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Presynaptic modulation of synaptic efficacy in the rat hippocampus

Thesis submitted for the degree of *Doctor Philosophiae*

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NOTES

The work described in this dissertation was carried out at the International School for Advanced Studies, Trieste, between November 1998 and July 2002. All work reported, with the exceptions listed below, arises from my own experiments and data analysis.

The study on silent synapses (Part 1 of Results) was done in collaboration with Sonia Gasparini (SISSA), who did most of the experiments from CA3 neurones and fEPSPs recordings from CA1.

The data reported in the present thesis have been published or submitted in the articles listed below:

Gasparini S., Saviane C., Voronin L.L. & Cherubini E. (2000). Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? *PNAS* 97: 9741-9746.

Saviane C., Savtchenko L.P., Raffaelli G., Voronin L.L. & Cherubini E. Frequency-dependent shift from paired-pulse facilitation to paired-pulse depression at unitary CA3-CA3 synapses in the rat hippocampus. *J. Physiol.* (In press).

Saviane C. & Cherubini E. An I_D-like conductance down-regulated by calcium modulates temporal coding and transmitter release at unitary CA3-CA3 connections in the rat hippocampus. Submitted.

ABBREVIATIONS

4-AP:	4-aminopyridine
ABC:	avidin-biotinilated complex
ACSF:	artificial cerebrospinal fluid
AHP:	afterhyperpolarisation
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AP5:	2-amino-5-phosphopentanoic acid
BAPTA:	Bis(2-amino-5-bromophenoxy)ethane-N,N,N',N'-tetraacetic acid
$[Ca^{2+}]_i$:	intracellular calcium concentration
CaMKII:	α-calcium-calmodulin-dependent protein kinase II
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
CNS:	central nervous system
CPP:	3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid
CTZ:	cyclothiazide
CV:	coefficient of variation
DCG-IV:	(2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine
DG:	dentate gyrus
DIV:	days *in vitro*
EGTA:	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EPSC:	excitatory postsynaptic current
GABA:	γ-aminobutyric acid
G-protein:	GTP-binding protein
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I_D:	"delay" current
LFD:	low-frequency depression
LTD:	long-term depression
LTP:	long-term potentiation
MF:	Mossy fibres
mGluR:	metabotropic glutamate receptor
N:	number of releasing sites
NMDA:	N-methyl-D-aspartate
NMJ:	neuromuscular junction
NSF:	N-emthylmaleimide-sensitive ATP-ase

p:	probability of release
P:	postnatal day
PBS:	phosphate buffer saline
PFA:	paraformaldehyde
PPD:	paired-pulse depression
PPF:	paired-pulse facilitation
PPR:	paired-pulse ratio
Q:	*quantal size*
QX-314:	N-(2,6-dimethylphenylcarbamoylmethyl) triethylammoniumbromide
RRP:	readily releasable pool
SC:	Schaffer collaterals
TTX:	tetrodotoxin

ABSTRACT

Modifications of synaptic efficacy play a crucial role in information processing in the brain. In particular, they are thought to be very important for the refinement of neural circuitry, information storage, learning and memory. Therefore, investigating the mechanisms that modulate synaptic transmission is of fundamental importance for understanding brain functions. In the present study, patch-clamp recordings were performed in order to further investigate synaptic transmission in the hippocampus, focusing on different presynaptic mechanisms that may affect synaptic efficacy.

In the last decade, silent synapses and their activation during postnatal development and plasticity processes, such as LTP, have attracted particular attention. These synapses are called silent because they do not respond at rest but are active at positive membrane potentials and can be converted into functional synapses by pairing presynaptic stimulation with postsynaptic depolarisation. A widely accepted interpretation is that N-methyl-D-aspartate (NMDA) but not α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are functionally expressed on the subsynaptic membrane. Thus, wakening of silent synapses during LTP induction would be due to the activation of new functional AMPA receptors. However, most of these studies have been done in the CA1 region of the hippocampus. Therefore, the first part of this study was focused on testing whether a similar kind of connections is present also in the CA3 region during postnatal development. Excitatory postsynaptic currents (EPSCs) were recorded from CA3 pyramidal neurones, following minimal stimulation (0.05 Hz) of Mossy fibres (MF) inputs in acute hippocampal slices from new-born rats. Although 16% of synapses appeared silent at 24°C, AMPA receptors were found to be functionally expressed on CA3 neurones. In fact, different tools known to increase the probability of release, such as paired-pulse stimulation (50 ms interval) or increasing the temperature (from 24°C to 32°C), were able to activate silent synapses. Similar results were obtained at Schaffer collateral (SC)-CA1 connections where 36% of synapses were found silent, but AMPA-mediated synaptic responses could be detected after increasing the probability of release by paired-pulse stimulation, by raising the temperature or by the application of cyclothiazide (CTZ), a drug known to block AMPA receptor desensitisation and to increase transmitter release. Overall, these results show that, in both CA3 and CA1 region of the hippocampus, AMPA receptors are expressed and functional on the subsynaptic membrane already at early stages of postnatal development. However, some synapses

may appear silent because of a very low probability of glutamate release, as they can be converted into functional ones by factors that enhance release probability. Although it cannot be excluded that "latent AMPA receptors" can become functional following activity dependent processes, these results clearly indicate that in the neonatal hippocampus a proportion of glutamatergic synaptic connections are "presynaptically" rather than "postsynaptically" silent. Moreover, conducting synapses could be switched off by increasing the frequency of stimulation from 0.05 to 0.1-1 Hz, suggesting a critical role for use-dependent modulation of synaptic efficacy.

To better investigate how activity can regulate synaptic efficacy, eventually leading to synaptic depression and silencing, hippocampal organotypic slice cultures were used. Taking advantage of the high connectivity between neurones, double-patch clamp recordings between interconnected pairs of CA3 pyramidal cells were performed to study the properties of short-term depression occurring in these synapses under different frequencies of presynaptic firing. In stationary conditions (0.05-0.067 Hz) pairs of presynaptic action potentials (50 ms apart) evoked EPSCs whose amplitude fluctuated from trial to trial with occasional response failures. Increasing stimulation frequency from 0.05-0.067 Hz to 0.1-1 Hz induced low-frequency depression (LFD) of EPSC amplitude with a gradual increase in the failure rate, suggesting the involvement of presynaptic mechanisms. Overall, 75% of cells became almost "silent" at 1 Hz, whereas recovery from depression could be obtained by lowering the frequency of stimulation to 0.025 Hz. Surprisingly, when the firing rate was sequentially shifted from 0.05 to 0.1 and 1 Hz, changes in synaptic efficacy were so strong that the paired-pulse ratio (PPR) shifted from paired-pulse facilitation (PPF) to paired-pulse depression (PPD). These results can be explained by a model that takes into account two distinct release processes, one dependent on the residual calcium and the other on the size of the readily releasable pool (RRP) of vesicles. According to this model, the depletion of the RRP of vesicles accounts for LFD and for the unexpected shift from PPF to PPD.

The possibility of switching between functional and non-functional synapses might play a crucial role in controlling the communication between neurones as well as network synchronisation. The CA3 hippocampal region is known to act as the pacemaker for the generation of synchronised activity, mainly because of the dense network of collaterals of axons interconnecting pyramidal neurones. This network is under control of both extrinsic factors, such as neurotransmitters and neuromodulators, and active conductances. Thus, in

the last part of the study, the role of a voltage-dependent, fast activating and slowly inactivating potassium current similar to I_D in controlling temporal coding and synaptic strength at CA3-CA3 connections was investigated. As I_D, this current is responsible for the delayed appearance of the first spike upon prolonged membrane depolarisation and for action potential repolarisation. Moreover, it could be blocked by low concentrations of 4-aminopyridine (4-AP), a drug known to generate interictal discharges that can propagate from the CA3 region to the entire hippocampus. Interestingly, the I_D-like current, was down-regulated by intracellular calcium, as demonstrated by the observation that Cd^{++}, a blocker of voltage-dependent calcium channels, significantly increased the delay of the first spike generation. Suppressing I_D by low concentration of 4-AP reduced this delay and, by broadening the presynaptic action potential, increased synaptic strength. Thus, it is likely that modulation of this current by fluctuations in resting membrane potential or intracellular calcium concentration, for example during activity-dependent processes, may play a critical role in determining information coding and network synchronisation in the CA3 region.

INTRODUCTION

1. Synaptic transmission

Multicellular organisms depend on the ability of individual cells to communicate with each other. The nervous system and especially the brain consist of ensembles of cells that communicate in a fast, accurate and modifiable way. Electrical signals can travel along the processes of a single neurone over considerable distances (from tens of micrometers up to meters). Therefore, information is transferred from the signal-generating cell (presynaptic) to the receiving one (postsynaptic) at specific contact sites termed synapses (from the Greek "syn" meaning "together" and "haptein" meaning "to clasp"). Synaptic contacts between neurones occur primarily between small swellings, known as *boutons*, either at the terminal or along (*en passant*) axonal profiles of the presynaptic neurone and small fingerlike processes (spines) of postsynaptic dentrites (axospinus synpases). However, other types of synapses have been characterised and have been termed depending on the contact elements (for example axosomatic, axoaxonic, dendrodendritic). Two structurally and functionally distinct forms of synapses exist: electrical and chemical. At electrical synapses, specialised channels (gap junctions) form a direct electrical connection between the presynaptic and the postsynaptic neurones. At chemical synapses, the cells are electrically disconnected from one another: the electrical signal is translated into a chemical one in the presynaptic neurone and only afterwards is re-converted to an electrical signal in the postsynaptic cell. Electrical synapses have the virtue that transmission occurs without delay, but they are far less rich in possibilities for adjustment and control than chemical synapses, that, in fact, represent the predominant way of communication between neurones.

1.1 Chemical synapses

Chemical synapses operate through the release, from the presynaptic neurone, of a neurotransmitter that diffuses in the synaptic cleft and provokes electrical changes in the postsynaptic cell. Neurotransmitter molecules are initially stored in the synaptic vesicles described, long time ago, by ultrastructural studies (Palade 1954; Palay, 1954). Most vesicles are retained in a large pool behind the plasma membrane, whereas a small part of them approaches the presynaptic membrane, and eventually fuse with it, through a

particular cycle that can be divided in several steps and involves a very large number of proteins (reviewed in Südhof, 1995). The vesicles immediately available for release belong to the so-called readily releasable pool of vesicles (Rosenmund & Stevens, 1996; Südhof, 2000). Synaptic vesicles are usually anchored to a network of cytoskeletal filaments by synapsins, a family of protein presenting phosphorylation sites for both cAMP and calcium-calmodulin dependent protein kinases. Phosphorylation is able to free vesicles from the cytoskeleton constrain, allowing them to move into the active zone, where they dock through the interaction between proteins in the vesicular membrane and proteins in the plasma membrane. Candidates for these protein-protein interactions include the vesicular membrane proteins synaptotagmin and Rab3 (Benfenati *et al.*, 1999). After docking, synaptic vesicles go through a maturation process, known as priming, during which a highly stable core complex is formed between the synaptic vesicle protein VAMP/synaptobrevin and the presynaptic membrane proteins syntaxin and SNAP-25. These three proteins are known as SNAP receptors or SNAREs, as they form a high affinity binding site for cytosolic α-SNAP (soluble NSF attachment protein), which itself becomes a receptor site for NSF (N-emthylmaleimide-sensitive ATP-ase). Under steady-state conditions, specific protein interactions, between VAMP and synaptophysin at the vesicle membrane and between syntaxin and munc 18 (or munc 13) at the plasma membrane, inhibit the formation of the core complex that would otherwise assemble outside the active zones (Thomson, 2000). The "unlocking" of these proteins occurs during docking and is modulated by phosphorylation and by calcium, suggesting that it may be an important rate-dependent step in the supply of fusion-competent vesicles during repetitive activity. Once the fusion core complexes are formed, they have to be disassembled for release to occur. The hydrolisis of ATP by NSF provides the energy required and regenerates the SNARE monomers that will be used in the next cycle. Rapid fusion and exocytosis are triggered by high local calcium concentration during the action potential invasion. Ca^{2+} ions act in a co-operative way, as judged from the steep relationship between change in intracellular calcium concentration ($[Ca^{2+}]_i$) and transmitter release, thus accounting for a considerable margin of safety for synaptic transmission (Dodge & Rahamimoff, 1967). Different lines of evidence suggest that synaptotagmins function as Ca^{2+} sensors in the final fusion step (Südhof, 1995). According to the classical model, during exocytosis, vesicles collapse completely into the plasma membrane ("total fusion") and release neurotransmitter in the synaptic cleft.

During this process Rab proteins dissociate from the vesicle membranes and may retard the activation of neighbouring vesicles, resulting in release site refractoriness (Geppert *et al.*, 1997). Therefore, empty vesicles internalise slowly (endocytosis) at sites distant from the active zones and translocate into the interior for endosomal fusion. New vesicles accumulate again neurotransmitter by means of an active transport driven by an electrochemical gradient created by a proton pump. Finally, filled vesicles translocate back to the active zones and the vesicle cycle ends. In recent years, this cycle model has been revisited and an alternative fusion process has been proposed. It suggests a transient state of the vesicle fusion, known as "transient fusion" or "kiss and run", a concept already proposed in the 1970s (Ceccarelli *et al.*, 1973). According to this hypothesis, vesicles fuse with the plasma membrane by forming a transient fusion pore while preserving vesicle integrity. After detachment, vesicles can fuse again without any preceding endosomal fusion. The advantages are a rapid cycling between fusion and non-fusion state and a reduced energy consumption. Applications of fluorescent and atomic force microscopy have provided evidence for the kiss and run model in non-neuronal and neuronal cells (Schneider, 2001).

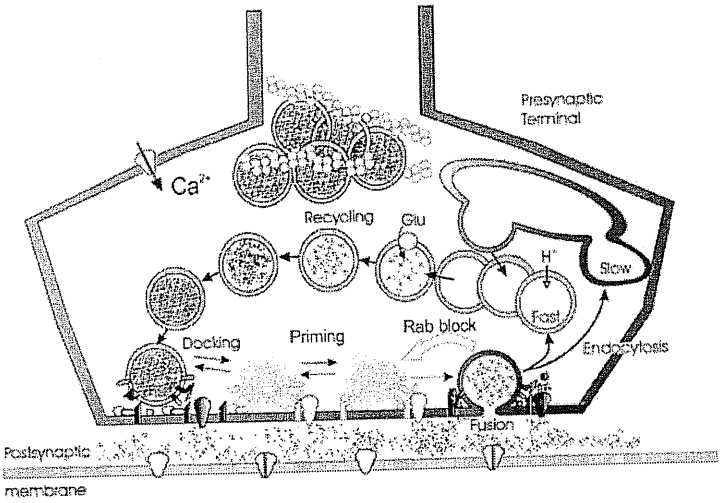

Figure I. Main steps in neurotransmitter release at chemical synapses. A small part of synaptic vesicles approaches the plasma membrane and, through docking and priming processes, gets ready for being rapidly released. The influx of Ca^{2+} ions during action potential invasion triggers vesicle fusion. Thus, the neurotransmitter released diffuses across the narrow synaptic cleft and acts on postsynaptic receptors. Following or during fusion, Rab proteins dissociate from the vesicle and may in some way retard the activation of neighboring vesicles (resulting in release site refractoriness). Synaptic vesicles are therefore recycled, in either slow or fast way, and may accumulate neurotransmitter again. (Modified from Thomson, 2000)

Once neurotransmitter is released in the cleft, it diffuses to act mainly on ionotropic or metabotropic receptors that are clustered in an electron-dense thickening of the postsynaptic membrane, known as postsynaptic density. Then neurotransmitter is rapidly eliminated by diffusion, enzymatic degradation or by uptake into nerve terminals or glial cells. Ionotropic receptors are ion channels that open when they are bound by neurotransmitter molecules, allowing ions to flow within the cell membrane. This process changes the membrane potential of the postsynaptic neurone either in the positive (depolarisation) or in the negative direction (hyperpolarisation). When receptors are permeable to calcium ions, this flow can also activate intracellular processes. After opening, postsynaptic receptors normally desensitise, i.e. they remain in an "open state" without allowing ion flux, until they recover and restore their initial condition. Alternatively the transmitter may bind to metabotropic receptors that are linked to ion channels through GTP-binding proteins (G-proteins). In the mammalian brain, glutamate and γ-amonibutyric acid (GABA) represent the principal excitatory and inhibitory neuotransmitters, respectively (see section 2.3).

1.2 Time course of synaptic signalling

The time course of postsynaptic responses depends on the synchrony of transmitter release, on the time course of transmitter concentration in the cleft as well as on the gating properties of receptors (Jonas, 2000). As previously described, evoked release of transmitter involves several steps, therefore it would not be surprising that the synaptic delay is somewhat variable from trial to trial at the same release site and between different releasing sites. The diffusion of neurotransmitter itself depends on several factors, such as the diffusion coefficient, the geometry of the cleft, the distribution and affinity of transmitter binding sites and the transporter uptake rate (Clements, 1996). It has been estimated that transmitter concentration during a synaptic event has a peak ranging between 1 and 5 mM and decays in a biphasic manner with time constants of approximately 100 μs and 2 ms (Clements, 1996). The rising phase of postsynaptic signals is determined by the binding process between neurotransmitter molecules and receptors. Therefore it depends on the transmitter concentration time course and on the receptors opening rate. The decay phase, on the contrary, is mainly determined by the unbinding process, if desensitisation is considerably slow as in the case of nicotinic acetylcholine receptors. It depends also on the kinetics of desensitisation when

desensitisation itself occurs at time scale comparable to the one of transmitter concentration, as in the case of AMPA receptors at most of glutamatergic synapses (Jones & Westbrook, 1996; but see also Jonas, 2000).

1.3 Synaptic efficacy

The neuromuscular junction (NMJ) represents the standard model for studying synaptic transmission. At the beginning of the 1950s del Castillo and Katz (1954) proposed the quantal hypothesis of transmitter release, according to which neurotransmitter is packaged in discrete quantities of fixed size, called *quanta*, which are generally identified with synaptic vesicles. Thus, when a nerve impulse reaches the terminal, an integer number of *quanta* release their content in the synaptic cleft. In line with this idea, miniature currents, which can be recorded in the absence of action potential, are due to the release of a single *quantum*. Each site operates in an all-or-none fashion, meaning that it can release either zero or one *quantum* and each *quantum* is released probabilistically and independently of the others. If the probability of release is uniform among the different sites, synaptic transmission can be fit with a simple binomial model. Thus, in a synapse with N functional releasing sites and a probability p of release at each individual site, the probability that n *quanta* are released by a single action potential is:

$$P(n) = \frac{N!}{n!(N-n)!} p^n (1-p)^{N-n} \tag{1.1}$$

While the probability of detecting no response (failure rate) is:

$$P(0) = (1-p)^N \tag{1.2}$$

The mean number of *quanta* released, known as quantal content, is given by:

$$m = Np \tag{1.3}$$

If Q represents the magnitude of the postsynaptic response to a single transmitter *quantum* (quantal size), the mean response I and its standard deviation σ are:

$$I = NPQ \tag{1.4}$$

$$\sigma = Q\sqrt{Np(1-p)} \tag{1.5}$$

Interestingly the coefficient of variation (CV), defined as the ratio between the standard deviation and the mean, is independent of Q:

$$CV = \frac{\sigma}{I} = \sqrt{\frac{(1-p)}{Np}} \tag{1.6}$$

In case of a very low probability of release ($p<<1$) and large N, the binomial distribution approximates the Poisson one and eqns.1.1-1.2-1.6 convert respectively into:

$$P(n) = \frac{e^{-m} m^n}{n!} \tag{1.7}$$

$$P(0) = e^{-m} \tag{1.8}$$

$$CV^2 = \frac{1}{m} \tag{1.9}$$

Thus, strength or efficacy of a synaptic connection depends on N, p and Q. Changes in one or more of these parameters account for modifications in synaptic strength. It is clear that, while Q depends on both pre and postsynaptic mechanisms, N and p are controlled only by presynaptic factors. On the basis of the previous equations, quantal analysis has been developed to find estimates of these parameters and/or to define the site of changes in case of modifications in synaptic efficacy, for example through the evaluation of failure rate or CV (Katz, 1969).

Later on, the basic concepts of neurotransmitter release have been extended to central synaptic transmission, despite the obvious differences, both functional and morphological, that distinguish the two systems. In particular, most of central synapses are characterised by a very small quantal size, with a change in conductance that is two orders of magnitude smaller than at the NMJ and is due to the opening of tens rather than thousands of postsynaptic receptors (but see calyx of Held; Borst *et al.*, 1995). Moreover the distribution of miniature currents is found surprisingly skewed (Bekkers *et al.*, 1990; Edwards *et al.*, 1990; Bekkers & Stevens; 1995). A first interpretation suggests that the small quantal size could be due to a small number of postsynaptic receptors. Thus, because of the high number of transmitter molecules released and because of the small volume of the synaptic cleft, glutamate reaches a concentration high enough to saturate all the receptors (Edwards *et al.*, 1990). According to this interpretation the skewed distribution could be due either to multiple quantal clusters of receptors on the postsynaptic membrane or to synapses consisting of multiple active zones (Edwards, 1995a). Alternatively, the variability observed could be accomplished by differences in miniature amplitudes between different releasing sites (Edwards *et al.*, 1990) or by fluctuations in the number of functional receptors or in their affinity for the transmitter. Obviously, receptor saturation has many important implications in synaptic physiology and in its possible modifications during synaptic plasticity (Scheuss *et al.*, 2002). Indeed,

it seems that certain heterogeneity exists among different types of synapses. High receptor saturation has been described at certain hippocampal connections (Edwards *et al.*, 1990; Clements *et al.*, 1992; Jonas *et al.*, 1993; Tang *et al.*, 1994), whereas more recent studies have shown that variation in the concentration of the agonist is the main source of variability of responses (Liu *et al.*, 1999; Hanse & Gustafsson, 2001). Moreover, an incomplete occupancy of AMPA receptors has been suggested by recordings of miniature excitatory postsynaptic currents from single synaptic boutons in hippocampal cultures (Forti *et al.*, 1997). These experiments have also shown that the large variability of miniature events recorded from the soma may arise from the summed behaviour of many synaptic terminals, rather than to intrinsic properties of single synapses. Furthermore, it is not clear yet whether at small central synapses different releasing sites behave independently as observed at the NMJ. Very recently, the analysis of the stochastic properties of miniature excitatory postsynaptic currents at individual hippocampal synapses has shown a clear divergence from the Poisson's law (Abenavoli *et al.*, 2002). This result, together with the morphological observation of spontaneous pairs of omega profiles at active zones by fast freezing, suggests that functional releasing sites might be clustered.

However, modifications of the original quantal hypothesis have been developed. They take into account the possibility of a non-uniform probability of release as well as the existence of both intra and intersite quantal variability, i.e. the variability in the quantal size at individual releasing site and between different releasing sites, respectively (Redman, 1990; Korn & Faber, 1991; Frerking & Wilson, 1996; Bennett & Kearns, 2000; Scheuss & Neher; 2001).

2. The hippocampus

2.1 Anatomy of the hippocampus

The hippocampus is among the best characterised brain structures, mainly because its layered organisation (Andersen *et al.*, 1971) is particularly suitable for anatomical and physiological investigations, but also because, since the early 1950s, it has been recognised to play a fundamental role in some forms of learning and memory (Kandel, 2001).

The hippocampus is an elongated structure located on the medial wall of the lateral ventricle, whose longitudinal axis forms a semicircle around the thalamus. Due to its layered organisation through this axis, when the hippocampus is cut across its transverse axis (the septotemporal one), it is possible to identify a particular structure that is preserved in all slices taken with this orientation. The hippocampus *proper* and its neighbouring cortical regions, the dentate gyrus (DG), subiculum and enthorinal cortex, are collectively termed "hippocampal formation". As shown in Figure II, the hippocampus *proper* is divided in *stratum oriens* (1), *stratum pyramidale* (2), *stratum radiatum* (3) and *stratum lacunosum-moleculare* (4). Excitatory neurones (pyramidal cells) are arranged in a layer that forms the *stratum pyramidale*, traditionally divided in four regions CA1-CA4. In general, CA4 is considered the initial part of CA3 and the small CA2, which is indistinct in some species, is included in CA1. All pyramidal neurones bear basal dendrites that arborise and form the *stratum oriens* and apical dendrites that are radially oriented in the *stratum radiatum* and *lacunosum-moleculare*. In the DG, granule cells represent the principal neurones, while the area between DG and the CA3 region is called the *hilus*.

2.2 The trisynaptic circuit

The main inputs to the hippocampus come from the enthorinal cortex, the septum and the contralateral hippocampus, whereas a unique unidirectional progression of excitatory pathways links each region of the hippocampus, creating a sort of trisynaptic circuit (Figure II). The perforant path, originated from the enthorinal cortex, passes through the subicular complex and terminates mainly in the dentate gyrus, making synapses on granule cells. Then, the distinctive unmyelinated axons of the granule cells (Mossy fibres) project to the *hilus* and to the *stratum lucidum* of the CA3 region. Here they make

synapses *en passant* on CA3 pyramidal neurones showing the large, presynaptic varicosities typical of Mossy fibres-CA3 contacts. These presynaptic expansions form a unique synaptic complex with equally intricate postsynaptic processes called *thorny excrescences* and may contain tens of releasing sites (Jonas *et al.*, 1993; von Kitzing *et al.*, 1994). Moreover, MF appear to be particularly interesting because their synapses have a postnatal development. In fact, although immature axons may contact pyramidal cells from the first postnatal day, full development of these characteristic *thorny excrescences* is reached only by the end of the second postnatal week (Amaral & Dent, 1981). Information is therefore transferred, through Schaffer collaterals, from CA3 to CA1 pyramidal neurones, which therefore send their axons to the subiculum and the deep

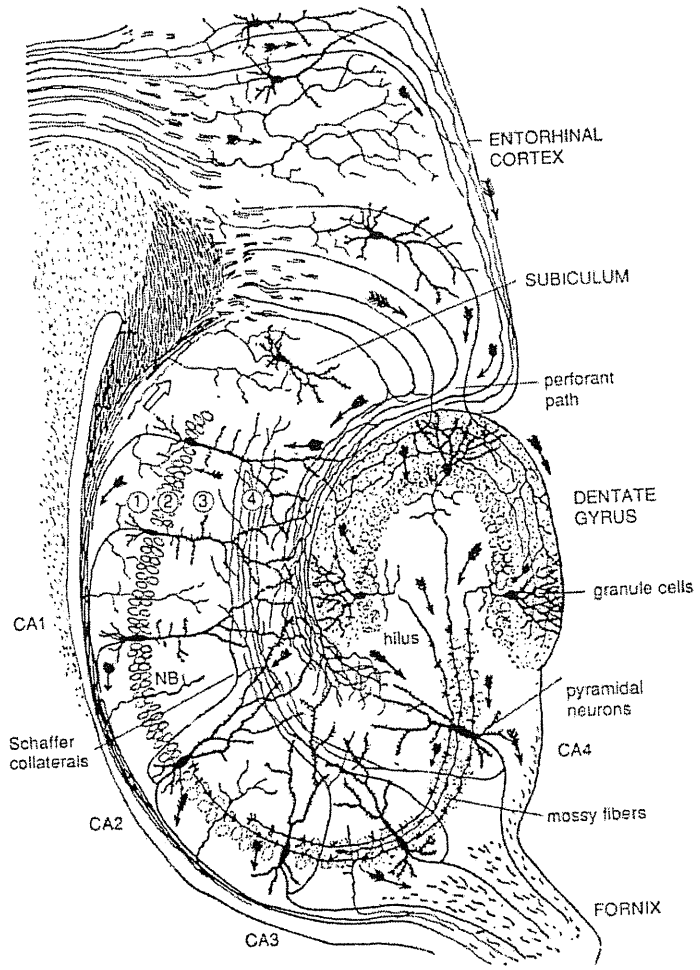

Figure II. Neuronal elements of the hippocampal formation. Labelled areas include the subiculum, part of the enthorinal cortex, the fornix, the dentate gyrus and the region CA1 to CA4. The hippocampus *proper* is divided into *stratum oriens* (1), *stratum pyramidale* (2; cell bodies drawn as ovals), *stratum radiatum* (3) and *stratum lacunosum-moleculare* (4). (Modified from Ramon y Cajal, 1911)

layers of enthorinal cortex. Then, signal is sent back to many of the same cortical areas. Thus, information entering the enthorinal cortex from a particular cortical area can traverse the entire hippocampus and return to the cortical area from which it originated. The transformations that take place during this process are presumably essential for information storage (Johnston & Amaral, 1998). Furthermore, commissural associative fibres provide synaptic contacts between CA3 pyramidal neurones and between the two hippocampi, via the fornix. Excitatory postsynaptic currents or potentials elicited by MF stimulation can be discriminated from those evoked by commissural fibers, on the basis of their faster kinetics (Yeckel *et al.*, 1999) and their sensitivity to the selective agonist for metabotropic glutamate receptors 2/3, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV; Kamiya *et al.*, 1996). The recurrent connections between pyramidal neurones are particular of the CA3 region and are responsible for making this region quite unstable. The simultaneous activation of a certain percentage of these connections is sufficient for generating epileptiform activity, characterised by spontaneous, synchronised and rhythmic firing in a large number of neurones (Miles & Wong, 1986; Traub & Miles, 1991). This feature accounts for the selective generation of seizures in this region following the application of convulsive agents (Ben-Ari & Cossart, 2000). A critical role in controlling the communication between pyramidal neurones, especially but not only in this case, is accomplished by local inhibitory interneurones and a balance is set between excitation and inhibition. In contrast to the rather uniform population of excitatory neurones, interneurones, which are distributed in the entire hippocampus, show a great variability and are classified in several families according to their morphology and axonal and dendritic arborization (for a review see Freund & Buzsaki, 1996). Among all the differences in morphology and in active and passive membrane properties between neurones, a particular feature of pyramidal cells, known as accomodation, is the possibility to limit the maximal firing frequency upon injection of a depolarising current step. This property distinguishes pyramidal neurones from interneurones that, on the contrary, are able to fire up to 400 Hz (Lacaille, 1991).

