There are some holes in the argument and the current evidence suggests that LTP is, at most, only partly responsible for memory in the mammalian brain. But most neuroscientists remain convinced that it is worth studying. Perhaps the single most important reason is the elegant way in which the NMDA receptor confers associativity on LTP. It simply seems too good not to be true.

What of STP? It shares many properties with its better-known relative, including associativity, but it has attracted much less attention. As I described in Chapter Five, this is largely because of confusion over exactly what STP is. Now that we know a little about it, we might be encouraged to find out still more. In some ways the brevity of STP makes it more difficult to study but there is also the prospect that it can tell us more about LTP. Indeed, a complete theory of LTP will almost certainly have to encompass STP and long-term depression (LTD) as well.

Whether STP has any more or less to do with memory than LTP is debatable. STP is induced by milder procedures so it might be more common *in vivo* than LTP is. But its shorter duration seems to make it less attractive as a physiological basis for remembering. I have two reservations about this view. The first is that, even if STP is responsible for comparatively transient memories, ignoring it in favour of LTP is a blinkered approach because short-term memories are as much a part of remembering as long-term memories are. In any case, LTP can hardly be said to explain memories that are long-term in the psychologist's sense of the word. In these terms, memories can last for anywhere between fractions of a second and decades. Within this broad

spectrum, the difference between the durations of STP and LTP looks less profound and it becomes obvious that they actually cover very similar ground.

The second is a tentative suggestion that we don't know enough about how memories are stored in the brain to assume with any confidence that the duration of a change in the strength of a particular synapse necessarily determines the duration of the associated 'memory'. Given the fact that memories appear to be able to move from one site in the brain to another over periods of weeks (e.g. from the hippocampus to the neocortex, see Chapter One), it seems not inconceivable that they might do so over much shorter periods as well. If this is the case, the time it takes for STP or LTP to decay need not determine the persistence of a particular memory any more than the length of an action potential determines the duration of a particular 'thought'. Thus STP is likely to be just as important as LTP when it comes to understanding how synapses and, by extension, whole brains behave.

## 7.4 The Broader Picture

### *7.4.1 The importance of quantal transmission in brain function*

Although random quantal transmission was discovered at the NMJ, it is unlikely to have much functional importance there. The role of the NMJ is not to process information but to send an unambiguous all-or-nothing signal. Perhaps because of this, large trial-to-trial variations in the postsynaptic response are only seen under conditions in which transmitter release is artificially suppressed (see Chapter One). In the CNS, however, transmission seems to vary greatly between successive trials, even under conditions similar to those in living animals. This is not just useful for scientists who want to study synaptic plasticity, it is likely to have profound implications for brain function. This is a poorly understood but potentially important point. The elements that make up the brain (synapses) behave in an unpredictable way but, in

contrast, their collective output (behaviour) is often stereotyped and highly predictable, even in higher animals.

The random, quantal nature of synaptic transmission usually seems to be ignored for the purposes of neural network simulations. This could be a fundamental oversight. In order to understand how important it might be, it is essential to know whether or not random synaptic transmission results in any significant amount of randomness at the level of the whole cell. In other words, do the vagaries of individual synapses lead to unpredictability in the firing of action potentials. This depends on the number of quanta needed bring the cell to the threshold for firing action potentials. If this number is very large then the amount by which a near-threshold response will vary from trial to trial is likely to be quite small. If, however, the number is relatively low then the variability (or, to be precise, the coefficient of variation) of a near-threshold response will be correspondingly larger and, as a result, the firing of the neurone will, to some extent, be random.

This is difficult to calculate because quanta vary in size and may add together non-linearly. We also have little idea of the resting potentials or input resistances of neurones *in vivo*. Andersen (1987) has estimated that about 100 incoming axons need to be stimulated to make a CA1 pyramidal cell reach threshold. Assuming that the mean quantal content of each single-axon response is not very small (i.e. not  $\ll 1$ ), this suggests that of the number of quanta needed to bring the neurone to threshold is quite high. But the input resistance of neurones *in vivo* is likely to be much higher than it is during intracellular recordings (Spruston & Johnston, 1992) so Andersen's figure could be an overestimate. Otmakhov *et al.* (1993) estimated that only about 14 quanta are needed to bring a CA1 pyramidal neurone to threshold and showed that near-threshold responses can be variable enough to cause apparent randomness in the firing of action potentials.

### *7.4.2 The importance of presynaptic and postsynaptic changes in brain function*

How learning affects the behaviour of networks is an important extension to these kinds of investigations. If the CNS really does use processes similar to STP and LTP to store information then finding out whether they are presynaptic or postsynaptic might be important for understanding networks and therefore large-scale brain function. As McNaughton (1993) has pointed out, an increase in quantal size simply increases the average strength of a synapse but an increase in release probability (or the number of release sites) changes the behaviour of the synapse so as to make it both stronger and more reliable. Otmakhov *et al.*, (1993) find LTP to be wholly or mainly presynaptic and show that, at the level of single neurones, this has the effect of keeping the trial-to-trial variability at threshold almost constant even though the threshold itself has changed. My results suggest that changes in release probability and quantal size depend on the initial setting of the synapses and further suggest that the initial release probability of the synapse is a particularly important factor in determining the degree of presynaptic and postsynaptic change. It would be interesting to investigate what effects this kind of rule has on the behaviour of neural networks.

### *7.4.3 Tackling the intangible*

I began this thesis with a quote from an 19th century novelist who expressed wonder at the human memory but was pessimistic about ever understanding it. Though our sense of awe is undimmed after 180 years, 20th century neuroscientists are more hopeful that we can understand (or at least think we understand) what memory is and how it works. Unravelling a psychological phenomenon at the cellular and molecular level would be an intellectual triumph with few equals and could also have important medical implications. Perhaps uniquely among scientific disciplines, neuroscience has the potential both to improve people's lives and to tackle the most perplexing

philosophical issues. Even as they struggle to find answers to the small questions that confront them every day, neuroscientists should not lose sight of the larger questions that make their subject so fascinating and important.

The mind-body problem has occupied some of the greatest minds since at least the time of Plato and Aristotle and is at the heart of neuroscience. Dualism, in which the mind is proposed to be metaphysical and beyond the scrutiny of science (e.g. Popper & Eccles, 1977), is almost dead. The struggle now moves on to replace it with a coherent materialist alternative that can adequately describe not only how the brain produces behaviour but also how it gives rise to the subjective mental world and unitary 'self' that we each experience. The ultimate goal of neuroscience must be to provide an answer. Whether or not this is possible is in doubt. The brain struggling to understand the world around it is one thing, the brain struggling to understand itself quite another. But to concede this is not to give up the hunt. In fact, it is what makes the brain a uniquely fascinating object of study. Insignificant though it may be in these terms, this thesis is my contribution.

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