2.3 Excitatory synaptic transmission in the hippocampus

Excitatory synaptic transmission in the hippocampus is mainly mediated by the action of glutamate on both ionotropic and metabotropic receptors (reviewed by Ozawa *et al.*, 1998). Three kinds of ionotropic glutamate receptors, all selective for cations but

distinguishable on the basis of agonist specificity, are present in the central nervous system: NMDA, AMPA and kainate receptors.

2.3.1 NMDA receptors

NMDA receptors show very slow kinetics, with synaptic currents lasting tens or even hundreds of ms (Randall & Collingridge, 1992). They are characterised by a voltage-dependent block by extracellular Mg^{2+} (Nowak *et al.*, 1984) and a high permeability to Ca^{2+} (Mayer & Westbrook, 1987). Thus, NMDA receptors have the particular feature to work as "coincident detectors" as they allow ions flowing into the cell only if glutamate release occurs in coincidence with the depolarisation required for the relief from Mg^{2+} block. NMDA receptors are also blocked by competitive antagonists, such as 2-amino-5-phosphopentanoic acid (AP5) and 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), and are sensitive to other endogenous ligands and modulators, such as Zn^{2+} and protons. In particular the co-agonist glycine must bind to the receptor for its activation (Johnson & Ascher, 1987). Over the past decade, a variety of NMDA receptor subunits have been identified (reviewed in Cull-Candy *et al.*, 2001): the ubiquitously expressed NR1 subunit, a family of four distinct NR2 subunits (A, B, C and D) and two NR3 subunits (A and B). Functional NMDA receptors are tetramers formed by the combination of NR1 subunits with at least one type of NR2, while the NR3 subunits can co-assemble with the NR1-NR2 complexes. The different subunit composition accounts for different properties of receptors, such as the kinetics, the single-channel conductance (between 20 and 50 pS) and the Mg^{2+} sensitivity. Interestingly, *in situ* hybridisation studies have shown a differential distribution of mRNAs for the various subunits throughout the brain and during development, suggesting the influence of NMDA receptor subtypes on the properties of synaptic transmission. At the hippocampal level, both NR2A and NR2B mRNAs are highly expressed, while NR2C and NR2D transcripts are found only in a subset of hippocampal interneurones. The developmental regulation in the expression of NR2 subunits shows that NR2B and NR2D mRNAs appear prenatally, whereas NR2A and NR2C are first detected around birth. In particular, a gradual replacement of NR2B by NR2A subunits during development seems to account for the speeding of the decay phase of NMDA receptor responses, a phenomenon itself linked to synaptic plasticity (Tang *et al.*, 1999).

2.3.2 *AMPA receptors*

AMPA receptors show faster kinetics than NMDA receptors, with synaptic currents lasting a few ms (Ozawa *et al.*, 1998). They are voltage independent and have a Ca^{2+} permeability that depends on their subunit composition. AMPA receptors are homomeric or heteromeric tetramers assembled by four different subunits, named GluR1-4 (Hollmann *et al.*, 1989; Keinanen *et al.*, 1990; typical conductance <20 pS). *In situ* hybridisation studies have shown that all GluR1-3 mRNAs are abundantly expressed in the hippocampus, both in the *pyramidal layer* and dentate gyrus. Expression of GluR4 mRNA is much less abundant and is relatively higher in CA1 and DG than in CA3. Each of the GluR1-4 subunit exists in two different form, "flip" and "flop", created by alternative splicing of a 115-base pair region immediately preceding the last putative transmembrane region (M4). These two forms are differentially distributed in the brain and have distinct developmental profiles: AMPA receptor subunits are expressed predominantly in the "flip" form in embryonic brain and switch to the "flop" form during development (Sommer *et al.*, 1990).

A critical role in the determination of AMPA receptors properties is accomplished by the GluR2 subunit, that, when present in the receptor, strongly reduces its Ca^{2+} permeability (Burnashev *et al.*, 1992). This unique feature is due to the change of a glutamine in an arginine by RNA editing at a specific site (Q/R site) in the second transmembrane region (M2). At early developmental stages, a small percentage of GluR2 does not undergo editing and therefore the two forms coexist. Postnatally, however, virtually all GluR2 subunits exist in the edited form. Due to the ubiquitous expression of this subunit in the central nervous system (CNS), AMPA receptors in most central neurones are Ca^{2+} impermeable. However, native AMPA receptors slightly permeable to Ca^{2+} have been detected in a variety of cells, including hippocampal and neocortical interneurones (Jonas *et al.*, 1994), where, by providing an alternative, even if less efficient, route for Ca^{2+} influx, they are supposed to play a role in modulating synaptic plasticity (Ozawa *et al.*, 1998).

2.3.3 *Kainate receptors*

AMPA and kainate receptors are often collectively classified as non-NMDA receptors. This is due to the absence of selective agonist or antagonist that, until few years ago, prevented from distinguishing between them. Thus, despite the epileptogenic action of

kainate has been well known for decades, the real involvement of specific kainate receptors has only recently started to get clarified (reviewed in Lerma *et al.*, 2001). A particular interest is addressed to the hippocampus, and mainly to the CA3 region, where kainate receptors activation generates a seizure and brain-damage syndrome that provides an animal model for temporal-lobe epilepsy (Ben-Ari, 1985).

Kainate receptors are supposed to be homomeric or heteromeric tetramers formed by the assembling of five different subunits: GluR5-7, KA1 and KA2. GluR5-7 represent the low-affinity binding site for kainate (K_d~50 nM), while KA1 and KA2 correspond to the high affinity one (K_d~5 nM). In expression studies, kainate-evoked currents are observed in homomeric receptors of GluR5-7 subunits, while KA1 or KA2 alone do not form functional channels. Since *in situ* hybridisation studies have shown co-localisation of functional and non-functional subunits, the native kainate receptors are supposed to be heteromers composed of different subunits. As in the case of GluR2, RNA editing occurs at the same site in GluR5-6 subunits, regulating the Ca^{2+} permeability and the single channel conductance (<10 pS). However, in this case both edited and unedited version of GluR6 form channels with a high Ca^{2+} permeability. The analysis by *in situ* hybridisation of kainate receptors distribution has shown that they are widely expressed throughout the CNS. In particular, at the level of the hippocampus, GluR6 and KA1 mRNAs have been detected in the CA3 region and dentate gyrus, while KA2 mRNA is found essentially in every part of the nervous system. Moreover, co-expression studies revealed the expression of both GluR5 and GluR6 in many hippocampal interneurones.

Non-NMDA receptors antagonists, such 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), have always precluded the possibility to isolate kainate receptor mediated responses. However, in the last decade selective AMPA receptor antagonists (in particular 2,3 benzodiazepins, e.g. GYKI 53655) have been synthesised. Thus, in the presence of GYKI 53655, a slow postsynaptic current due to the direct activation of postsynaptic kainate receptors localised on CA3 pyramidal neurones could be obtained upon repetitive stimulation of MF (Castillo *et al.*, 1997). In line with a role of GluR6 subunits, no postsynaptic currents could be evoked by the same kind of stimulation in GluR6-deficient mice (Mulle *et al.*, 1998). Moreover, several studies have focused on the role of presynaptic kainate receptors in the modulation of both glutamate and GABA release. Evidence exists for both enhancement and depression of release of both neurotransmitters (reviewed in Kullmann, 2001). Although there are important differences in location,

mechanism, and subunit composition of these receptors, both metabotropic and ionotropic cascades have been implicated.

2.3.4 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) belong to a family of G-protein coupled receptors that can be activated by glutamate. Eight different receptors subtypes have been cloned up to now (mGluR1-8) and can be classified in three different groups according to their sequence homology, transduction mechanisms and pharmacology (reviewed in Pin & Bockaert, 1995). In general members of group I (mGluR1 and mGluR5) activate phospholipase C, while members of group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) are linked to the inhibition of the cAMP cascade. The most used agonist and antagonist of mGluRs are *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) and α-methyl-4-carboxyphenylglycine (MCPG), respectively, even if the efficacy of these drugs differs among different subtypes. Immunohistochemistry studies showed that in the hippocampus group II and group III are predominantly localised to presynaptic elements, whereas group I is localised to postsynaptic ones. In particular, the MF were labelled for mGluR2, mGluR7a, and mGluR7b, whereas the Schaffer collateral only for mGluR7a (Shigemoto *et al.*, 1997).

Excitability of hippocampal neurones is modulated directly through mGluRs of group I coupled with various calcium, potassium (I_{AHP}, IK_{leak}, I_M) or non-selective cation channels. Recently a new modulation of the slowly inactivating potassium current I_D by mGluRs of group I and II has been reported in cultured hippocampal neurones (Wu & Barish, 1999). It has also been demonstrated that in synaptic activation of mGluR1 at MF-CA3 connections evokes an excitatory postsynaptic response independent of G-protein function, while inhibiting an afterhyperpolarization current through a G-protein-coupled mechanism. These results represent the first functional evidence for a dual signaling mechanism associated with mGluRs, in which intracellular transduction involves activation of either G proteins or tyrosine kinases (Heuss *et al.*, 1999). In addition to the excitatory postsynaptic effects, mGluRs also behave as "autoreceptors" at the presynaptic site (Scanziani *et al.*, 1995). Namely, they are responsible for presynaptic suppression of excitatory and inhibitory transmission through inhibition of Ca^{2+} currents and/or interfering directly with the release machinery. In the hippocampus, a particular role

involves mGluRs of group II in the CA3 region, where their selective activation by DCG-IV inhibits synaptic transmission at the MF-CA3 synapse (Kamiya *et al.*, 1996). On the contrary, group I and III mGluRs mediate inhibition at the SC-CA1 synapse (Gereau & Conn, 1995). Moreover both pre and postsynaptic mGluRs seem also to be involved in different plasticity processes (see section 3.2).

3. Modulation of synaptic efficacy

Modifications of synaptic efficacy are thought to be very important for the refinement of neural circuitry, information processing and storage. Thus, the possibility to find out the mechanisms and the sites of changes of synaptic strength is really important for understanding brain functions. At central synapses it is quite difficult to find appropriate experimental approaches and models for the application of quantal analysis. Easier approaches, such as the analysis of apparent transmission failures or *CV*, can be used to have an idea on whether pre and/or postsynaptic changes are responsible for particular modifications of synaptic efficacy (Katz, 1969).

Even if changes in Q and N may be involved in the modulation of synaptic strength and have always to be taken into account, probability of release seems to be a major factor that influences the pattern of transmitter release (Thomson, 2000). A strong variability in p has been found when comparing both synapses from different preparations and the same kind of synapses. p can vary from less than 0.01 at specific cortical connections (Thomson *et al.*, 1995) up to 0.9 at the calyx of Held (von Gersdorff *et al.*, 1997) and a similar range of variability has been detected in the CA1 region of the hippocampus (Dobrunz & Stevens, 1997). It is still difficult to define p in an unambiguous and precise way, as a lot of different processes are involved in transmitter release. This particularity can probably account for the high variability in p values as well as for the many opportunities in controlling and modulating it. In general, p represents the probability that Ca^{2+} entering the nerve terminal during action potential triggers the release of fusion-competent vesicles. Thus, we could imagine that different values of p are due to differences in local calcium affinity or binding at the level of Ca^{2+} sensors. Alternatively, the shape of the presynaptic action potential may be of fundamental importance for determining the strength of synapses, since the degree and the duration of depolarisation control the opening of voltage-gated Ca^{2+} channels, as well as the driving force for calcium influx itself. Therefore, also the number, location and properties of calcium channels are important in modulating the shape and the size of the Ca^{2+} transients themselves. On the other hand, a differential expression of proteins involved in release or differences in their phosphorylation could account for further variability in the probability of release. The interplay between all these mechanisms determines p and a modification at any of these levels might affect synaptic strength (Thomson, 2000).

The minimal requirement for release to occur is the presence of fusion-competent vesicles

and this is not so obvious especially in the case of activity-dependent processes. In fact, it appears that, as previously observed at the NMJ (Betz, 1970), after exocytosis each releasing site is refractory for a certain time, which may vary between types of synapses. This can be explained by the partial depletion of the readily releasable pool of vesicles, but also through the retarding action of Rab proteins (Thomson, 2000). This phenomenon accounts for the observed PPD at many central synapses. In order to consider all the releasing sites, whatever is their previous history, the probability of release can be expressed as the balance between two processes, one strictly related to the release of available vesicles and the other to vesicle availability itself (Zucker, 1973; Quastel, 1997; Scheuss & Neher, 2001). With this approach a direct correlation between the probability of release and the size of the RRP has been suggested (Dobrunz & Stevens, 1997).

At the postsynaptic level, receptor desensitisation and saturation have been shown to play crucial role in modulating synaptic strength (Trussel *et al.*, 1993; Otis *et al.*, 1996; Neher & Sakaba, 2001; Scheuss *et al.*, 2002). However, possible changes in the expression, function and localisation of both NMDA and AMPA receptors are now deeply investigated (Liao *et al.*, 1995; Roche *et al.*, 2001; Borgdorff & Choquet, 2002; Tovar & Westbrook, 2002). These kinds of processes are, in fact, thought to participate in a particular kind of synaptic plasticity that involves changes in the number of functional synapses.

3.1 Short-term plasticity

Short-term plasticity refers to use-dependent synaptic changes that are restricted to brief periods of time (reviewed in Zucker & Regehr, 2002). These processes are crucial for regulating temporal coding and information processing between neurones in the brain (Tsodyks & Markram, 1997), where, in fact, information is conveyed by spike train rather than by isolated action potentials (Lisman, 1997). These modifications vary from synapse to synapse and in the same synapse according to its previous history (Debanne *et al.*, 1996; Markram & Tsodyks, 1996; Canepari & Cherubini, 1998; Markram *et al.*, 1998). Thus, most synapses in the CNS, including neocortex and hippocampus, undergo dynamic bidirectional regulations of their efficacy following activity-dependent processes (Thompson & Deuchars, 1994).

3.1.1 *Facilitation, augmentation and potentiation*

Increases in transmitter release by repeated stimulation fall into two categories: those that act over short interval (facilitation) and those that accumulate significantly during prolonged stimulation, augmentation and potentiation (McLachlan, 1978). These phenomena have been shown to be presynaptic in origin, with a strong correlation between elevation in $[Ca^{2+}]_i$ and enhancement of synaptic strength (Zucker & Regehr, 2002; Thomson, 2000). In particular, the "residual calcium hypothesis" has been suggested for explaining facilitation. According to this hypothesis, the small fraction of calcium remaining in the terminal after a conditioning pulse increases the probability of transmitter release to a second stimulus (Zucker, 1989). More complex mechanisms, involving Na^+ accumulation and intracellular buffering of Ca^{2+}, modulate the dynamics of Ca^{2+} removal upon brief and long trains of stimulation, thus accounting for augmentation and potentiation, respectively (Magleby & Zengel, 1976; Regehr, 1997; Tang & Zucker, 1997). A particular form of short-term potentiation has been recently described at the Mossy fibres boutons upon high frequency stimulation (Geiger & Jonas, 2000). In this case the cumulative inactivation of fast-inactivating potassium channels accounts for activity-dependent spike broadening, resulting in an increased influx of calcium and enhanced synaptic strength.

3.1.2 *Short-term depression*

Another common form of short-term plasticity lasting from seconds to minutes is depression upon repeated use (Thomson & Deuchars, 1994; Nelson & Turrigiano, 1998). This may provide a dynamic gain control over a variety of presynaptic afferent firing action potentials at different rates (Markram & Tsodyks, 1996; Abbott *et al.*, 1997; O'Donovan & Rinzel, 1997). This form of plasticity is supposed to reflect mainly presynaptic depletion of the RRP of vesicles (Rosenmund & Stevens, 1996; Wang & Kaczmarek, 1998; Oleskevich *et al.*, 2000; Meyer *et al.*, 2001). However, many other mechanisms may be involved. Presynaptic mechanisms of depression may include a reduction in the Ca^{2+} influx as well as the adaptation of the Ca^{2+} sensor for release (Hsu *et al.*, 1996) or activity-dependent inactivation of the release machinery (Betz, 1970). Therefore changes in the action potential shape, calcium channels inactivation (Gingrich & Byrne, 1985) and calcium depletion from the synaptic cleft (Borst & Sakmann, 1999) may be involved. Synaptic depression may be due to homosynaptic (Forsythe &

Clements, 1990; Takahashi *et al.*, 1996; von Gersdorff *et al.*, 1997) or heterosynaptic (Dittmann & Regehr, 1997) inhibition via metabotropic receptors, activation of presynaptic ionotropic receptors (MacDermott *et al.*, 1999), release of retrograde messengers from the postsynaptic neurone (Zilberter *et al.*, 1999; Kreitzer & Regehr, 2002) or release-independent mechanisms such as propagation failures (Debanne *et al.*, 1997; Brody & Yue, 2000). Postsynaptic mechanisms of depression include mainly receptor desensitisation (Trussell *et al.*, 1993; Otis *et al.*, 1996; Scheuss *et al.*, 2002), but also receptor saturation (Neher & Sakaba, 2001; Sun & Wu, 2001).

Repeated use can either enhance or decrease synaptic efficacy, but in some cases multiple processes are present and the result is a combination of facilitation and depression. A particular example is obtained through the application of a paired-pulse protocol within a short time interval. In this case, the ratio between the mean amplitudes of the second response over the first one, known as paired-pulse ratio, is inversely related to the initial release probability. This means that either paired-pulse facilitation or paired-pulse depression can be observed in case of low or high release probability, respectively (Debanne *et al.*, 1996; Dobrunz & Stevens, 1997).

3.2 Long-term plasticity

Long-lasting activity dependent changes in synaptic efficacy are thought to play a fundamental role in the development of the neural circuitry, learning and memory (Stevens, 1998; Martin *et al.*, 2000). This is the reason why many groups are interested in understanding the cellular and molecular mechanisms that account for long-term potentiation (LTP) and long-term depression (LTD).

3.2.1 Long-term potentiation

Long-term potentiation refers to an increase in synaptic efficacy that can last for hours or even days and was at first obtained by repetitive stimulation at synapses between perforant path fibres and granule cells in the DG of the hippocampus (Bliss & Lomo, 1973). Although LTP has been shown to occur in other regions of the brain, it has been mainly studied in the hippocampus and, in particular, at SC-CA1 synapses (reviewed by Bliss & Collingridge, 1993; Larkman & Jack, 1995; Nicoll & Malenka, 1995; Malenka & Nicoll, 1999). Here LTP is input-specific (homosynaptic) and associative. This means

that repetitive stimulation of a synapse induces potentiation selectively at that input, whereas it may help in potentiating another synapse on the same cell if both synapses are activated within a certain temporal window. However, by means of a local superfusion technique, it has been suggested that input-specificity may depend on the distance from the activation site and that synapses in close proximity (<70 μm) may be potentiated too (Engert & Bonhoeffer, 1997).

This form of LTP is triggered by the influx of Ca^{2+} that occurs mainly through NMDA receptors, which act as coincident detectors, being activated by ligand and voltage at the same time. The requirement of co-activation of a presynaptic fibre and its postsynaptic neurone is a feature of Hebbian synapses (Hebb, 1949). However it is not clear yet whether Ca^{2+} itself is sufficient for the induction of LTP and a possible role for mGluRs has been proposed (Bashir *et al.*, 1993). The sequence of events that occurs following the rise in intracellular Ca^{2+} is still a matter of debate. Now it is generally accepted that the activation of Ca^{2+}-dependent protein kinases play a critical role. In particular α-calcium-calmodulin-dependent protein kinase II (CaMKII) seems to be of fundamental importance. A particular property of CaMKII is that, following activation, it autophosphorylates and converts to a form that remains active after the calcium concentration has returned to basal levels (Lisman, 1994). Injection of inhibitors of CaMKII or genetic deletion of its α-subunit blocks the ability to generate LTP (Malenka *et al.*, 1989; Malinow *et al.*, 1989; Silva *et al.*, 1992). However, it is not clear whether LTP expression is due to presynaptic or postsynaptic modifications (or both). The postsynaptic hypothesis requires an enhancement of AMPA receptors function by phosphorylation that could result in an increase in AMPA receptors conductance, or in the activation or insertion of new functional receptors. Indeed, it has been shown that CaMKII can directly phosphorylate the AMPA receptor subunit GluR1, increasing the single-channel conductance (Derkach *et al.*, 1999), and that such an increase in conductance occurs during LTP (Benke *et al.*, 1998). Moreover, an increase in the number of clusters of postsynaptic receptors containing the subunit GluR1 has been reported following LTP (Shi *et al.*, 1999; Antonova *et al.*, 2001). On the other hand, presynaptic modifications refer to changes in the probability of release, in line with a decrease in the failure rate and an increase in the quantal content often observed after LTP induction (Bekkers & Stevens, 1990; Malinow & Tsien, 1990; Stevens & Wang, 1994). Indeed, a raise in the probability of release could differentially affect AMPA and NMDA

receptors because of the much higher affinity of glutamate for NMDA than for AMPA receptors that would facilitate NMDA receptor saturation. This might partially account for the contradictory results obtained when investigating and comparing the potentiation of both NMDA and non-NMDA components (Astzely *et al.*, 1992; Kullmann, 1994; Clark & Collingridge, 1995). However, a presynaptic site of modification requires the activation of a retrograde messenger, such as nitric oxide or arachidonic acid, that transmits the induction signal from the postsynaptic cell to the presynaptic one (Bliss & Collingridge, 1993). It is interesting to notice that, although most forms of LTP studied are NMDA-dependent, a particular NMDA-independent form of potentiation characterises MF-CA3 synapses (Johnston *et al.*, 1992; Larkman & Jack, 1995; Nicoll & Malenka, 1995). In this case the mechanism of induction are still unresolved and multiple forms of LTP have been described (Zalutsky & Nicoll, 1990; Urban & Barrionuevo, 1996;Yeckel *et al.*, 1999). However, there is general agreement that its expression is due to presynaptic changes (Xiang *et al.*, 1994; Lopez-Garcia *et al.*, 1996; Castillo *et al.*, 1997). Very recently it has been suggested that the activation of the hyperpolarisation-activated cation current I_h by protein kinase A may be responsible for this form of LTP (Mellor *et al.*, 2002). It is likely that these two forms of LTP accomplish different roles during information processing and storage in the hippocampus (Nicoll & Malenka, 1995).

A novel approach for explaining long-term modification of synaptic efficacy deals with a change in the number of functional synapses. Namely the recruitment of silent synapses and silencing of previously active synapses have been suggested to account for LTP and LTD, respectively (Nicoll & Malenka, 1995; Voronin *et al.*, 1996; Malenka & Nicoll, 1999). However, also in this case both pre and postsynaptic mechanisms may be involved (see section 3.3).

The late phase of LTP, that accounts for the persistence in time of these modifications, requires genes transcript and new protein synthesis (Kelly *et al.*, 2000). This hypothesis is proposed by several groups who reported that the more persistent components of LTP are blocked by protein synthesis inhibitors (Frey *et al.*, 1988; Guzowsky *et al.*, 2000) and is supported by a significant literature which indicated that morphological changes, on both pre and postsynaptic site, accompanied LTP (Genisman *et al.*, 1993; Lisman and Harris, 1993; Edwards, 1995 a,b; Buchs & Muller, 1996).

3.2.2 Long-term depression

Long-term depression is a lasting activity-dependent decrease in synaptic efficacy that was at first described in CA1 neurones (Lynch *et al.*, 1977; for a review see Kemp & Bashir, 2001). It is widely accepted that LTD prevails in neonatal and young animals where it precedes the developmental onset of LTP (Dudek & Bear, 1993; Battistin & Cherubini, 1994). However, this developmental down-regulation of LTD is not due to a loss of the ability of synaptic transmission to depress, but rather to a change in the induction protocols required to access the synaptic machinery responsible for LTD. Both heterosynaptic and homosynaptic forms of LTD have been observed *in vitro* and, as for LTP, may be NMDA-dependent or independent (Dudek & Bear, 1992; Mulkey & Malenka, 1992; Domenici *et al.*, 1998). In particular, a role for mGluRs has been demonstrated in some forms of LTD (Bashir *et al.*, 1993; Bashir & Collingridge, 1994) and, a part for few exceptions, it seems that the requirement for NMDA and mGlu receptor activation is mutually exclusive (Bortolotto *et al.*, 1999). In some cases, an increase in postsynaptic Ca^{2+} is supposed to trigger LTD induction (Mulkey & Malenka, 1992; Bolshakov & Sieglebaum, 1994). Whether the synaptic activity is potentiated or depressed depends on the time course and the level of calcium itself (Lisman, 1989; Hansel *et al.*, 1997). Lower levels of Ca^{2+} entering during low-frequency stimulation may activate the phosphatase calcineurin, via CaMKII, that in turn dephosphorylates and inactivates inhibitor 1 resulting in the activation of protein phosphatase 1 and/or phosphatase 2 (Mulkey *et al.*, 1994). In particular, it has been shown that the calcineurin inhibitor FK506 reduces LTD (Mulkey *et al.*, 1994) and calcineurin-deficient mice display a lower threshold for LTP induction (Ikegami *et al.*, 1996). It is likely that different induction processes produce different expression mechanisms. Thus, LTD has been found to be accompanied by a dephosphorylation of GluR1, implying a reduction in single-channel conductance (Lee *et al.*, 1998), a decrease in receptor expression (Carroll *et al.*, 1999) or a decrease in glutamate release (Bolshakov & Sieglebaum, 1994; Domenici *et al.*, 1998). Similarly to LTP, LTD has been proposed to be accompanied by a selective postsynaptic change in AMPA receptors expression, with depression due to AMPA receptors endocytosis rather than exocytosis (Luscher & Frerking, 2001). Moreover, a possible role for protein synthesis and structural changes in LTD expression has been investigated (Huber *et al.*, 2000; Ahn *et al.*, 1999; Kirov & Harris, 1999).

3.3 Silent synapses

Silent synapses are synapses that do not respond at rest, but show pure NMDA-mediated responses at depolarised potentials (Figure III). They represent a common feature during postnatal development and have been observed in a variety of different structures including the hippocampus (Durand *et al.* 1996; Liao & Malinow, 1996; Wang *et al.*, 1996; Wu *et al.*, 1996; Isaac *et al.*, 1997; Li & Zhuo, 1998; Rumpel *et al.*, 1998; Losi *et al.*, 2002). Their conversion into functional synapses is thought to be crucial for stabilisation of synaptic contacts and for refinement of neuronal networks (Malenka & Nicoll, 1997; Atwood & Wojtowicz, 1999; Malinow *et al.*, 2000). In particular, it has been shown that the percentage of silent synapses is decreasing during postnatal development (Durand *et al.*, 1996; Liao & Malinow, 1996) and that they can be activated in an associative dependent way, such as during long-term potentiation (Isaac *et al.*, 1995; Liao *et al.*, 1995; Durand *et al.* 1996; Montgomery *et al.*, 2001; Poncer & Malinow, 2001; Figure III).

Different hypotheses have been suggested for explaining silent synapses (see Figure IV). The most common interpretation is that these synapses are "postsynaptically" silent, i.e. they do not express functional AMPA but only NMDA receptors, which at rest are inactive because of the Mg^{2+} block (Figure IVA). According to this idea, their activation requires either insertion of new AMPA receptor proteins in the subsynaptic membrane or activation of non-functional receptors (Isaac *et al.*, 1995; Liao *et al.*, 1995; Montgomery *et al.*, 2001; Poncer & Malinow, 2001). This hypothesis has been recently validated at ultrastructural level by the characterisation of a morphological correlate of silent synapses. Both immunocytochemistry and post-embedding immunogold electron microscopy have been used to examine the distribution of AMPA and NMDA receptors at hippocampal synapses. Indeed, some synapses has been found to contain only NMDA receptors both in adult (Takumi *et al.*, 1999; Racca *et al.*, 2000) and in young animals (Liao *et al.*, 1999; Petralia *et al.*, 1999). A selective acquisition of AMPA receptors has been detected during development and was found to be differentially regulated by chronic changes in synaptic activity (Liao *et al.*, 1999; Petralia *et al.*, 1999). However, although interesting, these results cannot be considered conclusive due to limits of the experimental technique, such as the use of serial sections or the stochastic nature of immunogold labelling. Moreover different results have been found, probably because of the differences in choice of the antibody, in the tissue processing or the immunoreaction conditions

(Racca *et al.*, 2000). Long-lasting potentiation of synaptic transmission in hippocampal cultures may be accompanied by an increase in the number of clusters of postsynaptic receptors containing the subunit GluR1, as observed by the introduction of a recombinant GluR1 tagged with a green fluorescent protein by virus expression system (Shi *et al.*, 1999). However, the postsynaptic origin of silent synapses is still a matter of debate and alternative interpretations, that involve presynaptic mechanisms, have been proposed. They mainly suggest that functional AMPA receptors are already expressed into the postsynaptic membrane and a reduced amount of transmitter is released in the cleft (Choi *et al.*, 2000; Renger *et al.*, 2001; Zakharenko *et al.*, 2001; Figure IVB). Thus, because of the higher affinity of glutamate for NMDA than for AMPA receptors (Patneau & Mayer, 1990), only NMDA can be activated and the connections appear "presynaptically" silent. Indeed the expression of functional AMPA receptors at silent synapses has been

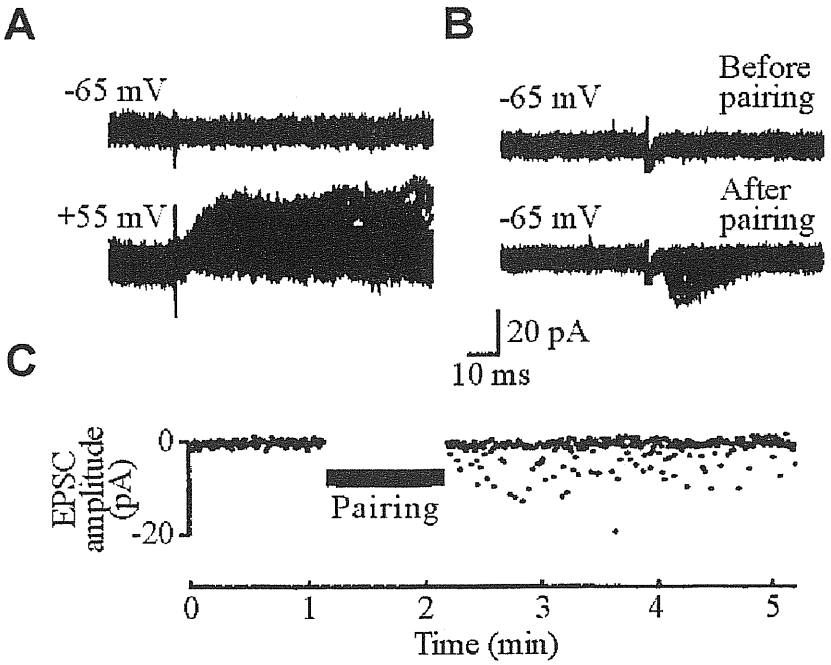

Figure III. Pairing presynaptic stimulation with postsynaptic depolarisation induces AMPA responses at synapses silent at rest but functional at positive membrane potentials. A) Subminimal stimulus produces all failure at -65 mV (upper) and clear responses at +55 mV (lower). **B-C)** Series of consecutive responses to a subminimal stimulus before (upper) and after (lower) the induction of LTP, by a pairing protocol. The intensity of stimulation was lowered until 100 consecutive trials produced only failures (C, before Pairing). Responses are visible at -65 mV after pairing protocol and persist for the duration of the experiment. Stimuli were delivered at 1.6 Hz continuosly (before placing the pipette in the bath) and EPSCs were recorded from CA1 pyramidal neurones in hippocampal slices from 10-18 days-old rats. (Modified from Liao *et al.*, 1995)

demonstrated by local glutamate application or by enhancing their responsiveness to glutamate by the addition of cyclothiazide (CTZ; Choi *et al.*, 2000; Renger *et al.*, 2001). Very recently an evaluation of the relative contribution of AMPA and NMDA receptors has been obtained by recording spontaneous EPSCs from CA1 neurones of neonatal rats (Groc *et al.*, 2002). AMPA/NMDA ratio for frequency was found close to one and to be constant during the first week of development, suggesting that newly formed glutamatergic synapses express both AMPA and NMDA receptors. In line with this presynaptic hypothesis, an increased concentration of glutamate in the cleft would account for switching on silent synapses during LTP. This concentration has been measured by the application of low affinity antagonist for NMDA receptors; indeed LTP could increase both the concentration of glutamate and its rate of rise (Choi *et al.*, 2000). This is in agreement with the observation that AMPA receptors activation depends on the instantaneous concentration of glutamate in the cleft, thus requiring a fast transmitter flux, while NMDA receptors activation is dependent on both the peak concentration and the persistence of transmitter (Renger *et al.*, 2001). The low concentration of glutamate in the cleft could be due, for example, to a vesicular fusion-pore that operates in a non-expanding mode and converts to a rapidly expanding mode upon induction of LTP (Choi *et al.*, 2000). Alternatively, neurotransmitter could spillover from a neighbouring synapse and reach the terminal at a very low concentration (Figure IVC; Asztely *et al.*, 1997; Kullmann & Asztely, 1998). Recently, a direct visualisation of changes in presynaptic function has been obtained by means of endocytotic markers, such as the fluorescent steryl dye FM1-43. The dye is taken up into synaptic vesicles in an activity-dependent manner as a result of endocytosis and is released upon subsequent exocytosis. Thus the rate of FM1-43 unloading provides a convenient and direct index of presynaptic function. An increase in the rate of FM1-43 release has been observed, both during development (Renger *et al.*, 2001) and during long-term synaptic plasticity (Zakharenko *et al.*, 2001), thus suggesting the involvement of modification in the process of vesicular fusion during synaptic transmission. Immunocytochemical studies in dissociated hippocampal cultures have shown an increase in the number of clusters of presynaptic protein and in the number of sites at which synaptophysin and GluR1 are colocalised following LTP induced by brief application of glutamate in the absence of Mg^{2+} (Antonova *et al.* 2001). These results show how redistribution of proteins at both pre and postsynaptic level can account for synaptic potentiation.

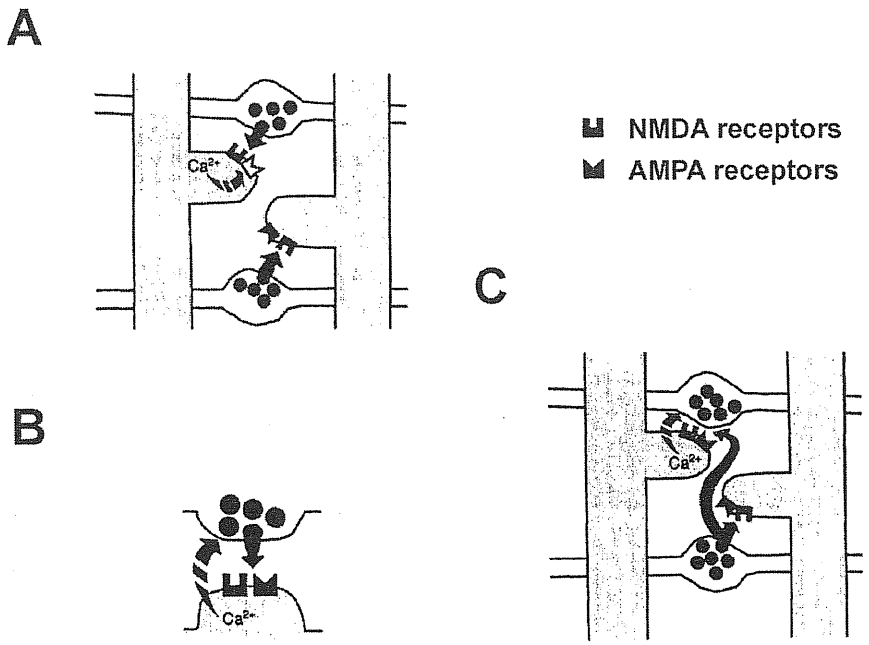

Figure IV. Three possible scenarios to explain silent synapses. A) Postsynaptically silent synapses. The synapse at the top is initially devoid of functional AMPA receptors, so glutamate released is sensed only by NMDA receptors. LTP induction, by pairing depolarisation with afferent stimulation, allows Ca^{2+} influx through NMDA receptors, thus triggering a cascade of intracellular events that culminate with the recruitment of a latent cluster of AMPA receptors. Therefore the synapse functions as a dual-component synapse (bottom). **B)** Low concentration of glutamate released in the cleft. According to this hypothesis the concentration of glutamate released from a single synaptic vesicle may be sufficient to open NMDA, but not AMPA receptors, because of their different affinity for glutamate. In this case, the Ca^{2+} influx during LTP induction would activate a retrograde messenger that would enhance or modify the dynamics of glutamate release, thus converting this synapse into a functional one. **C)** The spillover hypothesis. Glutamate released from one bouton (bottom) diffuses out of the synaptic cleft and reaches a neighbouring synapse (top), where exocytosis does not take place, in a concentration sufficient for activating NMDA but not AMPA receptors. LTP convert this silent synapse into a functional one by means of a retrograde messenger that increases the probability of glutamate release. Locally released glutamate, in contrast to spillover glutamate, can activate both AMPA and NMDA receptors. (Modified from Kullmann & Atszely, 1998)

3.4 Role of potassium conductances

Potassium channels constitute a large group of proteins that carry out a variety of different functions, including stabilisation of the resting membrane potential, repolarisation of the membrane after action potentials and regulation of the firing rate, both at the level of soma and nerve endings (Rudy, 1988; Storm, 1990; Roeper & Pongs,

1996; Meier *et al.*, 1999). Therefore, presynaptic K^+ channels localised on nerve terminals, as a result of these different actions, play a fundamental role in modulating temporal coding and synaptic efficacy.

3.4.1 Potassium channels

The multiple roles that K^+ channels play in the nervous system suggest that a wide variety of these proteins might exist and might be differentially expressed according to their specific function. Indeed, over 80 related mammalian genes for subunits of K^+-selective channels have been revealed (Hille, 2001). Although they come in several architectural forms, they are characterised by some common features, such as the presence of a pore-lining P-loop with a consensus amino acid sequence, called the K^+-channel "signature sequence" (Heginbotham *et al.*, 1992). These residues, repeated in all the principal subunits, line the selectivity filter. However, two transmembrane segments flanking the P-region are at least necessary for completing the pore-forming core. An example of this simple structure is given by the bacterial channel KcsA, which was the first one to be revealed at atomic resolution (Doyle *et al.*, 1998). In addition to this two-transmembrane core, most of the subunits of potassium channels have four additional transmembrane regions that, in voltage-activated channels, account for the voltage sensitivity, mainly through the fourth transmembrane segment (S4). Fully assembled potassium channels are formed by homo or heteromeric tetramers of principal subunits (named α) and are often supplemented by accessory subunits, known as β-subunits, that mainly seem to affect the voltage sensitivity and inactivation properties of the channels (Jan & Jan, 1997).

The first K^+ channel to be cloned was a rapidly inactivating one from the fruit fly *Drosophila* (Tempel *et al.*, 1987; Kamb *et al.*, 1988; Pongs *et al.*, 1988). Its identification started with mutant flies called *Shaker* that shake their legs while under ether anaesthesia, because of the lack of a fast transient potassium current in presynaptic terminals. Afterwards, alternative cDNAs related to the *Shaker* gene were cloned yielding three additional genes coding for K^+ channels and known as *Shal*, *Shab* and *Shaw*. These *Drosophila* genes code for K^+ channels ranging from rapidly inactivating "A-type" channels to slowly inactivating "delayed rectifier".

Later on, the cloning and sequencing of many mammalian α subunits have shown that most of them belong to two main families, encoding for voltage-activated (Kv) or inwardly rectifying (Kir) K^+ channels. Other classes of K^+ channels include, for example,

the Ca^{2+}-activated ones (Sah, 1996; Vergara *et al.*, 1998). Kv channel α subunits fall into different subfamilies Kv1-Kv9 according to their amino acid sequence similarity (Coeeze, 1999; Hille, 2001). Each of these subfamilies comprises several members, denoted Kvm.n thus indicating the subfamily (m) and the order of discovery (n) (Chandy, 1991). In particular, the four major subfamilies Kv1, Kv2, Kv3 and Kv4 are the diversified vertebrate homologous of the channels encoded by the four *Drosophila* genes previously introduced. However, according to this classification, channels that are fast inactivating, delayed rectifier and channels in between are found in the same subfamily. It is known that various α subunits belonging to the same subfamily can co-assemble, whereas those from different subunits cannot. The possibility of forming heteromultimeric channels and the existence of auxiliary β subunits account for the high variability and complexity of kinetics and pharmacology of K^+ currents (Rudy, 1988; Stuhmer *et al.*, 1989; Ruppersberg *et al.*, 1990; Storm, 1990; Bossu & Gähwiler, 1996).

3.4.2 Potassium currents in hippocampal pyramidal cells

Hippocampal pyramidal cells represent a particular example of how multiple potassium currents can co-exist and function in central mammalian neurones (Storm, 1990). Six principal voltage or calcium activated outward potassium current have been described mainly in CA1 pyramidal neurones. Four of them (I_A, I_D, I_K, I_M) are activated by depolarisation, whereas, the two others (I_C, I_{AHP}) are activated by voltage-dependent influx of Ca^{2+}. I_A is a fast activating (10 ms) and fast inactivating (15-50 ms) current that may be activated at membrane potential positive to -60 mV. It contributes to action potential repolarisation and can be blocked by millimolar concentration of 4-AP (Rudy, 1988; Storm 1990). The "delay" current (I_D) activates rapidly (within 20 ms) but inactivates slowly (seconds), causing a long delay in the onset of firing, and is already active at rest (Storm, 1988). Due to its slow recovery from inactivation (seconds), it may "integrate" separate depolarising inputs. As I_A, also I_D participates in spike repolarisation, but, in contrast to I_A, it is selectively blocked by micromolar concentration of 4-AP (Storm, 1988). Differently, I_K activates slowly (20-60 ms) in response to depolarisation positive to -40 mV and inactivates slowly too (seconds). It participates in spike repolarisation and may be blocked by millimolar concentration of tetraetylammonium. I_M has the particular feature of being suppressed by acethylcoline and other muscarinic agonist (from which the name M-current; Brown & Adams, 1980). It activates slowly (50

ms), but it does not inactivate at all. Although it does not seem to contribute to the stabilisation of the resting potential as much as in ganglia, in the hippocampus I_M still tends to reset the membrane potential towards the resting level in response to long-lasting depolarisations. In particular, it is involved in the modulation of firing properties, accounting for the early phase of the characteristic accomodation of pyramidal neurones and the medium afterhyperpolarisation (AHP) following sustained firing. A similar role is accomplished by I_{AHP} that is slowly activated by Ca^{2+} influx during action potentials, causing spike-frequency adaptation and the slow AHP. Finally, I_C has the particular characteristic of being both Ca^{2+} and voltage-activated and is thought to account for the final part of the spike repolarisation.

Several neurotransmitters have been found to modify neuronal excitability by modulating the function of K^+ channels (Rudy, 1988). Beside the already mentioned suppression of I_M by muscarinic agonists, for example it has also been shown that I_{AHP} is reduced by norepinephrine, serotonin or histamine (Madison & Nicoll, 1982). As already stated in paragraph 2.3.4, a novel modulation of I_D by mGluRs of group I and II has been recently reported in cultured hippocampal neurones (Wu & Barish, 1999). A critical role in the modulation of potassium currents is also accomplished by intracellular Ca^{2+} itself, that is well known to activate I_{AHP} and I_C, but has also been shown to suppress, and eventually block, I_A current in hippocampal neurones (Chen & Wong, 1991).

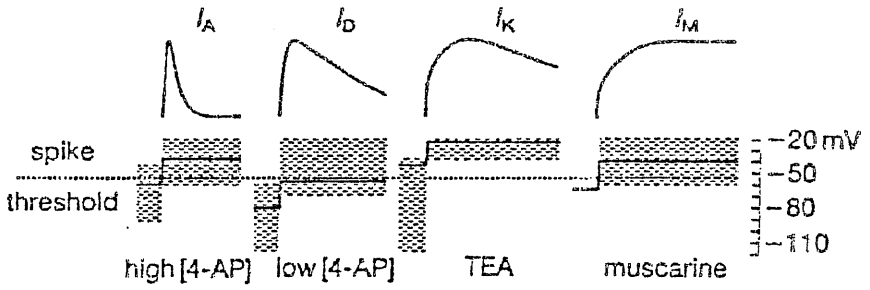

Figure V. Schematic representation of the four voltage-gated, Ca2+-independent K+ currents I_A, I_D, I_K, I_M. For each of these currents, the time course is roughly shown as drawing of idealised currents (top). The approximate voltage ranges of inactivation and activation (shaded areas) and typical voltage steps used to activate them (solid lines) are also indicated (middle). Effective blocking agents and their concentrations are listed on the bottom. (Modified from Storm, 1988)

3.4.3 *Modulation of neurotransmitter release by potassium currents*

As already mentioned at the beginning of this chapter, the shape of presynaptic action potential is of fundamental importance in determining transmitter release and synaptic efficacy. The duration of the depolarisation determines the calcium signal available to trigger fusion of synaptic vesicles with the plasma membrane, by controlling both the opening of voltage-activated Ca^{2+} channels and the driving force for Ca^{2+} itself (Augustine, 1990; Sabatini & Regher, 1997). Although modifications of presynaptic action potential have been shown to modulate neurotransmitter release in many systems (Klein & Kandel, 1980; Llinas *et al.* 1981; Gainer *et al.* 1986; Augustine, 1990; Sabatini & Regehr, 1997; Geiger & Jonas, 2000), most of our knowledge comes from the giant synapse of the squid (Katz & Miledi, 1967a; Llinas *et al.*, 1982; Augustine 1990) and much remains to be understood about the coupling between the presynaptic waveform and release. In fact, the enhancement produced by spike broadening depends on the properties of presynaptic calcium channels and the calcium sensitivity of the release machinery. For example, at the squid giant synapse, calcium entry is much more sensitive to action potential width than at central synapses. Spike broadening causes the same percentage increase in calcium influx and in the amplitude of postsynaptic responses supporting the idea that calcium entering through one single channel triggers vesicle fusion at each individual site (Augustine, 1990). Thus, action potential broadening increases neurotransmitter release primarily by opening more calcium channels. On the contrary, a different arrangement for central synapse has been suggested by different studies. They propose that the distance that Ca^{2+} ions must cover to reach the calcium sensor is relatively long and therefore Ca^{2+} entry through multiple channels is needed to release a single vesicle (Wu & Saggau, 1994; Mintz *et al.*, 1995; Borst & Sakmann, 1996). In the cerebellum, for example, spike broadening increases the open time of Ca^{2+} channels without opening many more channels (Sabatini & Regher, 1997). Most of the enhancement of synaptic efficacy occurs because of the superlinear relationship between neurotransmitter release and calcium influx that likely reflects the co-operativity between calcium ions in triggering release (Dodge & Rahamimoff, 1967). This non-linear relationship can be approximated by a power function whose exponent differs among synapses, but, a part for few exceptions, ranges between 3 and 4 (Dodge & Rahamimoff, 1967; Katz & Miledi, 1970; Wu & Saggau, 1994; Borst & Sakmann, 1996).

As stated in the previous paragraph, several K^+ currents are involved in shaping the action

potential and are therefore suggested to participate in the modulation of synaptic efficacy. As the spike shape may change between soma and terminals (Bourque, 1990; Geiger & Jonas, 2000), a proper estimate of electrical events that precede transmitter release should be based on direct recordings from presynaptic elements. As a direct examination of mammalian central synapses is hampered by the small size of presynaptic nerve endings, much of our present knowledge on the functional correlation between changes in spike shaping and transmitter release comes from studies on invertebrate models. For example, it has been shown that in the *Aplysia* serotonin modulates K^+ channels, thus producing long-lasting presynaptic spike broadening. This leads to increased influx of calcium, that contributes to the enhancement of neurotransmitter release which underlies behavioural sensitisation, a simple form of learning (Klein & Kandel, 1980; Hochner *et al.*, 1986; Byrne & Kandel, 1996).

In the last decades, some groups have succeeded in performing direct recordings from central nerve endings, few of them focusing on the specific role of presynaptic K^+ currents in modulating action potential shape and, consequently, Ca^{2+} influx and neurotransmitter release. Indeed, the first report of $[Ca^{2+}]_i$ signals from individual vertebrate nerve endings was performed on pituitary nerve terminals during high frequency stimulation (Jackson *et al.*, 1991). K^+ channels inactivation was found responsible for the observed increase in spike duration and the consecutive enhancement of AP-induced changes in $[Ca^{2+}]_i$. Similarly, patch-clamp recordings from Mossy fibre boutons in rat hippocampal slices have allowed the characterisation of voltage-gated rapidly inactivating potassium channels that, during repetitive activity, accumulate in the inactivating state, thus leading to spike broadening, increase in Ca^{2+} influx and enhancement of synaptic efficacy (Geiger & Jonas, 2000). Also at the calyx of Held synapse in auditory brainstem, that offers unique advantages because of the extraordinary size of its presynaptic terminals, a rapidly activating delayed rectifier K^+ current has been found involved in the modulation of presynaptic waveform (Forsythe, 1994). Namely, it allows the occurrence of fast action potentials with high firing rate, a feature that is crucial for the rapid and high-fidelity transmission typical of auditory system. At synapses between granule cells and Purkinje cells in rat cerebellar slices, the presynaptic action potential and calcium currents were monitored optically (Sabatini & Regehr, 1997). Broadening the presynaptic waveform, by blocking K^+ channels, was found to prolong the calcium current, without affecting significantly its peak. This increase in Ca^{2+} influx in

turn enhanced EPSCs amplitude in a supralinear way. Quite recently, the first recordings from mammalian CNS inhibitory nerve endings have been performed from cerebellar basket cells terminals (Southan & Robertson, 1998). Dendrotoxin-sensitive potassium channels have been identified using combined electrophysiological and fluorescence techniques. These channels, selectively localised in basket cell terminals, are supposed to modulate GABA release, as their block results in a dramatic increase of both number and amplitude but not kinetics of spontaneous inhibitory postsynaptic currents.

AIM OF THE STUDY

The development and functionality of the nervous system is based on the ability of neurones to communicate properly. The complex and fascinating machinery that accomplishes for synaptic transmission supports the critical role of an accurate connectivity and ensures the required variability and modifiability of synaptic strength. Despite the enormous advances made in the last decades in describing the mechanisms involved, a lot of knowledge is still missing for fully understanding how information is transformed and stored by neurones.

Aim of the present study was to go deeper in characterising synaptic transmission in the hippocampus, mainly focusing on different presynaptic mechanisms that may affect synaptic efficacy. I have used either acute or oganotypic hippocampal slices to address different questions.

Firstly, I have used acute hippocampal slices from new-born rats to investigate synaptic plasticity processes related to development. In particular, I have studied the possibility of switching between functional and non-functional synapses during postnatal development, focusing on the role of silent synapses both in the CA3 and in the CA1 region of the hippocampus.

To this aim, I have used minimal stimulation of afferent inputs, even if, with this method, it is impossible to ascertain that the same presynaptic axon is activated trial after trial as few axons may be stimulated. More convincing results on mechanisms involved in the modulation of synaptic efficacy may be obtained when recording from pairs of monosynaptically interconnected neurones, thus controlling both the pre and the postsynaptic cell.

Therefore, I set up the organotypic hippocampal slice cultures in order to take advantages of the high connectivity between neurones and perform paired recordings. With this approach, I have studied how active synapses can be switched off by activity-dependent form of synaptic plasticity, focusing on the frequency dependence of paired-pulse ratio at CA3-CA3 connections.

Finally, I have studied how synaptic efficacy can be modulated by a voltage-dependent potassium current, with pharmacological and biophysical properties similar to I_D, but down-regulated by intracellular calcium.

METHODS

1. Hippocampal slices preparation

Part of the experiments was performed on hippocampal slices obtained from postnatal day (P) 2-P7 Wistar rats as previously described (Domenici *et al.*, 1998). Briefly, animals were decapitated after being anaesthetised with i.p. injection of urethane (2g/kg). The brain was quickly removed from the skull and placed in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 130, KCl 3.5, NaH_2PO_4 1.2, $NaHCO_3$ 25, $MgCl_2$ 1.3, $CaCl_2$ 2, D-glucose 11, saturated with 95% O_2 and 5% CO_2 (pH 7.3-7.4; osmolarity 310 mOsm). Transverse hippocampal slices (300-400 μm thick) were cut with a vibratome (Vibracut) and incubated one hour at room temperature in a holding bath containing the same saline solution as above. After this recovery period, an individual slice was transferred to the recording chamber.

2. Organotypic hippocampal slice cultures

Other experiments were performed in organotypic hippocampal slice cultures as this preparation offers a lot of advantages for studying synaptic transmission and plasticity. In fact, more convincing results on these topics are obtained when recording from monosynaptically interconnected neurones. It is known that in acute hippocampal slices the probability of connectivity between excitatory neurones is rather low (less than 5% in adult rat; Miles, 1990) and therefore it is very hard to find pairs of interconnected cells. On the contrary, in organotypic hippocampal slices, where the cytoarchitecture of the tissue of origin is properly maintained, many connections are regenerated with time. Thus, 56% of CA3 pyramidal neurones and 76% of CA3-CA1 pyramidal cells are found connected after 2-4 weeks *in vitro* (Debanne *et al.*, 1995). In view of this high degree of connectivity, hippocampal slice cultures offer a unique opportunity to study mechanisms involved in the modulation of synaptic efficacy.

Moreover, organotypic slice cultures may be used for a broad spectrum of applications (for a review see Gähwiler *et al.*, 1997). In particular, they are very useful for experiments that require long-lasting preparations; for examples, studies that involve chronic applications of drugs or toxins, alterations in genes expression by viral vectors, regeneration of neuronal pathways, long-term effects of ischemia.

2.1 Preparation of organotypic hippocampal slice cultures

Several techniques are now available for preparing organotypic cultures depending on the particular requirements of a given experiment. The Maximov cultures (Crain, 1976) and interface cultures (Stoppini *et al.*, 1991) techniques, in which organotypic slices are incubated stationary, yield organised tissue cultures which remain many cell layer thick even after prolonged time of incubation. They offer clear advantages when a semi-three-dimensional structure is needed or for biochemical studies that require a large amount of tissue. The roller-tube technique, on the contrary, is suitable for experiments that require good optical conditions and access to neurones. In fact, after few weeks *in vitro*, remarkable sprouting and flattening are obtained and cells are almost arranged in monolayer.

For the present work, organotypic hippocampal slice cultures were prepared using the roller-tube technique (Gähwiler, 1981). The main steps of the preparation, carried out under a laminar flow hood in sterile conditions, are described below (Figure VI), while the properties of hippocampal slice cultures are discussed in paragraph 2.2.

2.1.1 Dissection

Young rats, between P4 and P7, were killed by decapitation. Brain was quickly removed from the skull and placed in a Petri dish, containing few drops of Gey's balanced salt solution (GBSS, Gibco) enriched with D-glucose (5 mg/ml) and kynurenic acid (1 mM). Hippocampi were carefully isolated and transverse 400 µm thick slices were cut with a tissue chopper (McIlwain). The sections were therefore placed in a second Petri dish containing the same dissection medium and were properly separated under the microscope (Olympus SZ40). The best slices were therefore selected and stored in a new Petri dish at +4°C.

D-glucose was dissolved at 50% in sterile distilled water (Gibco). Kynurenic acid was dissolved 100 mM in alkaline solution.

2.1.2 Embedding

After recovering for one hour at +4°C, single slices were placed in a drop (20 µl) of reconstituted chicken plasma (Cocalico) on a glass coverslip. To fix the slice, the plasma was coagulated by addition of 30 µl of thrombin (Merck). Coagulation took 30-45 min to occur. Then the coverslips were placed into tubes (Nunc).

Prior to be used, glass coverslips (12x24 mm^2, thickness 1 mm, Vitromed) had to be specially treated. They were inserted in a teflon holder, submerged in HCl 0.5 N for 24 h, repetitively washed with distilled water, and soaked for 30 min in absolute alcohol. After further repetitive washes in distilled water, coverslips were then dried and sterilised al +150°C in an oven overnight.

Lyophilised chicken plasma was diluted in distilled water and then centrifuged for 20 min at +4°C at 3500 rpm. Lyophilised thrombin was diluted in distilled water (200 u/ml).

2.1.3 Feeding of cultures and incubation

The coverslips bearing the slices were transferred to plastic tubes containing 0.75 ml of nutrient medium. The medium consists of 50% Basal medium (BME, Eagle, with Hanks's Salts, without L-Glutamine; Gibco), 25% Hanks' Balanced Salt Solution (HBSS; Gibco), 25% Horse Serum (Gibco). L-glutamine (Gibco, 1 mM) and D-glucose (5 mg/ml) were also added (osmolarity 310 mOsm, pH 7.4). The tubes were placed in a roller drum (6 revolutions h^{-1}, 5° tilted) located inside an incubator at 36°C, without any control in CO_2 and O_2 concentration. Through rotation, cultures were submerged in medium for half a turn and covered by a film of medium for the other half. This was essential for feeding, aeration and flattening of the cultures. The medium was replaced with fresh one after the first two days *in vitro* and, thereafter, once a week.

After at least 10-14 days *in vitro* (DIV), an individual coverslip was transferred to a particular recording chamber for electrophysiological experiments.

2.2 Properties of organotypic hippocampal slices

Organotypic hippocampal slice cultures have been widely studied to determine whether the properties of the tissue of origin were maintained and to compare them with those observed in living tissue *in situ* (reviewed in Gähwiler *et al.*, 1997). During the preparation of hippocampal slice cultures, afferent fibres are cut and therefore degenerate. This process might be supposed to account for synaptic rearrangement, but only few changes of this kind, similar to those obtained in lesion studies, have been observed only at the level of the DG. All nerve and glial cell types survive and the phenotypic morphology of neuronal types and the "gross" tissue organisation are similar to those *in situ*. Immediately after dissection, the density of synapses decreases, but then the development is comparable to synaptogenesis *in situ*. Pyramidal cells show normal

synaptic transmission and both short-term and long-term changes in synaptic strength occur, despite the absence of extra-hippocampal afferent. No loss of neurotransmitter receptors or major alterations of the voltage or ligand-gated membrane currents were found. Finally, the degree of connectivity between cells increases with time in cultures, up to at least ten times that reported for acute slices.

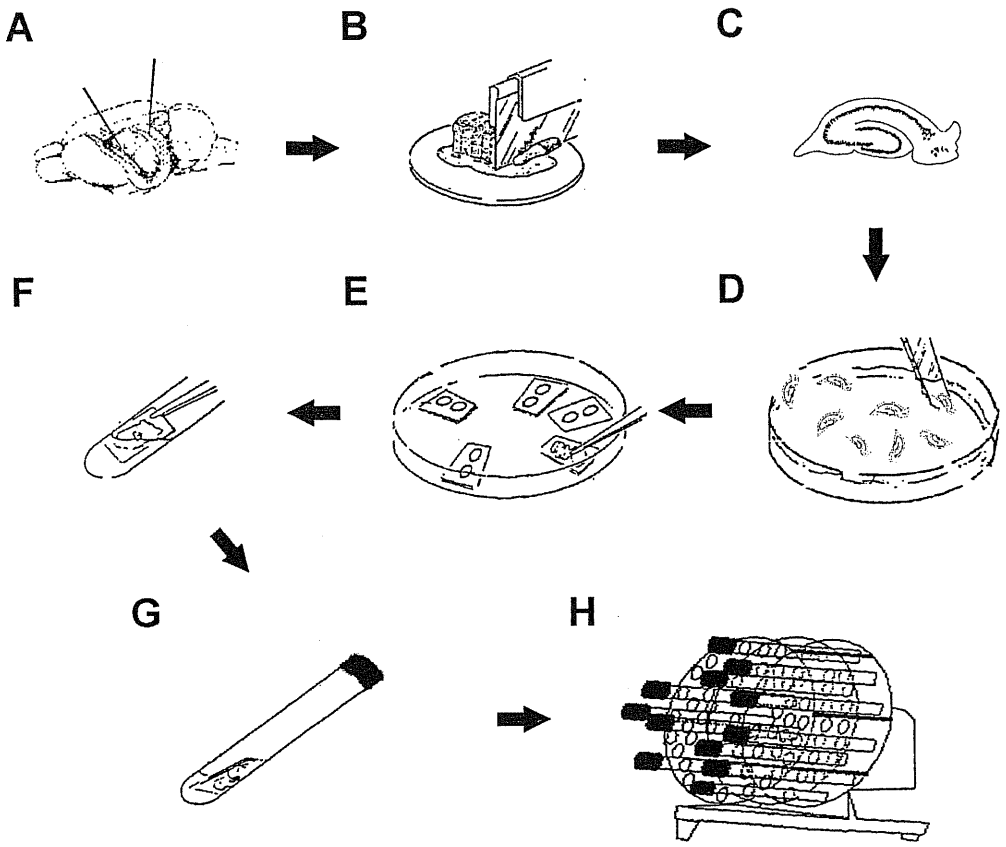

Figure VI. Preparation of organotypic hippocampal slice cultures. A) Following brain removal from the skull, hippocampi were carefully isolated. **B)** Transverse slices were obtained by means of a tissue chopper. **C-D)** After proper separation and selection, hippocampal slices were stored at +4°C in Petri dishes containing dissection medium, for recovery. **E)** Slices were attached to coverslips in a film of reconstituted chicken plasma clotted with thrombin. **F-G)** Coverslips with slices were transferred in plastic tubes containing nutrient medium. **H)** Tubes were inserted in the roller drum inside the incubator.

3. Identification of neurones

3.1 Staining with Cresyl violet

Some organotypic slices were stained with Cresyl violet in order to look at developmental changes in their structure. Cresyl violet binds to nucleic acid thus revealing nuclei, nucleoli, and cytoplasmic Nissl substance (cytoplasmic ribonucleoprotein) of neurones. It allowed *pyramidal layer* and DG at different stages of development to be distinguished (Figure VII).

Slices were fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) and kept at room temperature for 1 h. After few rinses in PBS, they could be stored in the same buffer at +4°C for few days. Before staining, they were washed twice for 10 min in distilled water. Then they were incubated in 0.5% of Cresyl violet (Sigma) for 10 min and rinsed twice briefly in distilled water. Slices were dehydrated by immersion in solutions containing an increasing concentration of ethanol up to 100%, then further soaked in

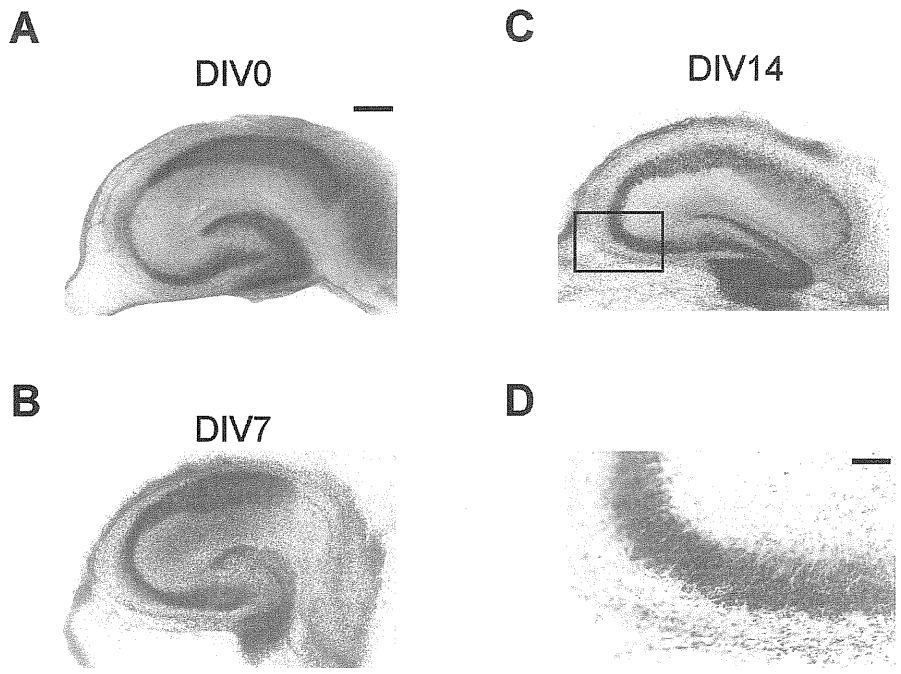

Figure VII. Organotypic hippocampal slice cultures at different days in culture (cresyl violet staining). A) An hippocampal slice was stained the day of culture preparation (DIV0). **B)** After one week *in vitro* the flattening of the culture could be already observed. **C-D)** Experiments were performed after about 2 weeks *in vitro* from CA3 pyramidal neurones (calibration bar is 400 or 100 μm in A-C or D, respectively). The part of the CA3 region included in the black rectangle in C is shown at higher magnification in D.

xylene-ethanol 50% and xylene 100% and finally mounted on coverslips with Eukitt mounting medium (O. Kindler Gmbh & CO).

3.2 Biocytin injection

Biocytin injection was often used to confirm the identity of cells as pyramidal neurones (Figure VIII). Biocytin (0.2-0.3 %; Sigma) was added to the intracellular solution just before use and injected into the cell during electrophysiological recordings. Then, the slices were fixed and stored as described in the previous paragraph. To label biocytin-injected cells, either the avidin-biotinilated alkaline phosphatase or the avidin-biotinilated horseradish peroxidase complex (ABC, Vector Laboratories) was used.

In the first case, after washing slices in PBS for 5 min, membranes were permeabilised by incubation in PBS 0.1% Triton (Sigma) for 30 min. After three washes in PBS (5 min each), slices were incubated in ABC kit (1:100 in PBS) for 40 min and washed again in PBS for three times (5 min each). Slices were then incubated in DIG developing buffer (100 mM TRIS-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM levamisolhydrochloride) for 10 min. The staining reaction was developed by incubation of slices in new DIG developing buffer where p-nitro blue tetrazolium chloride (NBT; 0.5 mg/ml, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP; 0.25 mg/ml, Sigma) were added. The reaction was stopped with P4DIG buffer containing (in mM): Tris-HCl 10 (pH 8), EDTA 1 (pH 8).

When the avidin-biotinilated horseradish peroxidase complex was used, slices were first washed in PBS for 5 min, then immersed in 1% H_2O_2 in 10% methanol for 10 min. After five washes in PBS (3 min each), membranes were permeabilised by incubation in PBS + 2% Triton (Sigma) for 1 h. Slices were incubated in ABC kit (1:100 in PBS) for 40 min and washed again in PBS for three times (5 min each). Slices were then incubated in 3,3'-diaminobenzidine (DAB) solution containing 0.03% $CoCl_2$, 0.02% nickel ammonium sulphate and 0.001 % H_2O_2. Once the cells were identified, the reaction was stopped by washing the slices in PBS three times (10 min each).

In both cases, after some washes in distilled water, slices were mounted on slides as previously described (see paragraph 3.1).

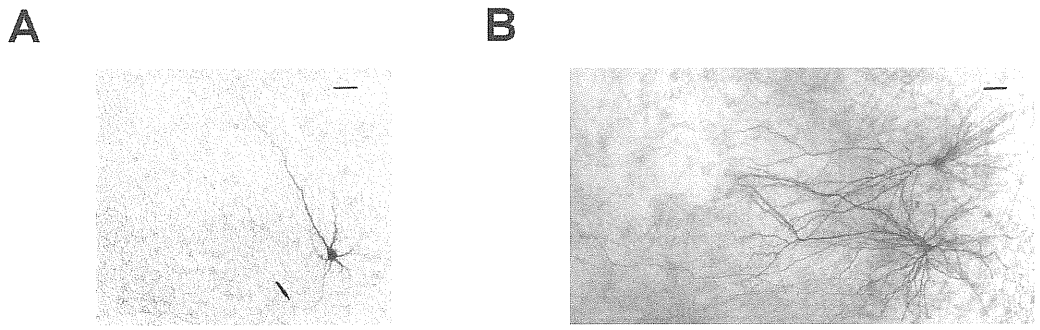

Figure VIII. Biocytin injected neurones. A) A CA3 pyramidal neurone was injected with biocytin during electrophysiological recording (alkaline phosphatase complex). Note the presence of a prominent apical dendrite and shorter basal dendrites. The thin process departing from the left side of the cell body might be the axon (see arrow). **B)** Pair of interconnected CA3 neurones labeled with biocytin (horseradish peroxidase complex). In this case, at the end of the experiment the slice was placed again in the incubator to allow a further diffusion of biocytin in the cells. In this way, a very high number of processes could be stained. (Calibration bar is 50 μm in A-B).

4. Solutions and drugs

Hippocampal slices were transferred to a recording chamber fixed to the stage of an upright microscope and continuously superfused at 2-3 ml/min with appropriate solutions.

4.1 Acute slices

Slices were superfused, either at room temperature (24°C) or at 32°C, with the same ACSF used for the dissection, containing (in mM): NaCl 130, KCl 3.5, NaH_2PO_4 1.2, $NaHCO_3$ 25 or 15 (at 24 or 32°C, respectively), $MgCl_2$ 1.3, $CaCl_2$ 2, glucose 11, saturated with 95% O_2 and 5% CO_2 (pH 7.3-7.4; osmolarity 310 mOsm).

Patch pipettes were filled with a solution containing (in mM): Cs-methanesulphonate 125, CsCl 10, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 0.6-2, N-(2,6-dimethylphenylcarbamoylmethyl) triethylammoniumbromide (QX-314, Alomone labs) 5, MgATP 2, Na_2GTP 0.3 (pH 7.3-7.4; osmolarity 290 mOsm).

Bicuculline methoiodide (10 μM, Sigma) and tetrodotoxin (TTX, 10 nM, Affiniti Research Products) were added to the bathing solution to block GABA receptors of type A and reduce polysynaptic activity, respectively. CPP (Tocris Cookson), CNQX (Tocris Cookson), DCG-IV (Tocris Cookson) or CTZ (Lilly) were added to the bathing solution when needed.

4.2 Organotypic slices

Cultured slices were superfused at room temperature with a bath solution containing (in mM): NaCl 150, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10 (pH 7.4, adjusted with NaOH; osmolarity 310 mOsm).

Different intracellular solutions were used for recordings. They contained (in mM): $KMeSO_4$ 135, KCl 10, HEPES 10, $MgCl_2$ 1, Na_2ATP 2, Na_2GTP 0.4 for the presynaptic cell or $CsMeSO_4$ 135, CsCl 10, HEPES 10, QX 314 5, EGTA 0.5, MgATP 2, Na_2GTP 0.3 for the postsynaptic cell; pH was adjusted to 7.3 with KOH and CsOH respectively (osmolarity 290 mOsm). When 10 mM Bis(2-amino-5-bromophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were added to the presynaptic intracellular solution, $KMeSO_4$ concentration was reduced to 125 mM in order to maintain the same osmolarity.

As for acute slices, TTX (10 nM) was added to the bathing solution to reduce polysynaptic activity. AP5 (50 µM, Tocris Cookson), $CdCl_2$ (200 µM) and 4-AP (10-100 µM, Sigma) was added to the superfusing solution when needed.

K^+ currents were recorded from the presynaptic neurones in the presence of extracellular CsCl (3 mM), Carbachol (50 µM, Sigma), $CdCl_2$ (200 µM, Sigma) and TTX (0.5 µM), in order to block the I_h, I_M, the calcium activated potassium channels and the sodium channels, respectively.

Stock solutions of the different drugs used were prepared in distilled water. Only CTZ was dissolved in dimethyl sulphoxide (DMSO) at the concentration of 1:1000. Test experiments using the same DMSO concentration demonstrated that it does not affect current or voltage responses. Stock solutions were appropriately stored either at +4°C or -20°C and the final concentration was obtained by dissolving an amount of the stock into the extracellular solution. At least 3-5 min of drug application were waited in order to achieve apparent equilibrium condition before data acquisition.

A liquid junction potential (Barry & Lynch, 1991; Neher, 1992) of around 9 mV between the intracellular and the extracellular solutions has been measured by means of a 3 M KCl-agar bridge.

5. Electrophysiological recordings

5.1 Patch-clamp recordings

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record, in voltage or current-clamp mode, from visually identified CA3 or CA1 pyramidal neurones (using infrared differential interference contrast video microscopy, Figure IX). Patch electrodes were pulled from borosilicate glass capillaries (Hingenberg) and had a resistance of 3-6 MΩ when filled with intracellular solution. Excitatory post-synaptic currents (EPSCs) were recorded at -60 mV by an EPC-7 amplifier (List-Medical), while an Axoclamp 2B amplifier (Axon Instruments) was used, after optimising the series resistance compensation, for current-clamp recordings. Action potentials were generated from rest, in CA3 neurones from organotypic slices, by short (5 ms, 0.05-0.1 Hz) depolarising current steps. Alternatively, long depolarising steps (800 ms, 0.05 Hz) were used to study their firing properties and to identify the cells as pyramidal neurones on the basis of their ability to accommodate. The resting membrane potential was maintained constant thorough any experiment by intracellular current injection. "Ensemble" outward potassium current was studied by voltage-clamp experiments at -60 mV. Depolarising steps were applied following specific conditioning prepulses for studying the activation and inactivation properties of the current (see part 3 of results for details).

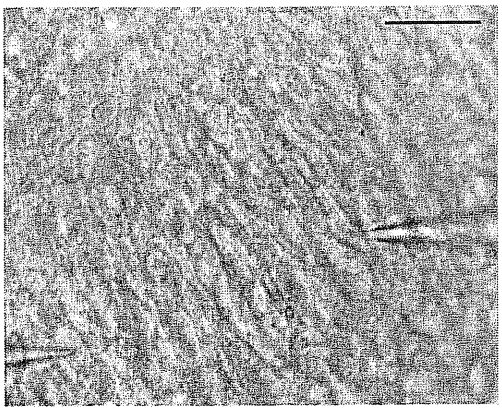

Figure IX. Visual identification of neurones. Pyramidal neurones were visually identified using an infrared differential interference contrast video microscope. The picture, acquired by frame grabber (ATI Technologies), shows an example of paired recording from CA3 neurones in organotypic slice. (Calibration bar is 50 μm)

Double-patch recordings from interconnected CA3 neurones were also performed in slice cultures. Single or pairs (50 ms interval) of presynaptic action potentials were evoked at resting membrane potential at different frequencies (from 0.025 Hz to 1 Hz), while EPSCs were recorded from the postsynaptic neurone at a holding potential of -60 mV.
Data were filtered with a cut off frequency of 1 kHz and acquired at a sampling rate of 20-100 kHz using a pClamp software (Axon Instruments).

5.2 Extracellular recordings

Field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode filled with NaCl (2M) placed in *stratum radiatum* of the CA1 area (DAM 80, World Precision Instruments). fEPSPs were evoked by pair of stimuli (50 ms interval, 100 μs duration, 0.05 Hz) delivered to the Schaffer collaterals with bipolar twisted NiCr-insulated electrodes. Input-output curves were constructed by plotting the amplitude of the afferent volley, normalised to their maximum values versus stimulation intensity, expressed as a fractional increase over threshold.

5.3 Extracellular stimulation

Bipolar twisted NiCr-insulated electrodes or glass pipettes, filled with the extracellular solution, were placed in the *stratum radiatum* or in the *stratum lucidum*, to activate SC or MF, respectively (Figure XA). They were moved around the cell until a response was found. Paired stimuli (50 ms interval, 100 μs duration) were usually applied at 0.05 Hz. In order to look for silent synapses, the minimal intensity necessary to evoke a response at -60 mV was found and then slightly decreased in order to obtain only failures. If no response could be detected at +40 mV, the stimulation intensity was slightly increased until a response was obtained at positive, but not negative potentials. Inputs were considered to be silent when 20-60 consecutive failures of AMPA responses were detected at -60 mV whereas, for the same input, outward NMDA-mediated synaptic currents were recorded at positive potentials.
For the experiments performed from CA3 neurones, the stimulation of the Mossy fibres input was ascertained by the fast rise time (1.4 ± 0.5 ms, n=45; Yeckel *et al.*, 1999) of EPSCs. Moreover, their sensitivity to the selective agonist for mGluRs 2/3 DCG-IV was investigated (Figure XB; Kamiya *et al.*, 1996). In comparison with previous data obtained from older animals (Domenici *et al.*, 1998), DCG-IV was able to significantly ($P<0.001$)

reduce MF-EPSCs only in 13 out of 21 cells tested. This could be attributed to a low expression of mGluRs on presynaptic terminals at early postnatal stages or to different transduction mechanisms. However, the possibility that associative commissural fibres, inducing unusually fast rising EPSCs, were stimulated cannot be completely ruled out.

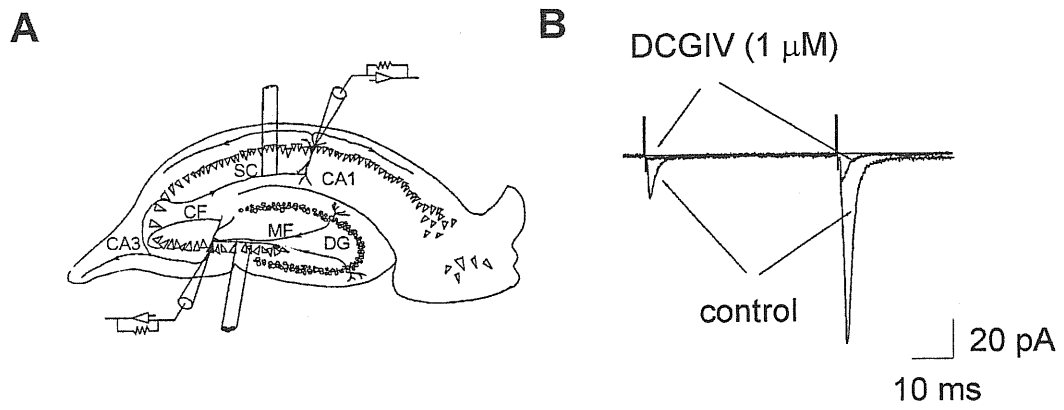

Figure X. Experimental configuration and MF input identification. **A)** A schematic representation of an hippocampal slice with stimulating and recording electrodes. The stimulating electrode was placed either in the *stratum lucidum* or in the *stratum radiatum* to stimulate MF or SC, respectively. EPSCs were recorded with the patch-clamp technique from CA3 or CA1 neurones, respectively. **B)** Average responses to pairs of MF stimuli (50 ms apart) are shown in control and in the presence of DCGIV. Note the disappearance of EPSC1 and the strong reduction of EPSC2 during DCGIV application.

5.4 Data analysis

5.4.1 Excitatory postsynaptic currents

EPSCs were analysed with AxoGraph 4.6 Program (Axon Instruments) which uses a detection algorithm based on a sliding template. Failures (N_0) were usually estimated by visual discrimination. In order to control the adequacy of the visual selection, in some experiments the fraction of responses with amplitude >0 pA, corresponding to failures, was calculated. Since failures should be symmetrically distributed around zero, N_0 was calculated by doubling this fraction (Nicholls & Wallace, 1978). A similarity and a high correlation between N_0 estimated by the two respective methods were found both in the case of extracellular stimulation (mean failure rates were 86 ± 4 and 83 ± 5%, n=28, r = 0.92; P<0.0001) and in the case of double patch recordings (mean failure rates were 85 ± 5 and 82 ± 6%, n=12, r=0.95; P<0.001).

Rise (10-90%), onset and decay time constants were evaluated on the averages of successes: the onset of the EPSC was given by the intersection of a line through the 10 and 90% of EPSC rise time with the baseline, while the decay time constant was obtained by fitting with a monoexponential function. In the case of paired recordings, EPSC analysis was performed after aligning presynaptic action potentials at their peaks. In general, mean EPSC latency was calculated as the time gap between the onset of the mean EPSC and the peak of the presynaptic spike. An estimate of the jitter of the responses was obtained by calculating the standard deviation of the latencies of all the responses. According to Debanne *et al.* (1995), criteria for monosynaptically connected pairs consisted of short latency responses with small fluctuations.

Mean EPSC amplitude was obtained by averaging both successes and failures. PPR was always calculated as the ratio between the mean amplitude of EPSC2 over EPSC1. The coefficient of variation (CV) of response amplitude was determined as the ratio between standard deviation and mean and its inverse squared value (CV^{-2}) was calculated.

5.4.2 Time course of frequency depression

The time course of frequency depression and recovery could be fitted with a monoexponential function using SigmaPlot 2001. Data points were obtained by averaging for any given frequency EPSC amplitudes over 4-8 consecutive trials and normalising them to the first value in the train (I/I_{1st}, in the case of depression) or to the mean EPSC amplitude obtained in control conditions ($I/I_{control}$, in the case of recovery from depression).

5.4.3 Current-clamp recordings

Current-clamp recordings were analysed with Clampfit software. Action potentials were characterised by their firing threshold, their amplitude (from threshold to peak) and their width at the threshold level. The delay to the first spike generation was evaluated as the time interval between the spike peak and the beginning of the depolarising current step. Input resistance was calculated by measuring the amplitude of voltage responses to hyperpolarising steps.

5.4.4 Voltage-dependence of the "ensemble" potassium current

Following appropriate off-line capacitance and leak corrections, the activation and inactivation properties of the "ensemble" outward potassium current were determined by fitting the normalised current amplitude according to a Boltzmann equation of the form: $I/I_{max} = 1/[1 + \exp^{+/-(V-Vh)/k}]$ for activation (+) and inactivation (-), respectively. In the equation, Vh is the voltage at which the current is half of its maximum and k indicates the slope of the voltage-dependence of the processes.

All values are given as mean ± SEM. Significance of differences was assessed by Student's *t*-test or Wilcoxon test. The differences were considered significant when P was <0.05.

RESULTS

1. Silent synapses in the developing hippocampus: lack of AMPA receptors or low probability of glutamate release?

Silent synapses have been widely studied at the level of the CA1 region of the hippocampus, where they are believed to represent the majority of glutamatergic synapses at early developmental stages. On the contrary, no information is available regarding the CA3 region.

Thus, aim of this part of the work was to study whether, at the MF-CA3 synapses, glutamatergic AMPA receptors are present and functional since early postnatal days. Like in the CA1 neurones, a percentage of synapses were silent at rest. However, both MF-CA3 and SC-CA1 connections were found to be presynaptically silent since they could be converted into functional ones by factors that enhance release probability such as paired-pulse stimulation, increase of temperature or cyclothiazide, a drug that blocks AMPA receptor desensitisation and increases transmitter release.

1.1 Paired-pulse stimulation at Mossy fibres-CA3 synapses

Although most of the experiments on silent synapses have been done at room temperature, the first set of the present experiments was done at 32°C. The whole-cell configuration of the patch-clamp technique was used to record from CA3 pyramidal neurones in hippocampal slices from rats between P2 and P7. EPSCs were elicited by minimal stimulation of the MF.

In Figure 1A, when the cell was held near its resting potential (-60 mV), no synaptic responses could be recorded to the first stimulus. However, occasional (8/25) EPSCs, fluctuating in amplitude from trial to trial, were seen after the second stimulus. These EPSCs were completely blocked by CNQX (10 μM, not shown) implying that they were generated by glutamate acting on non-NMDA receptors. When held at +40 mV the cell showed EPSCs with slow kinetics after both the first and second stimulus (Figure 1B, top traces). The contribution of a NMDA-mediated component to the EPSCs was ascertained by applying the selective NMDA receptor antagonist CPP (20 μM). As shown in Figure 1B (bottom traces), CPP revealed a fast AMPA-mediated component. Thus, it appears that this synapse was silent to the first stimulus not because of the absence of AMPA

receptors but rather because of the low probability of glutamate release. Overall, only 2 out of 29 cells were silent to the first stimulus under the present experimental conditions (32°C, 0.05 Hz). On average, the percentage of successes to the first stimulus at -60 mV was 34 ± 4%. When held at +40 mV, all neurones showed slow kinetics EPSCs with significantly higher percentage of successes (56 ± 5%, $P<0.001$; Figure 1D). The presence of AMPA-mediated EPSCs after the second stimulus at -60 mV and +40 mV allows the conclusion that, at 32°C, postsynaptic AMPA receptors can be revealed at MF-CA3 synapses even at early developmental stages.

1.2 Temperature-dependent activation of silent synapses at Mossy fibres-CA3 connections

It is known that the reliability of synaptic transmission decreases with decreasing temperature, apparently because of reduced transmitter release probability (Hardingham & Larkman, 1998). To examine this effect, EPSCs were recorded first at 24°C and then at 32°C (Figure 2). At 24°C, in two out of 12 cells held at -60 mV, responses to the first stimulus were absent whereas responses to the second one could be rarely recorded (in the cell shown in Figure 2, the second stimulus induced 3 responses out of 18 trials). When the temperature was raised to 32°C, EPSCs to the first stimulus appeared and the number of responses to the second was clearly enhanced (14/32; Figure 2A-B). On average in 7 cells, increasing the temperature from 24°C to 32°C produced a significant increase in the percentage of successes to the first stimulus (from 9 ± 4% to 21 ± 4%, $P<0.05$; Figure 2C). These results suggest that the absence of synaptic responses at MF-CA3 synapses in early postnatal life is not due to the absence of functional AMPA receptors, but rather to a low level of glutamate released, particularly at room temperature. According to this hypothesis, the conversion of silent synapses into functional ones, observed after raising the temperature from 24°C to 32°C, could be due to an increase in neurotransmitter release.

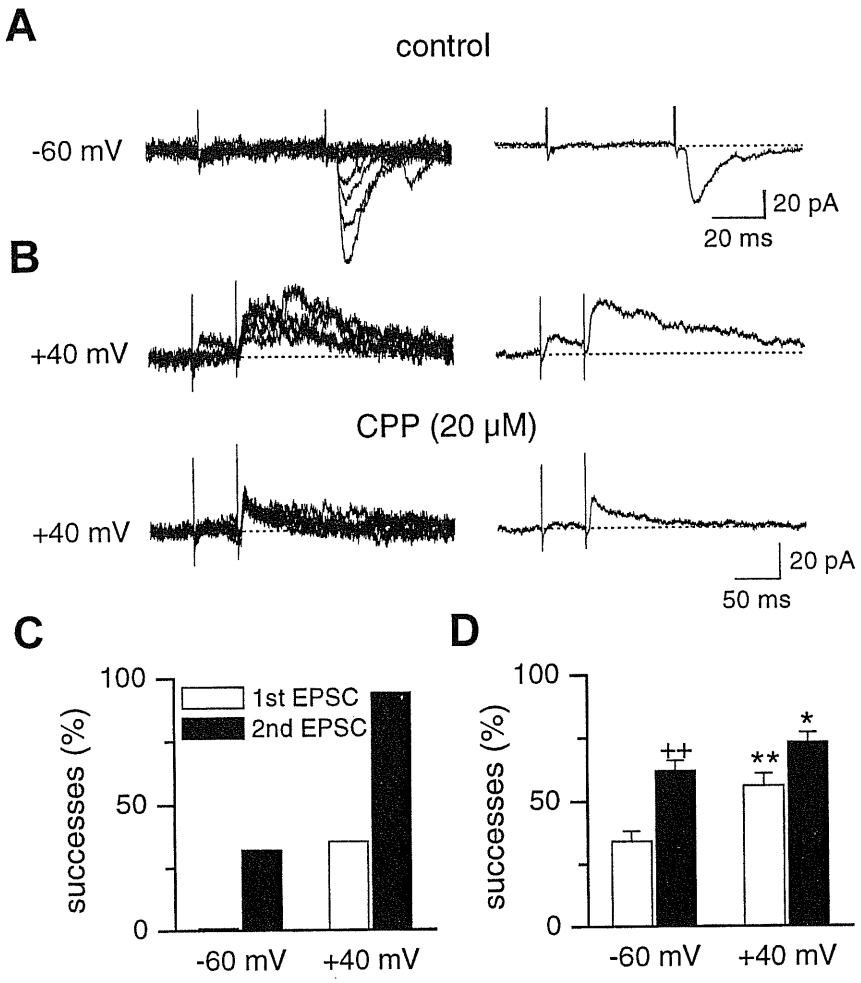

Figure 1. Neonatal MF-CA3 synapses, silent to the first stimulus, respond to the second in a paired-pulse paradigm. A-B) 8-10 individual traces (left) obtained at 32°C by pair of stimuli (50 ms interval, 0.05 Hz) delivered to the MF at -60 mV (A) and at +40 mV in control conditions and in the presence of CPP (B, P5 rat). Averages of successes to the second stimulus (A and B, bottom) or to the first stimulus (B, top) are shown on the right. **C)** Percentage of successes to the first (empty column) and to the second stimulus (filled column) at -60 and +40 mV for the neurone shown in A. **D)** Mean percentage of successes to the first and to the second stimulus at -60 and +40 mV for all P2-P7 CA3 pyramidal neurones examined at 32°C (n=29). At -60 mV the percentage of successes to the second stimulus is significantly higher than that to the first one (++ $P<0.001$), while at +40 mV the values are significantly higher than those at -60 mV for both the stimuli (* $P<0.05$, ** $P<0.001$).

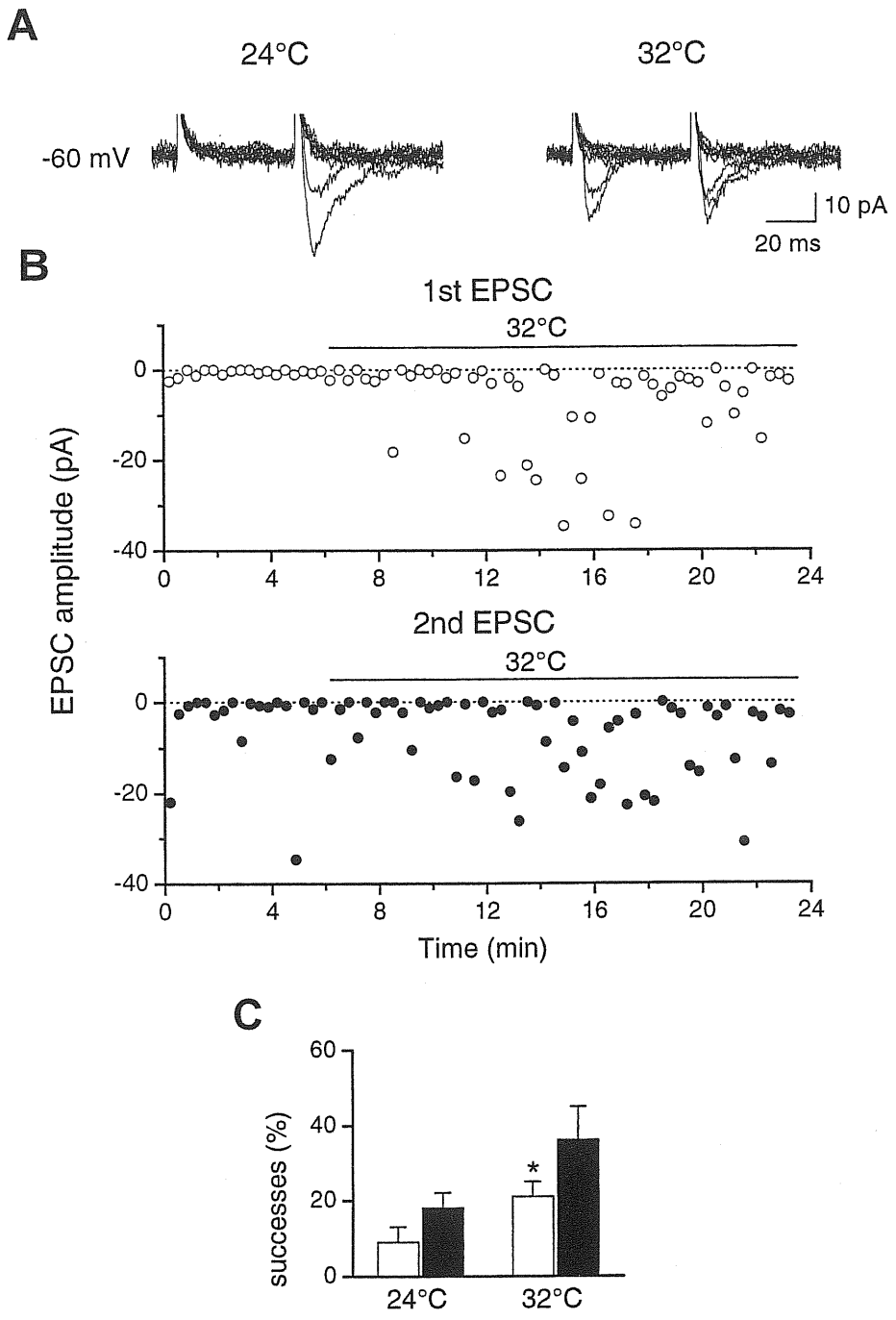

Figure 2. Silent MF-CA3 synapses become functional after increasing the temperature from 24°C to 32°C. A) Paired-pulse stimulation of MF at 24°C evoked no responses to the first stimulus but some to the second one (P4 rat). Raising the temperature from 24°C to 32°C resulted in the appearance of responses to the first stimulus and increased the number of successes to the second (ten traces are shown for both conditions). **B)** Peak amplitude of the first (top) and the second (bottom) EPSCs recorded at -60 mV, at 24°C and at 32°C (bars), plotted versus time from the same neurone as in A. **C)** Mean percentage of successes to the first and to the second stimulus at 24°C and 32°C for all P2-P7 CA3 pyramidal neurones examined (n=7). The percentage of successes to the first stimulus at 32°C was significantly higher than at 24°C (* $P<0.05$).

1.3 Effects of temperature at Schaffer collaterals-CA1 synapses

A similar temperature effect was seen when paired-pulse minimal stimulation of Schaffer collaterals was used to induce EPSCs in CA1 pyramidal neurones in P2-P4 hippocampal slices (Figure 3). At 24°C, 10 out of 28 CA1 neurones exhibited no successes to the first stimulus, but 7 of them showed sporadic responses to the second. However, as for MF input, NMDA-receptor mediated EPSCs could be detected at +40 mV (not shown). It should be stressed that, at 24°C, the probability of successes to the first stimulus was extremely low even in those synapses that were not silent, sometimes only a single response being detected in more than 20 trials. As shown in the example of Figure 3, raising the temperature from 24°C to 32°C resulted in the appearance of AMPA-mediated responses to the first stimulus and an increase in the number of second responses (from 6/20 to 12/26). On average, in 8 out of 10 cells recorded at both 24°C and 32°C, the percentage of successes to the first and second stimulus increased significantly ($P<0.05$) from $6 \pm 2\%$ and $13 \pm 5\%$ to $38 \pm 7\%$ and $48 \pm 8\%$, respectively (Figure 3C).

In previous studies, synapses were considered silent if they did not exhibit any AMPA-mediated responses in 25-100 consecutive trials (at stimulation frequencies of 0.3-2 Hz; Isaac *et al.*, 1995; Liao *et al.*, 1995; Choi *et al.*, 2000). To see whether the present results were consistent with previously published papers, additional experiments were performed, at the stimulation frequency of 0.5 Hz, using stricter criteria. Synapses were considered "silent" when they showed no responses to the first stimulus in >60 consecutive trials. The chance of observing >60 failures in a row would be <5% for a release probability <0.05. Out of 13 tested cells, 7 (54%) were silent to the first stimulus in 60-70 consecutive trials but showed rare responses to the second one (on average $8 \pm 3\%$; see Figure 4A and B). Among seven cells recorded at both 24°C and 32°C, four became conductive to the first stimulus at higher temperature while in the remaining three the percentage of successes to both stimuli increased considerably. On average, the mean percentage of successes to the first and second stimulus was significantly enhanced (from $1.6 \pm 0.9\%$ and $4 \pm 1\%$ at 24°C to $30 \pm 10\%$ and $27 \pm 7\%$ at 32°C, respectively; $n=7$, $P<0.05$; Figure 4C). These results suggest that, also at SC-CA1 connections, the existence of silent synapses in early postnatal life is not due to the absence of functional AMPA receptors, but to a low level of glutamate release. Indeed, rising the temperature from 24°C to 32°C could convert silent synapses into functional ones, even when using more strict criteria for their identification.

Although this process may be due to an increase in neurotransmitter release, it can not be excluded that increasing the temperature induces a change in presynaptic cell excitability, so that the same stimulus may recruit more presynaptic fibres. To test this hypothesis, possible changes in the amplitude of extracellular fibre volley, an index of presynaptic excitation, were monitored by recording fEPSPs evoked in the CA1 region by pairs of

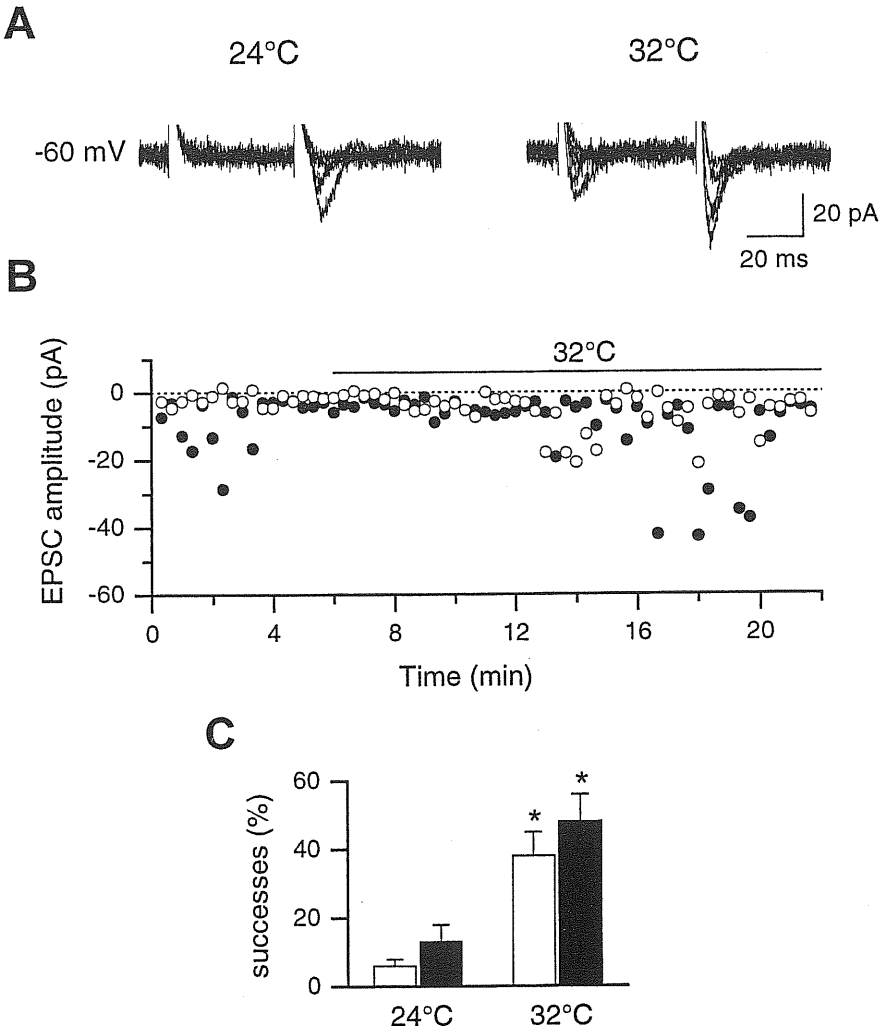

Figure 3. Temperature dependence of SC-CA1 synaptic transmission. A) Five individual traces of EPSCs evoked by paired stimulation of Schaffer collaterals at 24°C (left) and 32°C (right) (P2 rat). **B)** Peak amplitude of the first (empty circles) and second (filled circles) EPSCs recorded from the CA1 region are plotted against time. Note the absence of responses to the first stimulus at 24°C. Increasing the temperature from 24°C to 32°C resulted in the appearance of some responses to the first stimulus and enhanced the number of successes to the second. **C)** Mean percentage of successes to the first and to the second stimulus at 24°C and 32°C for all P2-P6 CA1 pyramidal neurones tested (n=8). The values at 32°C are significantly higher for both the first and the second EPSC (* $P<0.05$).

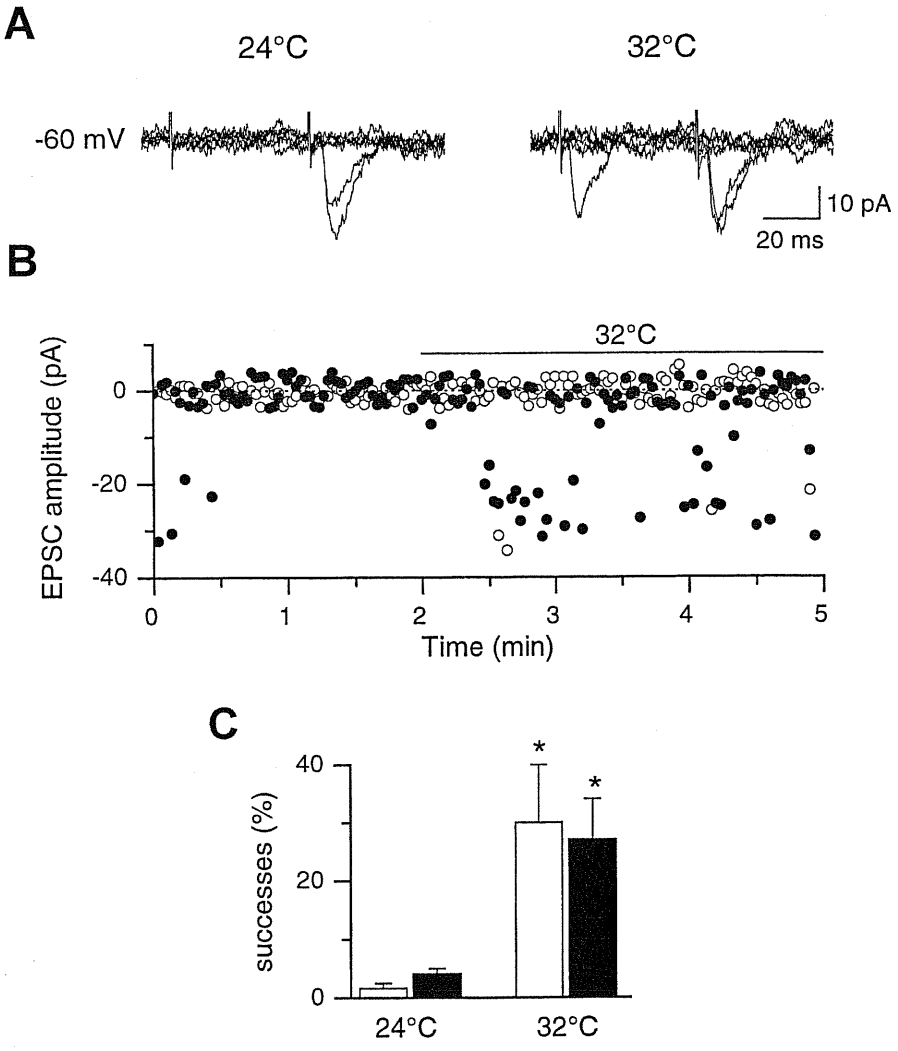

Figure 4. Effect of temperature on EPSCs elicited in CA1 pyramidal neurones at 0.5 Hz stimulation. A) Six individual EPSCs elicited at 0.5 Hz stimulation, at 24°C and 32°C. **B)** Time course of the first (open circles) and the second (filled circles) EPSCs rising the temperature from 24°C to 32°C. **C)** Mean percentage of successes to the first (white column) and to the second (black column) stimulus at 24°C and 32°C (n=7, * $P<0.05$).

stimuli. As shown in Figure 5A, the amplitude of the afferent volley preceding the first and second response was exactly the same. Increasing the temperature from 24°C to 32°C caused an increase in the amplitude of the fEPSP (from 140 ± 30 to 210 ± 50 μV, at 2-2.5 times the threshold, n=4) and a reduction in the peak latency (from 8.3 ± 0.5 to 5.6 ± 0.1 ms, see Figure 5A). Moreover, as shown in Figure 5A and B, a small but consistent

reduction in the amplitude of the afferent volley was observed. At 2.5 times the threshold, the normalised amplitude changed from 0.81 ± 0.06 at 24°C, to 0.70 ± 0.10 at 32°C (n=4). It is therefore likely that temperature-dependent conversion of silent synapses into functional ones is not due to recruitment of new afferent fibres but requires an increase in neurotransmitter release.

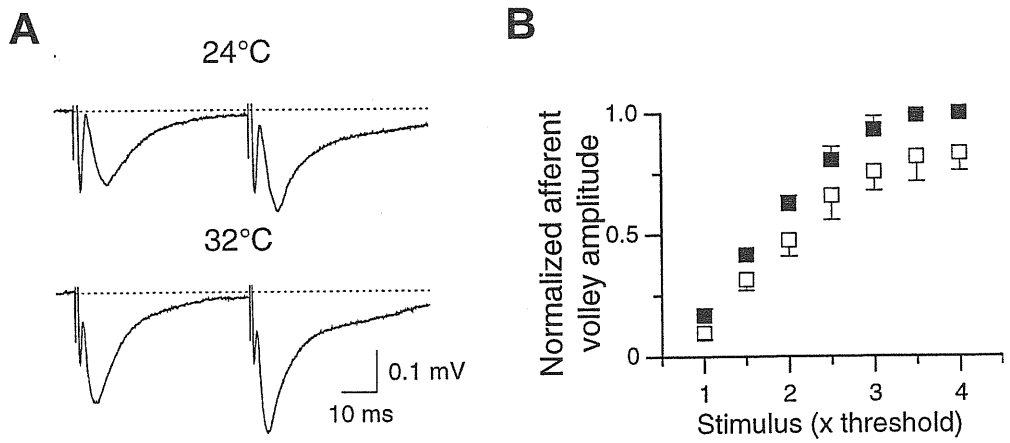

Figure 5. Rising the temperature from 24°C to 32°C does not increase presynaptic afferent volley. A) Pair of fEPSPs (average of three traces) evoked by SC stimulation at 24°C and 32°C from a P6 rat. **B)** Mean amplitude of the afferent volley (n=4), normalised to the maximum amplitude versus stimulus strength (expressed as x times the threshold), at 24°C (filled squares) and 32°C (open squares).

1.4 Effect of CTZ on synaptic responses at Schaffer collaterals-CA1 synapses

To further examine the possibility that the activation of silent synapses was due to an enhancement in glutamate release, bath applications of CTZ were performed. CTZ is a drug known to potentiate glutamate release and to selectively block AMPA receptor desensitisation (Diamond & Jahr, 1995; Bellingham & Walmsley, 1999; Ishikawa & Takahashi, 2001; but see Choi *et al.*, 2000). Before CTZ addition, 4 out of 10 cells were silent. During CTZ application (50 μM), two out of four silent synapses became conductive (Figure 6A-B). In other six cells, CTZ induced an increase in the percentage of successes to the first stimulus (from to 0.31 ± 0.08% to 0.45 ± 0.08%, Figure 7B) and a significant increase in the amplitude of the first response (from -26 ± 8 to -33 ± 8 pA, $P<0.05$). Moreover, in agreement with previous studies (Choi *et al.*, 2000; Rammes *et al.*, 1999), CTZ enhanced the rise time and the decay time constant (see Figure 7A). For the

first response the rise time varied from 1.23 ± 0.21 to 2.1 ± 0.2 ms (n=5, P<0.05) and the decay time constant from 7 ± 1 to 15 ± 2 ms (n=5, P<0.005). To see whether the potentiating effect of CTZ on minimal EPSCs was due to a presynaptic site of action, the PPR of synaptic responses elicited by stimulation of the Schaffer collaterals was measured before and during CTZ perfusion. CTZ reduced PPF from 2.0 ± 0.1 to 1.4 ± 0.1 (P<0.05, n=6; Figure 7C). In extracellular recordings, CTZ did not increase the amplitude of the afferent volley preceding the fEPSP (at 2.5 times the threshold, the normalised

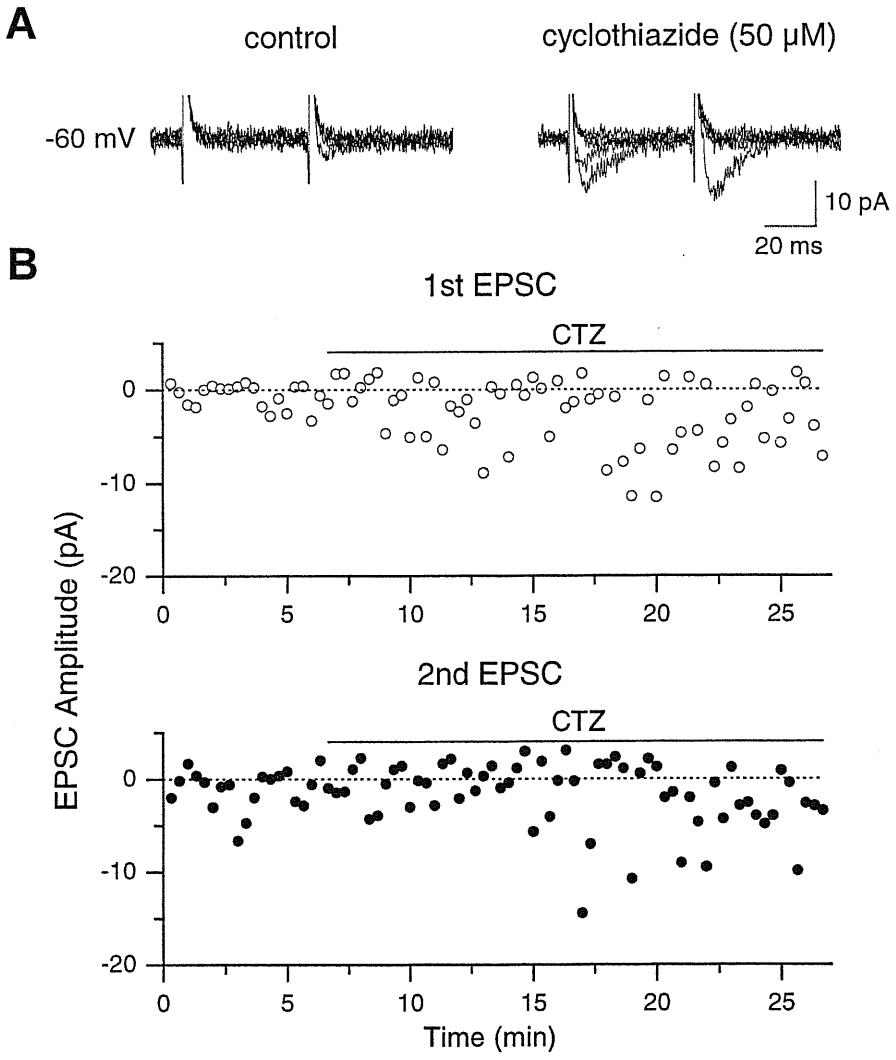

Figure 6. Cyclothiazide can activate silent synapses. A) EPSCs recorded at 24°C (P3 rat). Bath perfusion of CTZ (50 µM, bars) induced the appearance of successes to the first stimulus (in both conditions 5 traces are shown). **B)** Time course of CTZ effect on the amplitude of the first (open circles) and second (closed circles) EPSCs, before, during and after CTZ application (50 µM, bars) for the cell shown in A.

amplitude was 0.76 ± 0.04 before and 0.69 ± 0.05 during CTZ application, n=4). These experiments suggest that CTZ increases transmitter release without recruiting more presynaptic fibres.

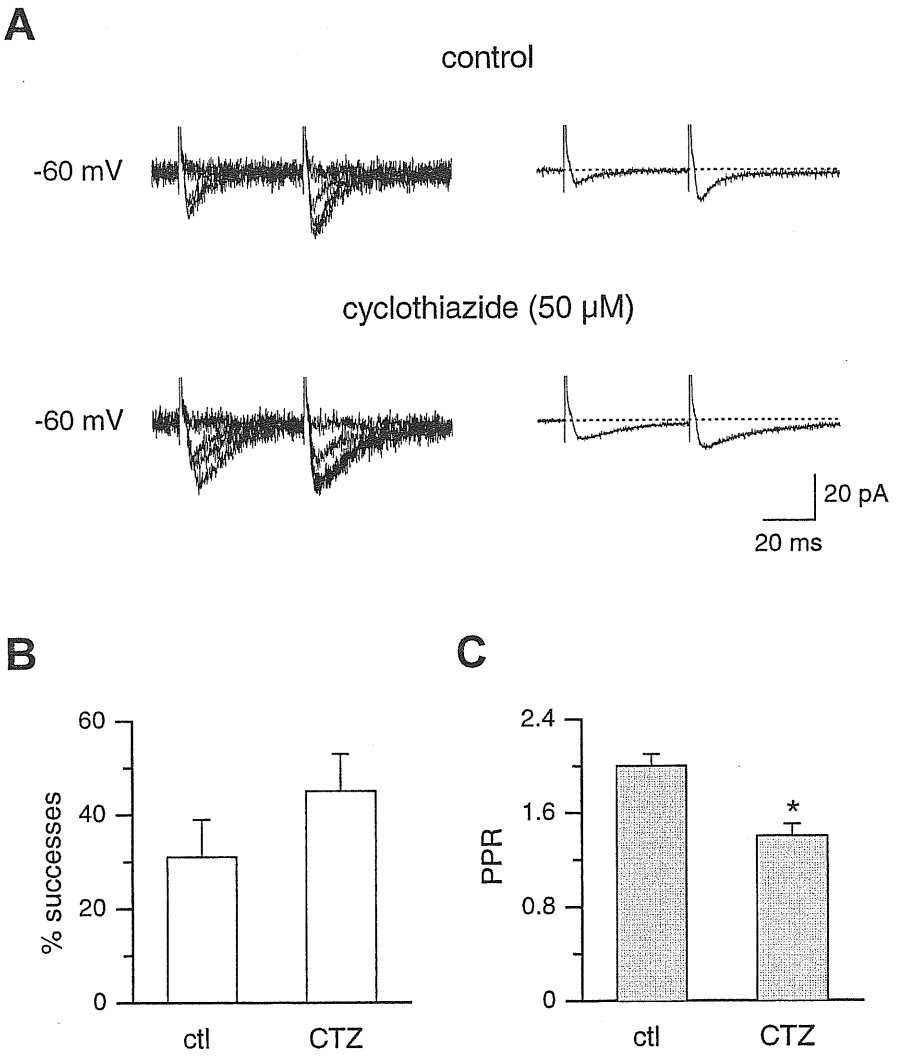

Figure 7. CTZ increases the percentage of successes to the first pulse and decreases the paired-pulse ratio. A) Five individual traces (left) and averages (right) recorded from a CA1 neurone in control conditions and in the presence of CTZ (P4 rat). **B)** Mean percentage of successes to the first stimulus in control conditions and in CTZ (n=6). **C)** Mean PPR measured before and during CTZ application (n=6; * $P<0.05$). PPR was calculated as the ratio between the amplitude of the second and the first response after averaging all the traces. Note changes in the kinetics of AMPA-mediated responses in the presence of CTZ.

1.5 Increasing frequency of stimulation switches off active synapses

In spite of the low frequency of stimulation used in the majority of the present experiments (0.05 Hz), EPSCs tended to disappear with time (see the sequence of second EPSC in Figure 3B). This synaptic depression, which occurred in the absence of any change in leak conductance of the cell, could be reversed by increasing the temperature or by slowing down the rate of stimulation. To see whether increasing stimulation frequency could convert functional synapses into silent ones, Schaffer collaterals were stimulated first at 0.1 and then at 1 Hz (Figure 8). At 0.1 Hz the percentage of successes was very

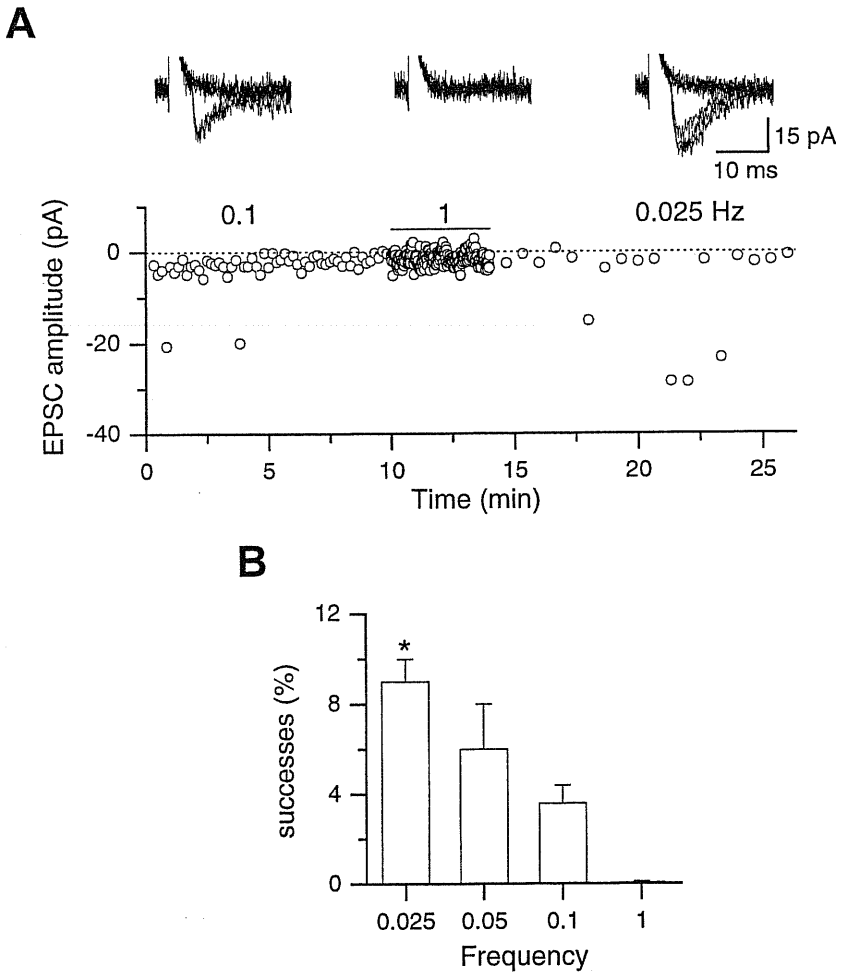

Figure 8. Effect of stimulus frequency on EPSCs elicited in CA1 pyramidal neurones by Schaffer collateral stimulation. A) Time course of the first EPSCs recorded while changing the stimulation frequency from 0.1 to 1 Hz (bar) and then to 0.025 Hz. Insets represent seven individual EPSCs elicited at each stimulation frequency. **B)** Mean percentage of successes obtained by changing the stimulation frequencies as shown in A (n=8). For comparison the mean percentage of successes obtained at 0.05 Hz stimulation in 8 other neurones is shown. The value obtained at 0.025 Hz is significantly different from that at 0.1 or 1 Hz (* $P<0.05$ for both).

low leading to "silent" synapses in 2 out of 8 cells. During 1 Hz stimulation, all synapses became silent so that in >60 trials only failures to both stimuli were recorded (Figure 8A and B). In all neurones tested, EPSCs reappeared after reducing the stimulation frequency from 1 to 0.025 Hz. These results indicate that, in early postnatal life, synaptic reliability strongly depends on stimulation rate and that at relatively modest frequencies of stimulation conducting synapses can be converted into silent ones.

1.6 Discussion

1.6.1 Activation of silent synapses

In this study it has been shown that both Mossy fibre-CA3 and Schaffer collateral-CA1 synapses express functional AMPA receptors since the first postnatal days, but they may appear silent at resting membrane potential because of a very low probability of glutamate release. Several lines of evidence suggest that these synapses are presynaptically silent: i) they could respond to a second stimulus in a paired-pulse protocol. Paired-pulse facilitation is a widely accepted index of presynaptic function (Zucker, 1989) and was observed also in those cases in which rare successes to the first stimulus were detected. It should be stressed that the paired-pulse stimulation procedure mimics repetitive firing, known to play a crucial role in information processing, thus making synaptic transmission more reliable (Lisman, 1997). ii) they could become conductive when the temperature was raised from 24°C to 32°C. Moreover, synapses already functional at 24°C showed an enhanced percentage of successes at 32°C. Temperature is known mainly to affect processes involved in synaptic release, for example increasing the mean number of transmitter quanta released per presynaptic action potential (Hardingham & Larkman, 1998). The possibility that temperature affects presynaptic cell excitability so that, at higher temperature, the same stimulus may recruit more fibres can be ruled out. In extracellular experiments, rising the temperature from 24°C to 32°C did not produce any significant increase in the amplitude of the afferent volley: a tendency towards a reduction was instead constantly observed. These data are in agreement with a previous study in which rising temperature caused a reduction in the duration of the directly evoked action potential in hippocampal slices from immature rabbits (Shen & Schwartzkroin, 1988). iii) they could become functional in the presence of CTZ, which is known to block AMPA receptor desensitisation and to enhance glutamate release (Diamond & Jahr, 1995;

Bellingham & Walmsley, 1999; Ishikawa & Takahashi, 2001; but see Choi *et al.*, 2000). In line with an increase in release probability, is the observation that CTZ not only considerably enhanced the percentage of successes to the first stimulus but also significantly decreased the PPF ratio. These results are in agreement with recent findings suggesting that in the immature hippocampus, silent synapses containing an appreciable number of AMPA receptors appear silent because the peak of glutamate concentration in the synaptic cleft is too low to activate low affinity AMPA receptors (Choi *et al.*, 2000). Low levels of glutamate could arise either from spillover of neurotransmitter from neighboring terminals (Astzely *et al.*, 1997; Kullmann & Astzely, 1998; Min *et al.*, 1998) and/or by the trickle of neurotransmitter from a fusion pore as occur in neuronal (Bruns & Jahn, 1995) or non neuronal secretory cells (Spruce *et al.*, 1990). According to the spillover hypothesis, only high affinity NMDA receptors, but not low affinity AMPA receptors would be activated by the low concentration of glutamate diffused from a neighbouring synapse (Patneau & Mayer, 1990; Kullmann & Astzely, 1998). An enhanced probability of neurotransmitter release, following PPF, increased temperature and CTZ, would allow glutamate to be released also locally close to the subsynaptic membrane so that its concentration would be high enough to activate both NMDA and AMPA receptors, thus converting silent synapses into functional ones. The higher percentage of silent synapses at 24°C rather than at 32°C, found in the present experiments, is also in favour of the spillover hypothesis (Astzely *et al.*, 1997; Kullmann & Astzely, 1998). However, at physiological temperature, spillover would be limited by the higher rate of glutamate clearance from the extracellular space (Astzely *et al.*, 1997; Min *et al.*, 1998) and therefore the contribution of this mechanism should be minimal. Alternatively, release of a small amount of glutamate from a non-expanding fusion pore may account for some of the above observations (Spruce *et al.*, 1990; Bruns & Jahn, 1995; Zhou *et al.*, 1996; Lollike *et al.*, 1998). Factors which increase the release probability would modify the gating properties of presynaptic fusion pores from the non-expanding mode to a rapidly expanding one in the same way as recently suggested for LTP at silent synapses (Choi *et al.*, 2000).

1.6.2 Conversion of functional synapses into silent ones by low-frequency stimulation

Additional evidence in favour of a presynaptic mechanism is given by the experiments performed at different stimulation frequencies. The short-term synaptic depression and

the appearance of silent synapses induced by increasing stimulation rate are most likely attributable to changes in presynaptic release mechanisms. Using multiple-probability fluctuation analysis at rat climbing fibre-Purkinje cell synapses, it was found that frequency-dependent depression was associated with a dramatic fall in release probability with no apparent changes in the number of functional release sites or in the quantal size, suggesting a pure presynaptic origin (Silver *et al.*, 1998). At SC-CA1 synapse of the adult hippocampus a reduction in quantal content due to changes in release probability was found during low-frequency depression, although it was sometimes accompanied by changes in the quantal size (Larkman *et al.*, 1997). The slow reappearance of responses observed in the present experiments may involve recovery from presynaptic calcium channel inactivation (Gingrich & Byrne, 1985) or distinct kinetic phases in vesicle recycling (Dittmann & Regher, 1998). Altogether, the above results strengthen the role of presynaptic mechanisms in controlling synaptic efficacy in the developing hippocampus. In comparison with previous work (Durand *et al.*, 1996; Liao & Malinow, 1996), the percentage of SC-CA1 synapses silent at rest was smaller. This apparent discrepancy could be attributed to differences in recording conditions. In particular, previously reported data were obtained at higher frequency of stimulation (0.1-0.3 Hz), conditions that, according to the present results, would favour the reduction in the percentage of successes and eventually would lead to the disappearance of synaptic responses. In keeping with this, also in the present experiments the percentage of silent synapses increased to 54% when the stimulation frequency was 0.5 Hz.

Although the above results do not preclude the possibility that functional AMPA receptors can be recruited at postsynaptic sites during LTP (Liao *et al.*, 1999; Shi *et al.*, 1999), they strengthen the role of presynaptic mechanisms in controlling synaptic efficacy in a proportion of hippocampal neurones during early postnatal development.

2. Frequency-dependent modulation of paired-pulse ratio at unitary CA3-CA3 synapses in the rat hippocampus

As also observed in the first section of the results, activity-dependent depression represents a common form of short-term plasticity. Whatever the mechanisms involved are, in most cases, synaptic depression should be dependent on previous release (Debanne *et al.*, 1996). Thus, in a paired-pulse protocol, PPR is inversely related to the initial release probability (Dobrunz & Stevens, 1997) and the smaller is the probability of release to the first pulse, the more facilitated is the response to the second one. It follows that, if repetitive stimulation of presynaptic neurone causes a reduction in the release probability and, therefore, in the amplitude of the first EPSC (Thomson *et al.*, 1993), it should induce a further facilitation of the second one, leading to an increase in PPR. Thus, the following experiments were undertaken to better investigate the mechanisms involved in a form of activity-dependent depression similar to the one previously observed in acute slices and to see whether the relation between PPR and probability of release was still maintained during this kind of plasticity.

In the hippocampus, synaptic depression has been studied mainly with minimal stimulation of afferent inputs (Voronin, 1993; Larkman *et al.*, 1997). With this method it is impossible to ascertain that the same presynaptic axon is activated trial after trial as different axons are likely to be stimulated. Moreover, failures of presynaptic release cannot be unambiguously distinguished from failures of the stimulus to trigger an action potential in the presynaptic fibre. To overcome these problems whole-cell recordings were performed from pairs of interconnected CA3-CA3 pyramidal neurones in hippocampal slice cultures (Debanne *et al.*, 1996; Pavlidis & Madison, 1999). In the majority of neurones, LFD was associated with a reduction in EPSCs amplitude. The magnitude of LFD varied between pairs and depended on the rate of presynaptic firing. Unexpectedly, PPR was reduced at higher presynaptic firing frequency (1 Hz) and, on average, PPF was converted into PPD suggesting that during LFD release probability results from the balance of at least two distinct processes, one depending on the residual calcium and the other on the number of available vesicles.

2.1 Paired-pulse modulation at CA3-CA3 connections

Stable long-lasting (>30 min) whole-cell recordings were performed from 22 CA3 pyramidal cell pairs. Interconnected neurones were identified as pyramidal cells both

visually and on the basis of their firing properties, i.e. their ability to accommodate in response to long (800 ms) depolarising current pulses. The identity of the connected cells as pyramidal neurones was confirmed in three experiments in which cell pairs were labelled with biocytin. In control conditions, pairs of presynaptic action potentials (50 ms apart), delivered at the frequency of 0.05-0.067 Hz, evoked two sequential EPSCs that fluctuated in amplitude from trial to trial with occasional response failures (Figure 9). Paired-pulse modulation was quantified by calculating the paired-pulse ratio as the ratio between the mean amplitude of the second and the first EPSC. In agreement with previous reports (Debanne *et al.*, 1996; Pavlidis & Madison, 1999) a strong heterogeneity in PPR across different connections was observed. The majority of cells (15 out of 20) exhibited PPF (1.4 ± 0.1, see Figure 9), while the remaining 5 PPD (0.7 ± 0.1).

2.2 Properties of low-frequency depression

Increasing frequency of presynaptic firing from 0.05-0.067 to 0.1-1 Hz induced depression of postsynaptic responses (n=14). The degree of synaptic depression was heterogeneous across different pairs but was clearly dependent on the testing frequency, being larger at higher frequencies. As shown in the example of Figure 9, consecutive changes in presynaptic firing frequency from 0.05, to 0.1 and 1 Hz induced a gradual reduction in the number of successes, leading only to transmission failures. Overall, in 9/12 (75%) cells at the end of 1 Hz low frequency train, synaptic transmission was almost completely abolished (Figure 9B and 10B). In the remaining 3 cells, 1 Hz stimulation induced a reduction of the mean amplitude of synaptic responses (of 60 ± 10% and 70 ± 10%, for EPSC1 and EPSC2, respectively), although only few failures were observed. Successes reappeared in all cells examined (n=8) after switching to a lower frequency (0.025 Hz).

2.3 Interaction between paired-pulse modulation and low-frequency depression

In the example of Figure 9, increasing the stimulation frequency from 0.05 to 0.1 Hz produced a reduction in the amplitude of both EPSC1 and EPSC2. This was associated with an enhanced number of failures and a slight decrease in PPR. The Figure shows also that a further increase of stimulation frequency to 1 Hz strongly depressed both responses. Unexpectedly, EPSC2 became silent before EPSC1, leading to the conversion of PPF into PPD. Responses reappeared after setting the frequency of stimulation to 0.025 Hz (Figure

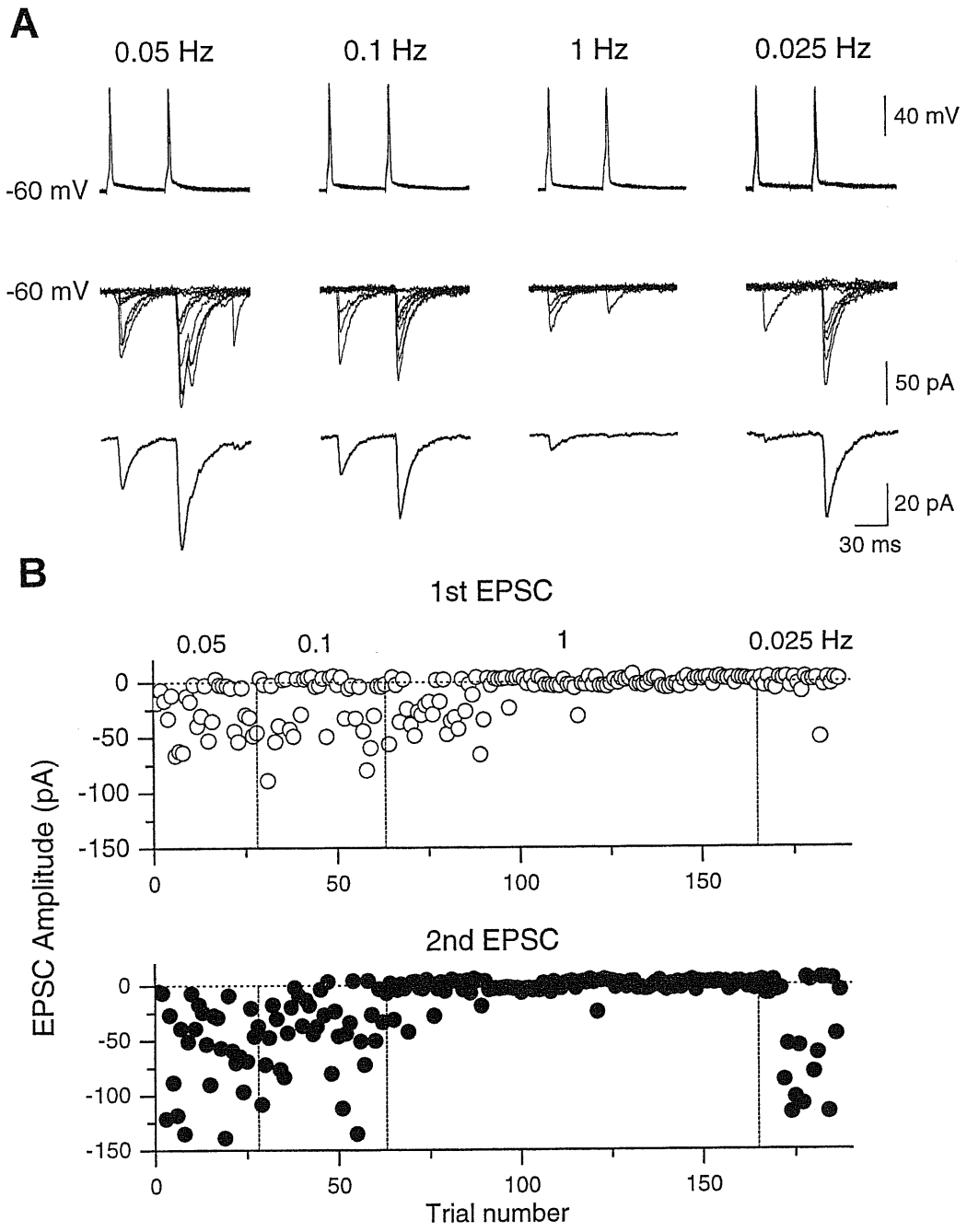

Figure 9. Low-frequency depression induced in a synaptically connected pair of CA3 neurones by increasing the presynaptic firing frequency from 0.05 to 1 Hz. A) Ten pairs of action potentials (50 ms apart; top traces) evoked in the presynaptic cell and the corresponding postsynaptic responses (middle traces) are superimposed for each frequency. All EPSCs evoked at a given frequency (including failures) are averaged (bottom traces). **B)** Time course of EPSC amplitude to the first (top) and second (bottom) pulse for the same neurone shown in A.

9B). In this particular case, EPSC1 recovered less than EPSC2 leading to a very strong PPF.

LFD was observed also in cases with initial high probability of release as in the example of Figure 10, where only successes were recorded in control condition (at 0.05 Hz).

Increasing the stimulation frequency led to a decrease in the mean EPSCs amplitude and to the appearance of some transmission failures. Again, at 1 Hz, the responses were almost abolished with sporadic responses to EPSC2. A complete recovery of both EPSC1 and EPSC2 was obtained at 0.025 Hz. (Figure 10B). Similar depression and recovery were observed in additional 4 neurones that were sequentially tested at 0.05, 0.1, 1 and0.025 Hz. Mean values of failure rate, changes in EPSC amplitudes and PPR are shown in Figure 11. While at 0.05 Hz the failure rate to the first spike was significantly

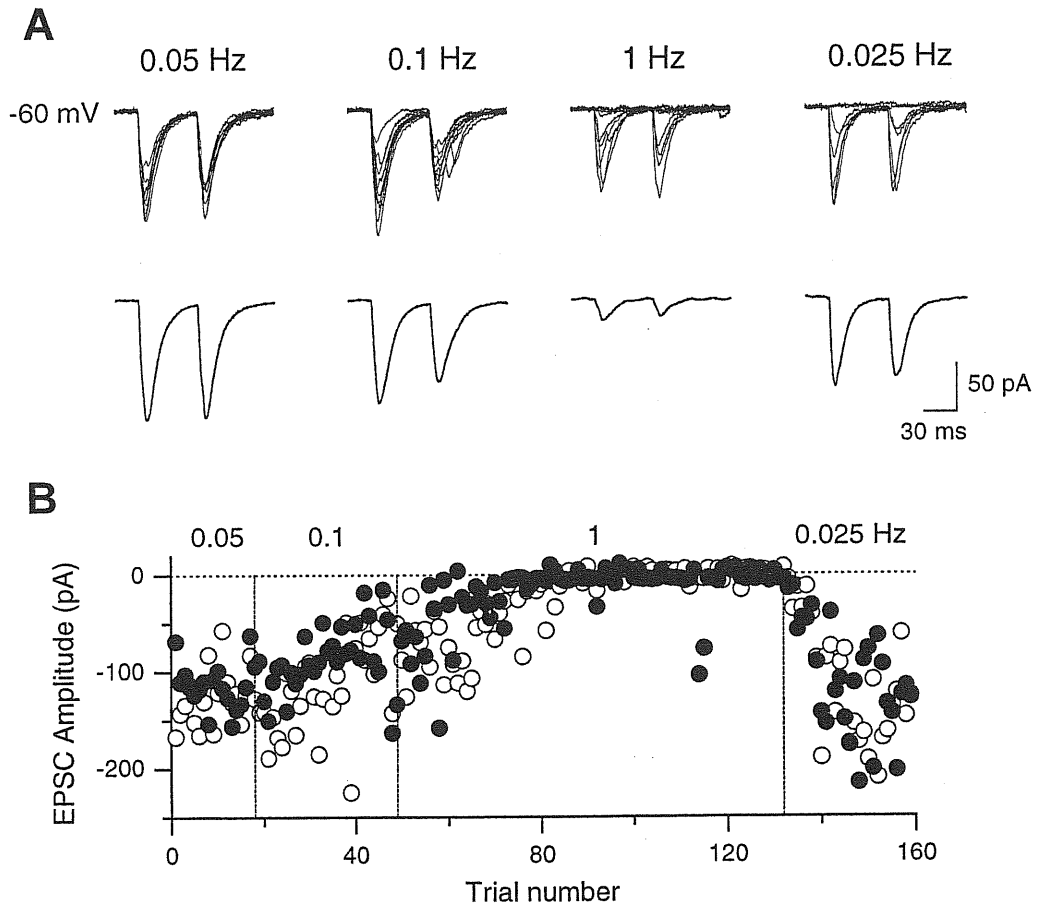

Figure 10. Low-frequency depression in a synapse with high release probability. A) Ten EPSC pairs evoked at different frequencies by presynaptic action potentials are superimposed (top traces). All EPSCs evoked at a given frequency (including failures) are averaged (bottom traces). **B)** Time course of EPSCs amplitude to the first (open circles) and second pulse (filled circles) for the same neurone shown in A.

higher than that to the second (black and white column of Figure 11A, P<0.05), at 0.1 Hz this difference was less pronounced. At 1 Hz, the failure rate of synaptic response to the second spike was significantly (P<0.05) higher than that to the first one. A partial recovery was observed at 0.025 Hz (n=4). The higher failure rate found at 0.025 Hz in comparison to 0.05 Hz reflects the slowness of the recovery process since, for any given frequency, responses occurring during the entire period of stimulation were analysed.

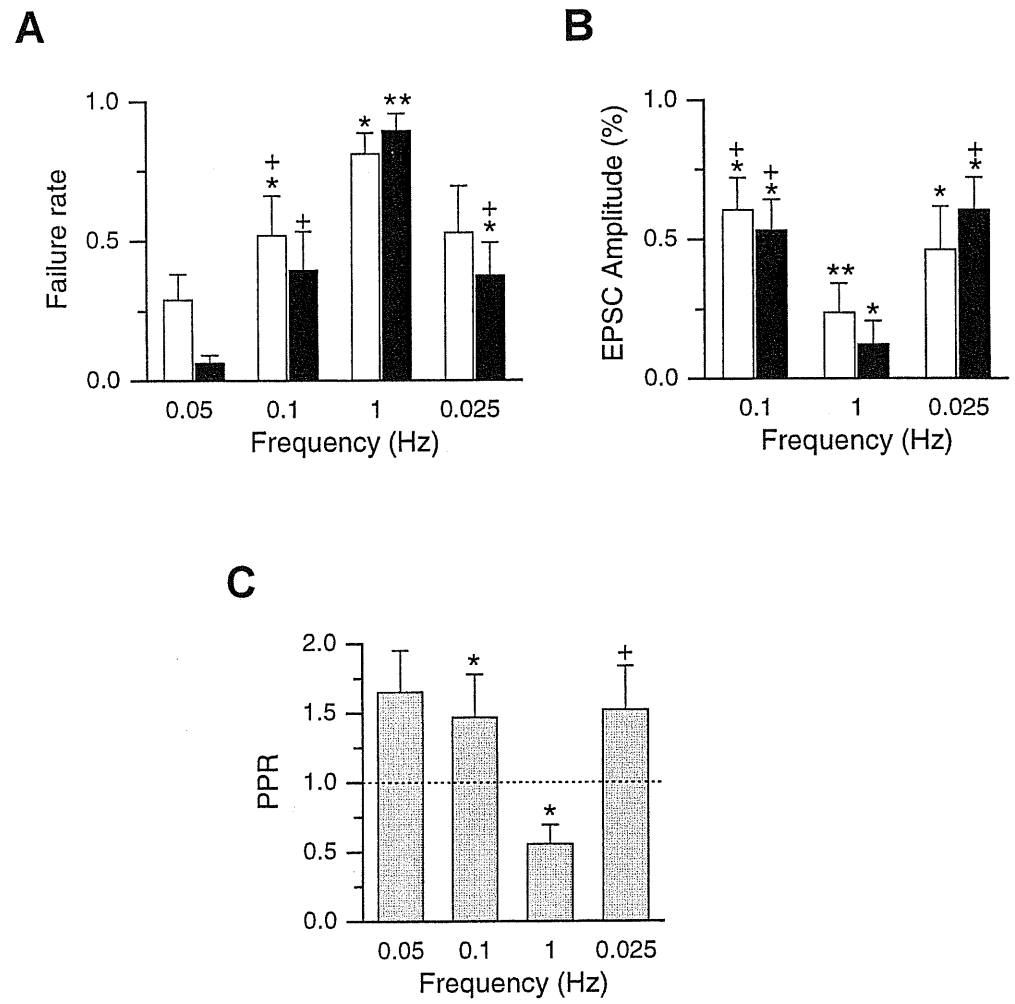

Figure 11. Summary data of changes in failures rate, amplitude and paired-pulse ratio of synaptic responses evoked at different frequencies. A) Failures rates to the first (white columns) and second (black columns) pulse under different frequencies of stimulation (n=5). **B)** EPSCs amplitude, as percent of their own value at 0.05 Hz, plotted for the other frequencies tested (six connected pairs). **C)** PPR calculated at different frequencies for the same six pairs of cells (0.025 Hz refers to 3 neurones). Signs * or + indicates significant differences from control or from 1 Hz values, respectively (*/+ P<0.05, **/++ P<0.001).

This also accounts for the smaller mean EPSC amplitude measured at 0.025 Hz with respect to controls (Figure 11B, see also Figure 10B and C). In fact, the percentage of recovery obtained after the first five minutes, was 70 ± 20% and 80 ± 20%, for EPSC1 and EPSC2, respectively (n=3). This rules out the possibility of long-term depression (Montgomery & Madison, 2002) or responses rundown. At 0.1 and 1 Hz, changes in failure rate were associated with a reduction in the mean EPSC amplitude (Figure 11B). The greater reduction of EPSC2 in comparison with EPSC1 is in line with the frequency-dependent PPR changes shown in Figure 11C. Similar results were found in additional 8 neurones in which use-dependent depression was investigated using different stimulation frequencies.

2.4 Time course of low-frequency depression

The amount of depression varied from cell to cell according to their previous history (i.e. to the duration and frequency of stimulation). In the graph of Figure 12, the time courses of depression and recovery are shown. While at 0.05 Hz, the mean EPSC amplitudes remained stable (Figure 12A), LFD appeared at the presynaptic firing frequency of 0.1 Hz and strongly increased at 1 Hz (Figure 12B-C). The time course of depression of EPSC1 could be fitted with a monoexponential function having a time constant of 98 s and 36 s at 0.1 Hz and 1 Hz, respectively. Interestingly, the time course of depression of EPSC2 did not follow that of EPSC1. While at 0.1 Hz, depression of EPSC2 was slower than EPSC1 (time constants of 145 s versus 98 s), at 1 Hz it was faster (7 s versus 36 s). All synapses slowly recovered from depression during 0.025 Hz stimulation. The time course of recovery was well described by a single exponential function having a time constant of 594 s and 603 s for EPSC1 and EPSC2, respectively (Figure 12D). The slowness of this process may account for the incomplete (70 ± 20%) recovery of EPSC1 after 5 min. In line with a presynaptic site of action, LFD did not modify EPSC kinetics (Figure 12E). As shown in Figure 12F, the decay time constants of single EPSCs evoked at different frequencies were not significantly different (P=0.89). Moreover, taking advantage of the double recordings technique, possible modifications of the presynaptic action potential were investigated. In spite of a small reduction in spike amplitude (from 92 ± 9 mV to 86 ± 9 mV), no change in spike half width value (from 1.7 ± 0.1 ms to 1.66 ± 0.07 ms) was observed between 0.05 and 1 Hz.

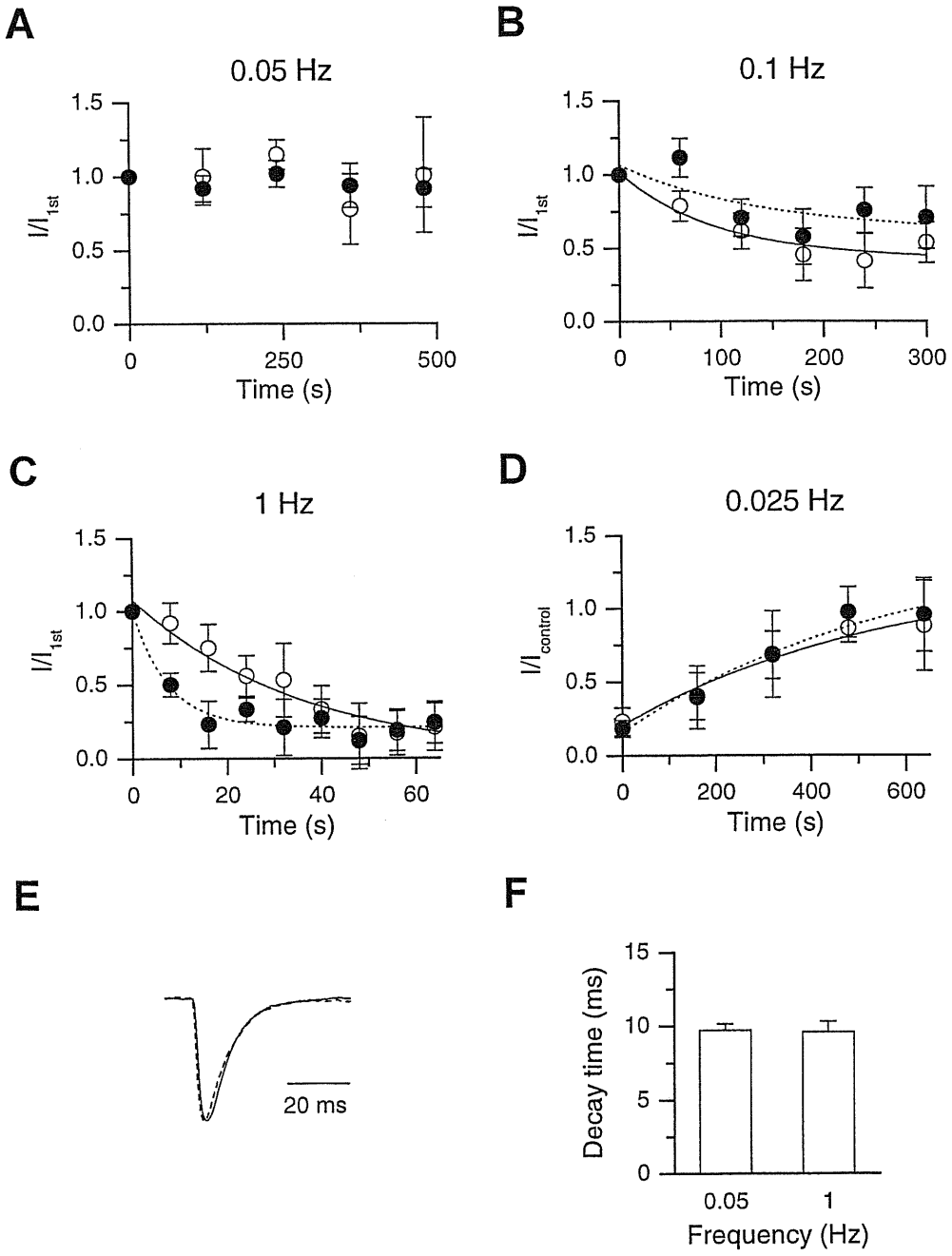

Figure 12. Time course of frequency depression and recovery. A-D) Time course of normalised mean EPSC amplitude evoked at different frequencies (n=5-6 neurones for A-C and 3 for D, see methods for normalisation). Open and filled circles refer to the first and second EPSC, respectively. Data points were fitted with one exponential function. Solid and dotted line represent the fit to the first and to the second EPSC, respectively. **E)** An example of normalised and superimposed mean EPSC evoked at 0.05 (solid line) and 1 Hz (dashed line) in one representative neurone. **F)** Mean decay time constant of EPSC evoked at 0.05 and 1 Hz (n=4).

2.5 NMDA receptors are not involved in short-term low-frequency depression

Recently it has been reported that in the same preparation at CA3-CA3 connections, presynaptic action potentials (600 pulses) at 1 Hz associated with a slight depolarisation (-55 mV) of the postsynaptic cell were able to induce a long-term depression (LTD) of EPSCs of almost 80% (Montgomery & Madison, 2002). This effect required the activation of NMDA receptors and was prevented by the NMDA receptor antagonist AP5 (Montgomery & Madison, 2002). To see whether a similar type of mechanism could account for the present results, in four experiments LFD was induced in the presence of the NMDA receptor antagonist AP5 (50 μM). A similar degree of depression of EPSC1was attained when the presynaptic firing was switched from 0.05 to 1 Hz (34 ± 6% and 40 ± 10% in control and AP5, respectively). Also in the presence of AP5, this effect was associated with a reduction of the paired-pulse ratio (from 1.3 ± 0.3 to 0.82 ± 0.03, data not shown). In two cases, LFD was studied in the same pairs before and after application of AP5. Thus, after EPSC depression at 1 Hz and recovery, presynaptic firing was set again at 1 Hz in the presence of AP5. Also in these cases a similar degree of depression was obtained in control (31 ± 9%) and in the presence of AP5 (28 ± 4%). These data allow exclusion of the involvement of NMDA receptors in LFD.

2.6 Discussion

The present results indicate that LFD is a common form of short-term synaptic plasticity at CA3-CA3 connections in hippocampal slice cultures. The reduction in synaptic strength varied across different connections according to presynaptic firing frequency and was associated with a modulation of paired-pulse ratio, with PPD prevailing over PPF at higher stimulation frequencies.

2.6.1 Mechanisms of low-frequency synaptic depression

Frequency depression constitutes the predominant form of short-term dynamics in many CNS structures including sensorimotor cortex (Thomson *et al.*, 1993; Abbott *et al.*, 1997; Tsodyks & Markram, 1997), auditory pathways (von Gersdorff & Borst, 2002), cerebellum (Silver *et al.*, 1998), hippocampus (Dobrunz & Stevens, 1997). Although in the present experiments comparatively low frequencies were used, these were able to produce a gradual reduction in EPSCs amplitude. Moreover, especially at 1 Hz, synaptic depression could be so strong to make synapses almost "silent" at –60 mV. LFD observed

here differed from LTD recently described in the same preparation by Montgomery & Madison (2002), since responses recovered after switching to a low frequency stimulation. Unlike LTD, the induction of LFD was NMDA-independent because it was insensitive to AP5. Moreover, LFD could be induced from a holding potential of -69 mV (including the correction for liquid junction potential), while LTD from -55 mV.

As in other CNS structures (Thomson *et al.*, 1993; Abbott *et al.*, 1997; Tsoddyks & Markram, 1997; Silver *et al.*, 1998), use-dependent depression was found to be presynaptic in origin as suggested by the increase in failure rate and changes in PPR. Presynaptic mechanisms interfering with synaptic vesicles release include changes in action potential waveform, inactivation of calcium currents, modulation of presynaptic calcium channels by G-protein coupled receptors, etc. Changes in action potential waveform may depend on sodium channel inactivation. However, this mechanism does not seem to play a crucial role in paired-pulse depression under basal conditions (He *et al.* 2002). Moreover, the observation that small changes in spike amplitude were not associated with modifications in action potential half width allows exclusion of a strong role of sodium channel inactivation in LFD (Brody & Yue, 2000; von Gersdorff & Borst, 2002). Presynaptic group II and III metabotropic glutamate receptors could potentially exert a strong inhibitory effect on transmitter release (Takahashi *et al.*, 1996; von Gersdorff *et al.*, 1997). These receptors however are not present on hippocampal associative commissural fibres (Berretta *et al.*, 2000).

Although the locus of depression is likely to be presynaptic, a postsynaptic effect, such as receptor desensitisation, cannot be completely ruled out. In the present experiments, EPSC kinetics was unchanged under different stimulation frequencies, suggesting that modifications in AMPA receptor gating are not involved in this form of plasticity.

2.6.2 Frequency-dependent modulation of paired-pulse ratio

Several independent approaches suggest that both frequency depression and paired-pulse modulation depend largely on changes in release probability (p). A reduction in p during LFD predicts an increase in the PPR. Indeed, PPR has been shown to be enhanced when the extracellular calcium/magnesium ratio was lowered (Debanne *et al.*, 1996; Canepari & Cherubini, 1998). Unexpectedly, in the present experiments PPF was converted into PPD. The reduction in PPR can be attributed to the activation of two temporally distinct processes: i) modulation of the release probability by the residual calcium and ii) changes

in size of the readily releasable pool of vesicles (Wu & Saggau, 1997; Dittman *et al.*, 2000). The idea of expressing release probability as the balance between two processes, one related to the residual calcium and the other to vesicle availability, is similar in many aspects to that put forward by Zucker (1973), Quastel (1997), Scheuss & Neher (2001). Unlike previously proposed models, the number of docked vesicles per release site was not constrained, even though the model proposed by Zucker (1973) could lead to equations similar to those presented in the appendix. Moreover, desensitisation was not considered (Scheuss & Neher, 2001). Finally, in the model the interaction between paired-pulse ratio and stimulation frequency was described by a differential equation for the mean population of docked vesicles. It should be stressed that, although the number of docked vesicles may be related to the release probability (Dobrunz & Stevens, 1997), this can not be generalised to all synapses (see Xu-Friedman *et al.*, 2001).

According to the model, the probability of release at a single active zone (p) can be written as:

$$p = Pr(Ca^{2+}) * Pr(Ves) \qquad (2.1)$$

where $Pr(Ca^{2+})$ and $Pr(Ves)$ are the probability of release dependent on residual calcium and on the size of the available pool, respectively. The interplay between $Pr(Ca^{2+})$ and $Pr(Ves)$ at the moment of arrival of the second spike would determine the direction of the paired-pulse modulation (PPF or PPD). Thus, PPF observed in the majority of neurones in stationary conditions would be mainly dictated by the residual calcium (Zucker, 1989), being $Pr(Ves)$ constant. In fact, at lower stimulation frequencies the size of the pool should be larger and any "loss" of vesicles during the first pulse will be negligible. At higher stimulation frequencies, when the available pool becomes too small, an additional depletion due to the release from the first presynaptic spike in the paired-pulse paradigm could diminish the size of the pool to such a critical level that at the moment of the arrival of the second spike $Pr(Ves)$ would be close to zero. In this case, according to eqn (2.1), p would be close to zero independently of the value of $Pr(Ca^{2+})$. Therefore, PPR is expected to increase at relatively low frequencies, and to decrease at higher frequencies when fewer vesicles are available for release. To validate these assumptions, a simple model has been developed (see Appendix). As predicted by the model in stationary conditions, when the time-dependent term of the process is neglected ($f(t)$ of eqn (A8) in

the Appendix), PPR is dependent on the frequency of stimulation (Figure 13A). It is clear from the Figure that the switch from PPF to PPD occurs between 0.1 and 1 Hz.

The time-dependency of the process is introduced by describing the dynamics of the population of docked vesicles *via* a first-order differential equation that considers both depletion and refilling of the pool. These two processes are characterised by time constants λ_d and λ_r (see also Scheuss & Neher, 2001). While λ_d depends on the stimulation frequency ω, the question on whether λ_r also depends on the same factor is still open. Recently it has been suggested that the endocytosis rate, which influences the refilling of the pool (λ_r), depends on the frequency of stimulation (Sun *et al.*, 2002). However, assuming λ_r independent on ω, the frequency-dependent decrease in the mean pool size was underestimated. The time course of the probability of release obtained at different frequencies (Figure 13B) is similar to that of EPSC amplitudes obtained in experimental conditions, including the switch from PPF to PPD at 1 Hz. This suggests that a decrease in p can account for the present observations, without requiring changes in quantal size or in the number of release sites. By averaging the probability of release over all trials (at each frequency) an estimate of PPR in non-stationary conditions was obtained (Figure 13C). As in experimental conditions (compare to Figure 11C) PPR decreased with increasing frequency leading to PPD at 1 Hz. This effect fully recovered after switching to 0.025 Hz.

In conclusion, from the present experiments it appears that presynaptic changes in p accounts for frequency-dependent synaptic depression. Release probability is directly correlated with a morphologically defined pool of docked vesicles (Dobrunz & Stevens, 1997; but see Xu-Friedman *et al.*, 2001). The size of this pool would vary enormously between different synapses. This may account for the different time course of depletion and replenishment and for distinct frequency-dependent modulation of paired-pulse ratio found in various brain structures. Thus, a large pool of vesicles at the calyx of Held would ensure reliable synaptic transmission even at high frequencies when only a small fraction of synaptic vesicles are available for release (von Gersdorff *et al.*, 1997).

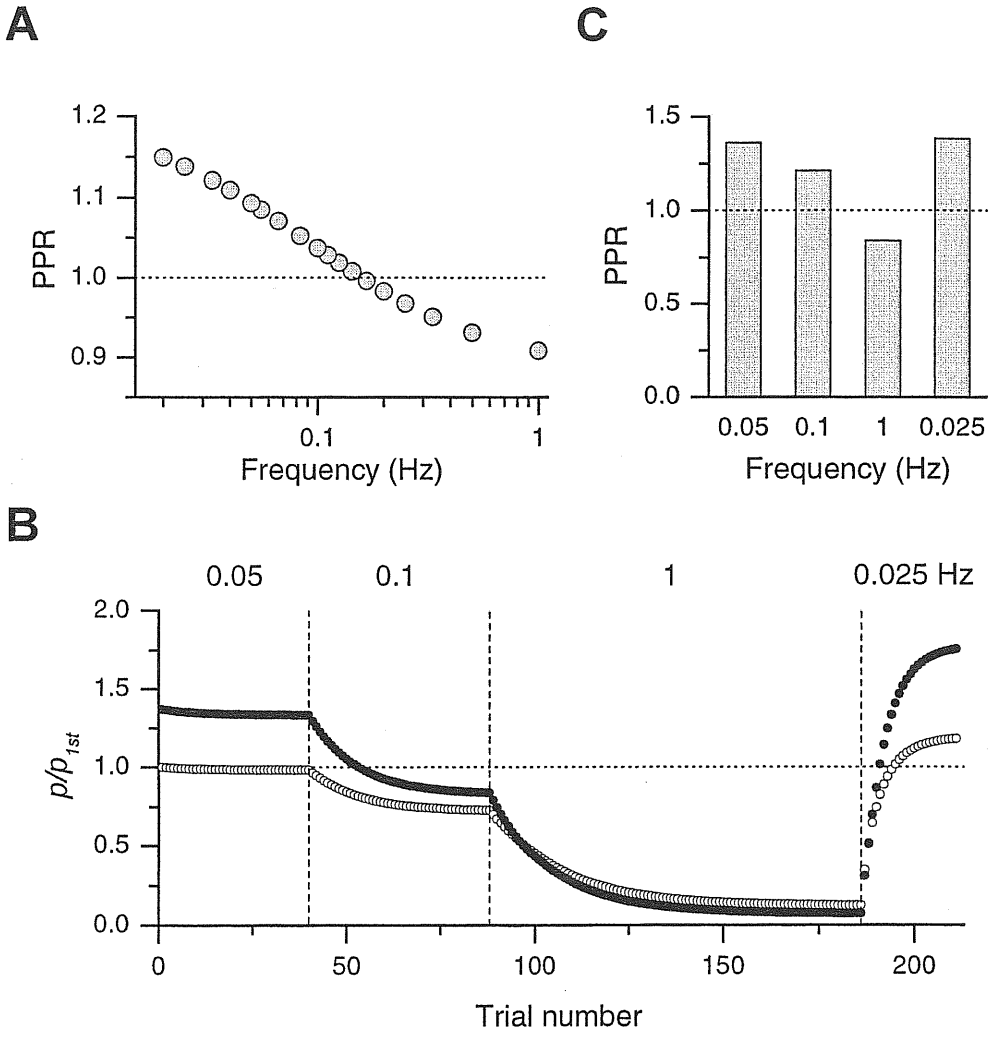

Figure 13. Expected frequency-dependent modulation of paired-pulse ratio. A) Stationary PPR obtained for different stimulation frequencies according to the proposed model (see eqn. (A6) in the Appendix). **B)** Predicted time course of the probability of release to the first (open circles) and second pulse (filled circles) at different stimulation frequencies. **C)** PPR calculated from the data of Figure 13B for respective frequencies. PPR was obtained as the ratio between the mean values of p_2 and p_1. Model parameters: α=0.48, γ=0.28, n_d=18, λ_r=0.0026 Hz, τ=100 ms, N_c=5.

2.7 Appendix

The dependence of p on the size of the available pool can be written as:

$$p_1 = k*(1 - exp(-\gamma*N^{3/2})) \quad \text{(A1)}$$

where k is a constant, p_1 is the probability of release to the first pulse, γ the fusion rate for a vesicle integrated over the duration of the presynaptic pulse (Dobrunz & Stevens, 1997) and N is the number of ready releasable vesicles.

The probability of release to the second pulse (p_2) is given by:

$$p_2 = p_1*P2(1) + (1 - p_1)*P2(0) \quad \text{(A2)}$$

where $P2(1)$ is the probability of release to the second pulse, after release to the first and $P2(0)$ the same probability after no release to the first pulse.

Taking into account also the effect of the residual calcium, these probabilities can be written as:

$$P2(1) = k*(1 + \alpha*exp(-t/\tau))*(1 - exp(-\gamma*(N - 1)^{3/2})) \quad \text{(A3)}$$

$$P2(0) = k*(1 + \alpha*exp(-t/\tau))*(1 - exp(-\gamma*N^{3/2})) \quad \text{(A4)}$$

Where α is a constant representing the sensitivity to residual calcium and τ is the time constant of decay of the higher probability of release, which is related to the rate of removal of residual calcium (Atluri & Regehr, 1996).

Hence the paired-pulse ratio is:

$$PPR = p_2/p_1 = P2(1) + P2(0)*(1 - p_1)/p_1 \quad \text{(A5)}$$

Thus, by substituting $P2(1)$, $P2(0)$ and $p1$ from eqns (A3) and (A4) into eqn (A5):

$$PPR = (1 + \alpha*exp(-t/\tau))*(1 - exp(-\gamma*(N - 1)^{3/2})) + (1 + \alpha*exp(-t/\tau))*exp(-\gamma*N^{3/2}) \quad \text{(A6)}$$

The number of available vesicles N can be modeled by writing

$$dN/dt = -\lambda_d*N + \lambda_r*(N_c - N) \quad \text{(A7)}$$

where λ_d and λ_r are the depletion and refilling constants, respectively, while N_c is the maximum size of the ready releasable pool of vesicles.

This equation states that there is a use-dependent depletion, whose rate depends on: i) the frequency of stimulation ω with $\lambda_d = \omega/n_d$, and ii) the refilling process controlled by λ_r. The solution of this first order differential equation gives the mean occupancy of each release site in the model synapse at time t:

$$N(t) = f(t) + N_{eq} \quad \text{(A8)}$$

where the time-dependent term $f(t)$ is equal to $a*exp(-(\lambda_d + \lambda_r)*t)$ and the equilibrium term N_{eq} is given by

$$N_{eq} = \lambda_r * N_c / (\lambda_r + \lambda_d) = \lambda_r * N_c * n_d / (\omega + n_d * \lambda_r)$$

3. Modulation of temporal coding and transmitter release at unitary CA3-CA3 connections by an I_D-like conductance down-regulated by calcium

The activity-dependent switch between functional and non-functional synapses described in the previous sections may play a fundamental role in network synchronisation. In this respect, it is known that the CA3 region acts as the pacemaker for the generation of epileptiform activity, mainly because of the dense network of collateral of axons interconnecting pyramidal neurones. Network activity is controlled by extrinsic factors, such as neurotransmitters and neuromodulators, as well as by active conductance. In particular, presynaptic K^+ channels localised on axon terminals are responsible for setting the resting potential, for repolarising the membrane after action potentials and for regulating the firing rate and information coding (Meir *et al.*, 1999). By combining these different effects, they play a crucial role in modulating transmitter release and in controlling synaptic efficacy in target neurones. In particular, by shaping presynaptic action potentials, K^+ channels control calcium signal necessary to trigger fusion of synaptic vesicles with the surface membrane, exocytosis and transmitter release (Sabatini & Regher, 1997). Although the shape of action potential may change between soma and terminals (Bourque, 1990; Geiger & Jonas, 2000), an indirect estimate on how spike repolarisation and presynaptic firing affect transmitter release and synaptic efficacy can be inferred using simultaneous recordings from two synaptically connected neurones. To this purpose, organotypic hippocampal slice cultures provide an ideal model. In this preparation, a fast activating and slowly inactivating voltage-dependent K^+ current, very sensitive to 4-AP, has been described in CA3 pyramidal neurones (Bossu *et al.*, 1996; Bossu & Gähwiler, 1996). In the present study this current has been further characterised and appeared to be similar to the "delay" current (I_D) described in hippocampal neurones by Storm (1988) and named "delay" because of its involvement in the delayed appearance of the first spike during membrane depolarisation. It was found that changes in membrane potential and intracellular calcium concentration up or down-regulate I_D in CA3 pyramidal cells. Moreover, blocking I_D by low concentrations of 4-AP resulted in increased cell firing, spike broadening and enhanced transmitter release suggesting that this conductance is of fundamental importance in determining timing and strength of synaptic transmission at CA3-CA3 synapses. Small changes in membrane potential or

intracellular calcium concentration may turn on and off this conductance leading to modifications in synaptic efficacy.

3.1 A voltage-dependent potassium current similar to I_D is responsible for the delayed appearance of action potentials during membrane depolarisation

Patch-clamp recordings, in whole-cell configuration and current-clamp mode, were performed from CA3 pyramidal neurones in organotypic hippocampal slice cultures. These neurones were identified as principal cells both visually (using infrared differential interference contrast video microscopy) and on the basis of their firing properties, i.e. their ability to accommodate in response to long depolarising current pulses from a resting membrane potential of -56.7 ± 0.7 mV (n=40). In some experiments (n = 16), cells were morphologically identified as pyramidal neurones by biocytin injection. As shown in Figure 14A, a long (800 ms) depolarising current step applied from the resting membrane potential induced the generation of a single spike followed by an afterhyperpolarisation. By increasing the current intensity, the firing of the cell increased showing a strong adaptation followed by a pronounced AHP (Figure 14B). Interestingly, a certain delay in the generation of the first spike was always observed (between 47 and 790 ms, with a mean of 320 ± 30 ms; n=40). The delay decreased by increasing the intensity of the current steps (Figure 14A and B) and increased when the membrane was hyperpolarised below the resting level (~10 mV, Figure 14C). In twelve neurones, changing the membrane potential from -56 ± 1 mV to -66 ± 1 mV induced a significant increase in the delay of the first spike from 100 ± 20 ms to 540 ± 60 ms ($P<0.001$, Figure 14E), when comparing the same current steps. At the single spike level, no changes in spike amplitude were observed (67 ± 3 mV at both potentials, n=8). However, action potentials evoked from the hyperpolarising level exhibited a longer time to threshold (from 2.1 ± 0.2 ms to 3.0 ± 0.3 ms, n=8; $P<0.05$) and a reduction in their width (from 3.3 ± 0.1 ms to 2.59 ± 0.09 ms, n=8; $P<0.001$). These results suggest the involvement of a voltage-dependent conductance in this phenomenon. One possible candidate is the fast activating, slowly inactivating voltage-dependent potassium current, known as "delay current" or I_D for its particular characteristic to retard the appearance of the first spike upon prolonged membrane depolarisation (Storm, 1988). This current is known to be very sensitive to micromolar concentrations of 4-AP (Storm, 1988; Rudy, 1988). To see whether I_D was responsible for the delayed appearance of action potentials, 4-AP (10-50

μM) was applied in the bath. 4-AP significantly reduced the delay of the appearance of the first spike from 330 ± 80 ms to 50 ± 20 ms, n=8 (P<0.05, Figure 14 E-G). This effect was associated with an enhancement of spontaneously occurring synaptic events in the absence of any significant change in input resistance (this was 220 ± 30 MΩ and 210 ± 30 MΩ, in the absence and presence of 4-AP, respectively). Often, 4-AP produced small fluctuations of membrane potential due to an increase in network activity. In these cases,

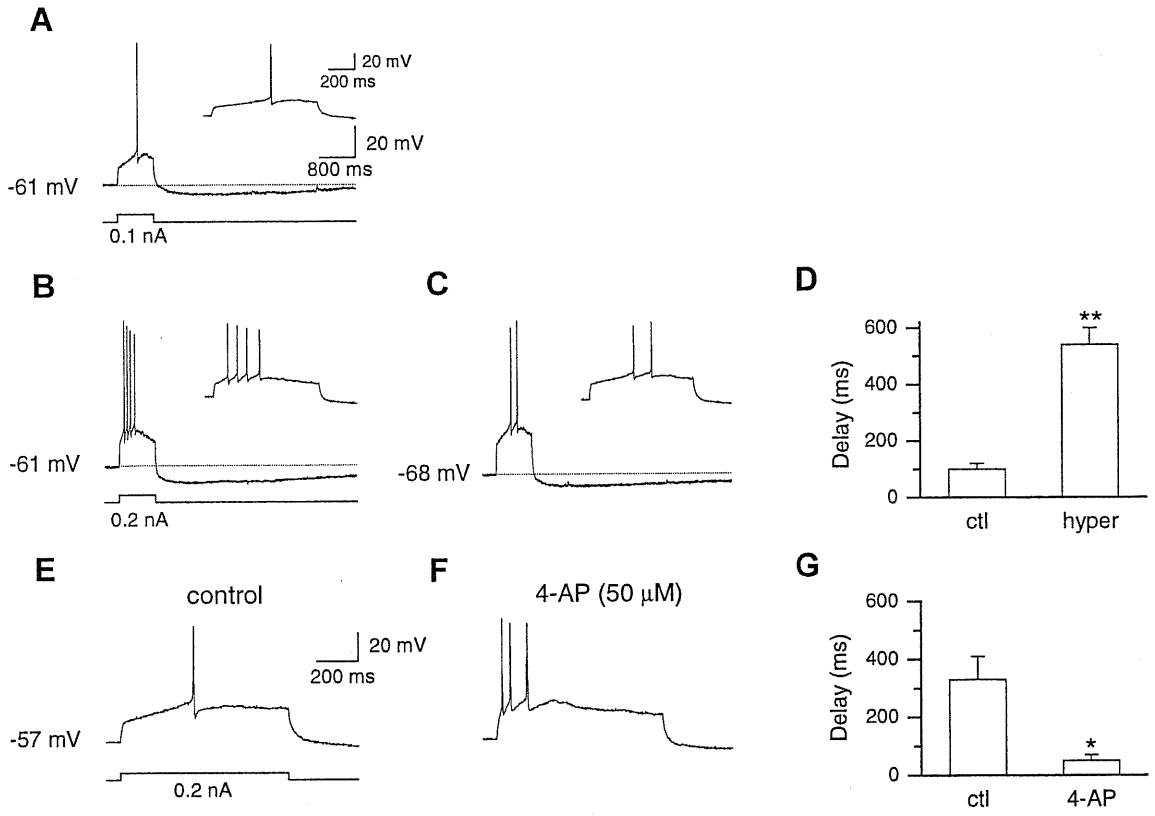

Figure 14. An I_D-like current activated from the resting membrane potential is blocked by low doses of 4-aminopyridine. A) Firing response upon injection of a steady inward current (0.1 nA for 800 ms) from the resting membrane potential of -61 mV. Notice the afterhyperpolarisation at the end of the pulse. **B)** Increasing stimulation intensity (0.2 nA) produced in the same neurone repetitive firing with strong adaptation followed by a prominent afterhyperpolarisation. **C)** Upon membrane hyperpolarisation (-68 mV), the same neurone responded to a current step of 0.2 nA with a reduced number of spike and with a larger delay in the appearance of the first action potential. Insets in A-C represent the same traces in an expanded time scale. **D)** Mean delay to the appearance of the first spike generation from resting membrane potential (ctl, -56 ± 1 mV; n=12) and from more hyperpolarised membrane potential (hyper, -66 ± 1 mV). **E-F)** Voltage responses to 0.2 nA current step in control (E) and in the presence of 4-AP (F) for a different neurone. **G)** Mean delay to the first spike generation in control and in the presence of 4-AP (n=8). (*P<0.05, **P<0.001)

appropriate current injections were applied in order to maintain the membrane potential at the same resting level and to compare the firing properties in the two different conditions. 4-AP significantly reduced the time needed to reach spike threshold (from 3.5 ± 0.2 ms to 2.8 ± 0.2 ms, n=16, P<0.001) and increased the spike duration (from 2.35 ± 0.09 ms to 3.2 ± 0.3 ms; n=16; P<0.001; see Figure 18A), but did not modify spike amplitude (69 ± 2 mV and 66 ± 3 mV, in the absence and presence of 4-AP, respectively; n=14). These data indicate that indeed an I_D-like current, activated from resting membrane potential, is responsible for regulating the appearance of the first spike. As expected (Storm, 1988), this conductance contributed also to spike repolarisation.

3.2 Voltage-dependence of the I_D-like current

An outward, fast activating, slowly inactivating voltage-dependent potassium current sensitive to low concentration of 4-AP has been already described in CA3 neurones from organotypic hippocampal slices using voltage-clamp recordings (Bossu *et al.*, 1996; Bossu & Gähwiler, 1996). Similar experiments in voltage-clamp mode were therefore performed in 9 cells in order to compare the I_D-like current with the previously studied one. "Ensemble" outward potassium currents were activated by 10 mV depolarising voltage steps from a holding potential of -60 mV, in the presence of extracellular CsCl (3 mM), carbachol (50 µM), $CdCl_2$ (200 µM) and TTX (0.5 µM), in order to block I_h, I_M, calcium activated potassium channels and sodium channels, respectively. As shown in the example of Figure 15 A-C, the 4-AP sensitive component was obtained by subtracting the outward currents evoked in the presence of the drug from the control ones. Like I_D, the 4-AP sensitive component showed fast activating and slowly inactivating kinetics (Figure 15C). Moreover, the activation and inactivation curves of the "ensemble" outward potassium currents gave potentials for half-inactivation and half-activation of -48.6 mV and 10.5 mV, respectively, while the slope factors were 9.4 mV and 12.7 mV for inactivation and activation, respectively (Figure 16). These values are very close to those previously obtained by Bossu *et al.* (1996). In addition, in agreement with Bossu *et al.* (1996) the existence of a window current above the resting membrane potential was disclosed from activation and inactivation curves suggesting the involvement of the I_D-like current in stabilisation of the resting membrane potential. Moreover, it appears that fluctuations around the resting membrane potential would strongly modify the inactivating state of the current.

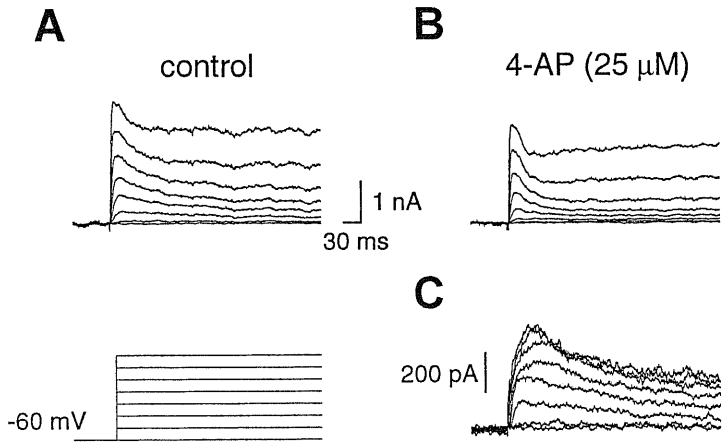

Figure 15. Voltage-dependence of the I_D-like current. A-B) Outward currents elicited by depolarising voltage steps (800 ms duration, 10 mV increment, bottom traces in A) from a holding potential of -60 mV in control (A) and in the presence of 4-AP (B). **C)** Traces showing the 4-AP sensitive current obtained by subtracting the traces in A from those in B.

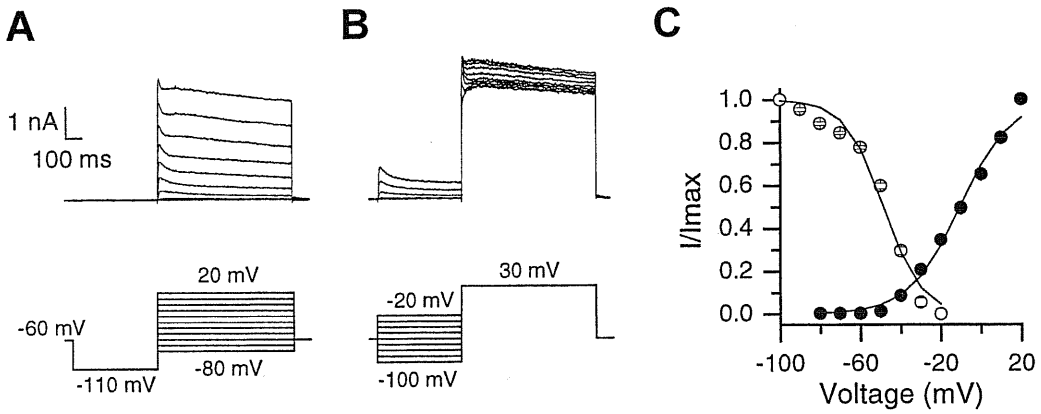

Figure 16. Activation and inactivation properties of the "ensemble" outward potassium current. A) "Ensemble" outward potassium current was activated by depolarising voltage steps from -80 to 20 mV (800 ms duration, 10 mV increment) after a conditioning pre-pulse to -110 mV (500 ms duration). **B)** Inactivation properties were studied by applying conditioning pre-pulses from -100 to -20 mV (10 mV increment) followed by a depolarising step to +30 mV. **C)** Activation (filled circles, n=9) and inactivation (open circles, n=5) curves for the I_D-like current. Data points were obtained following normalisation to the maximal current. Curves represent Boltzmann equation fits, obtained as described in paragraph 5.4.1 of the methods. The potentials for half inactivation and half activation were -48.6 mV and 10.5 mV, respectively, while the slope factors were 9.4 mV and 12.7 mV for inactivation and activation, respectively. All these experiments were performed in the presence of extracellular CsCl (3 mM), carbachol (50 μM), $CdCl_2$ (200 μM) and TTX (0.5 μM) to block I_h, I_M, calcium-activated potassium channels and I_{Na}, respectively.

3.3 Up-regulation of the I_D-like potassium current following blockade of calcium flux through voltage-activated calcium channels

Bath perfusion with the non-selective voltage-dependent calcium channel blocker Cd^{++} (200 μM) blocked the AHP and reduced spike adaptation (Figure 17), as usually observed in pyramidal neurones (Madison & Nicoll, 1984; Storm, 1989). However, in contrast to previous observations (Madison & Nicoll, 1984; Storm, 1989), in the presence of the divalent cation, the number of spikes evoked by long (800 ms) depolarising current pulses was reduced (Figure 17B and D). Surprisingly, application of Cd^{++} significantly increased the delay of the appearance of the first spike from 170 ± 40 ms to 570 ± 40 ms, with a mean increase of 400 ± 40 ms (n =10; P<0.001; Figure 17C). This effect was reversed by the addition of a low concentration (10-50 μM) of 4-AP (Figure 17E-F). In the presence of Cd^{++} and 4-AP, the cells were able to fire with much shorter delays (on average from 540 ± 60 ms to 12 ± 3 ms, n=6; P<0.001). Cd^{++} did not affect spike width (2.6 ± 0.1 ms

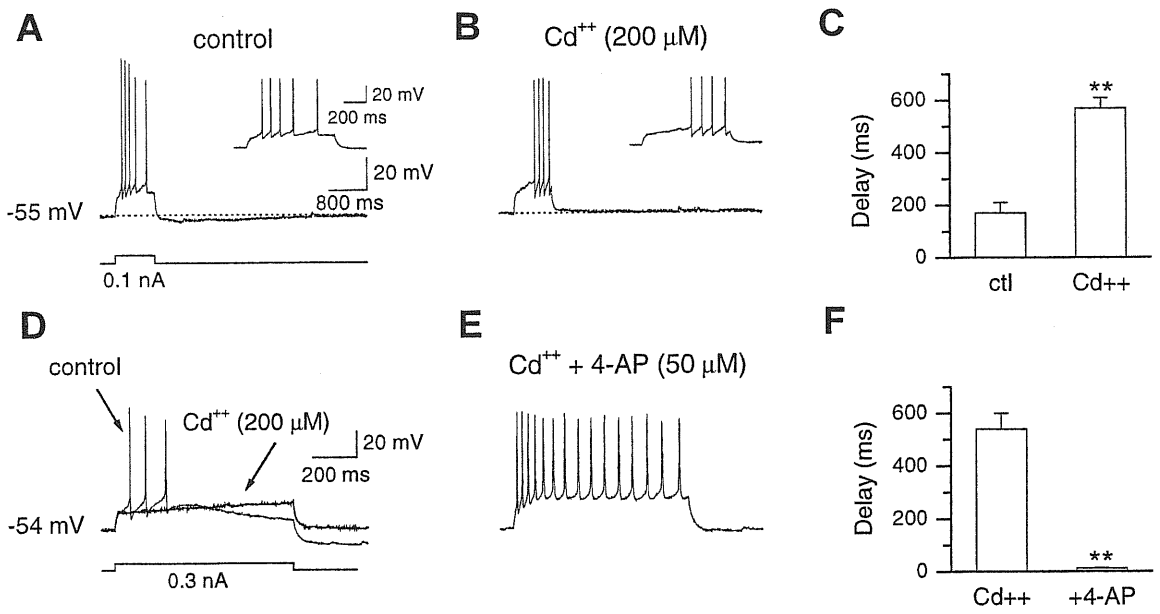

Figure 17. Intracellular calcium down-regulates I_D-like potassium current. A-B) Firing patterns upon injection of 0.1 nA current step in control (A) and in the presence of Cd^{++} (B). Insets represent the same traces in an expanded time scale. **C)** Mean delay to the first spike generation in control and in the presence of Cd^{++} (n=10). **D)** Two superimposed responses of another neurone to 0.3 nA current step in control solution (thin line) and in the presence of Cd^{++} (thick line). Note the lack of action potentials in Cd^{++}. **E)** Same neurone as in (D) in the presence of Cd^{++} and 4-AP. **F)** Mean delay in the generation of the first spike in the presence of Cd^{++} or Cd^{++} + 4-AP for the same current injections (n=6). (** P<0.001)

and 2.57 ± 0.09 ms, in control and in Cd^{++}, respectively; n=10; Figure 18B) or spike amplitude (70 ± 3 mV and 69 ± 2 mV, in control and in Cd^{++}, respectively; n=10), but significantly reduced the time to threshold (from 3.5 ± 0.2 ms to 2.9 ± 0.2 ms, n=10; $P<0.05$). Addition of 4-AP to Cd^{++} produced a significant broadening of the action potential (from 2.41 ± 0.09 ms to 3.5 ± 0.1 ms, n=5; $P<0.05$; Figure 18C). These results strongly favour the possibility that I_D-like current is down-regulated by a increase in intracellular calcium through voltage-dependent calcium channels, as the block of these channels by Cd^{++} influenced both the retard in spike generation and action potential repolarisation. To further investigate this point, some experiments (n=10) were performed using the calcium chelator BAPTA (10 mM) into the patch pipette. In accord with the calcium hypothesis, neurones loaded with BAPTA still exhibited a delay in spike generation (490 ± 90 ms) indicating that the I_D-like current was still active at rest (Figure 19A). However, in comparison with controls, significantly more hyperpolarised resting potentials (-63 ± 2 mV; $P<0.05$, Figure 19B) were observed. The difference in resting membrane potential could be attributed to the up-regulation of the I_D-like current, following the reduced concentration of intracellular calcium at rest. The effectiveness of BAPTA in chelating intracellular calcium was assessed by its capability to block the AHP in all cells tested (Figure 18A).

According to Bossu & Gähwiler (1996) the same channel operating in different gating modes would underline different inactivation kinetics of the "ensemble" 4-AP-sensitive outward potassium current. The gating mode would be controlled by intracellular factors such as kinases or phosphatases (Bossu & Gähwiler, 1996). These enzymes, whose calcium-dependent activation would turn on or off the delayed current, might provide the biochemical substrate for the switch. Thus, the possibility that the calcium calmodulin-dependent serine/threonine phosphatase calcineurin could change neuronal signaling by modifying the phosphorylation state of I_D-like channel was explored. To this purpose cells were recorded with patch pipettes containing the phosphatase 2B inhibitor FK506 (50 μM, Lu *et al.*, 2000). Also in this condition, application of Cd^{++} (200 μM) induced an increase in the delay of the first spike generation (from 170 ± 40 ms to 690 ± 40 ms, n=3) without any significant change in spike width. Further application of 4-AP to Cd^{++} reduced the delay of the appearance of the first spike upon membrane depolarisation (from 690 ± 40 ms to 19 ± 3 ms). This effect was associated with a broadening of the

action potential (from 2.57 ± 0.05 ms to 3.20 ± 0.03 ms). These data allow a role of calcineurin in modulation of the I_D-like current to be ruled out.

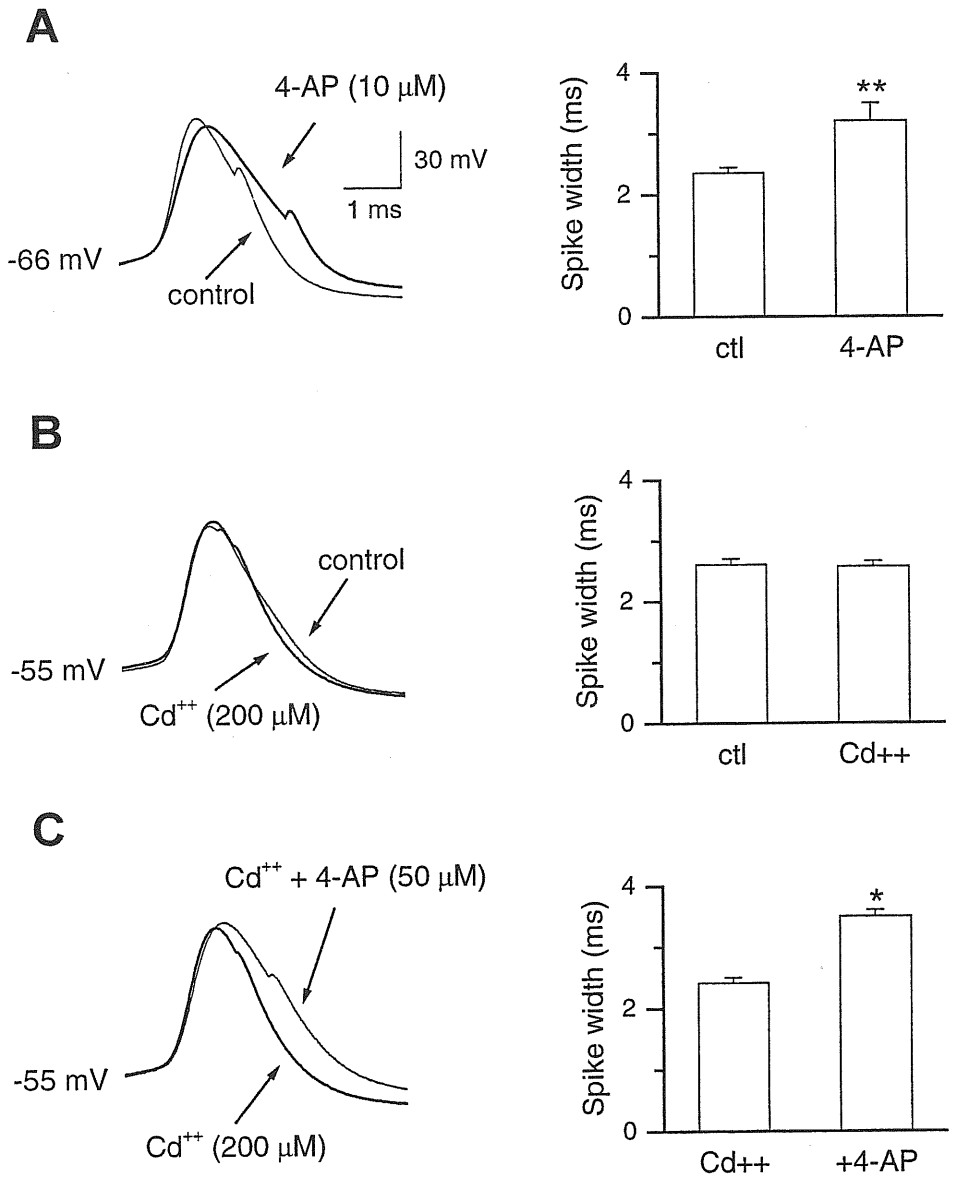

Figure 18. The I_D-like current is involved in action potential repolarisation. Action potentials were generated from rest by injection of short (5 ms duration) current steps and spike width was calculated at the threshold level. Action potentials generated in different conditions are aligned at the threshold and superimposed (left). The corresponding changes in width are shown on the right. **A)** Application of 4-AP significantly broadened the spike (n=16). Thick lines correspond to spike recorded in the presence of 4AP. **B-C)** Application of Cd^{++} did not affect action potential width (n=10, B), while further addition of 4-AP to Cd^{++} increased spike duration (n=5; C). In B-C thick lines correspond to action potential recorded in the presence of Cd^{++} alone. (* $P<0.05$, ** $P<0.001$)

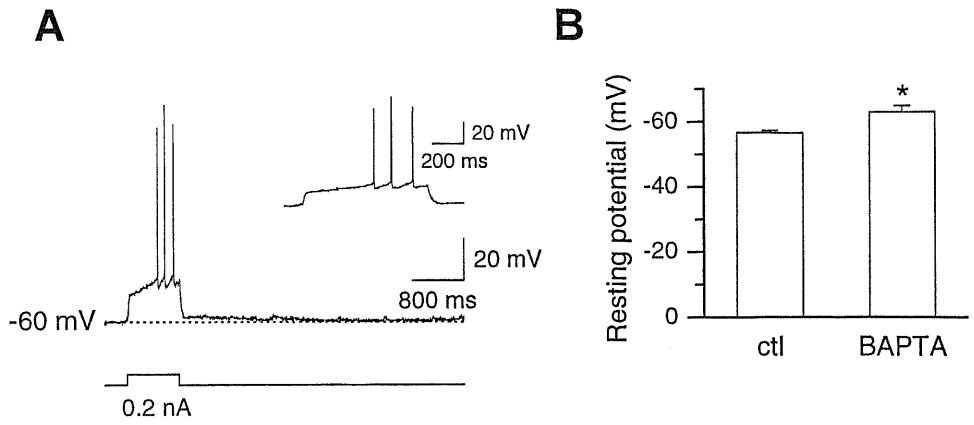

Figure 19. Buffering intracellular calcium with BAPTA shifts the resting membrane potential towards a more hyperpolarised value. A) Firing response of a CA3 pyramidal neurone loaded with BAPTA (10 mM), upon injection of 0.2 nA current step. Note the absence of afterhyperpolarisation at the end of the pulse. Inset represents the same trace in an expanded time scale. **B)** Mean resting membrane potential of neurones recorded with normal intracellular solution (ctl, n=40) or with one containing BAPTA (BAPTA, n=10). (* $P<0.005$)

3.4 I_D-like current modulates neurotransmitter release

The broadening of the action potential induced by low concentrations of 4-AP reveals a role of the I_D-like current in spike repolarisation and strongly suggests the involvement of this conductance in modulating transmitter release. In order to properly study this possibility, double patch-clamp recordings from interconnected CA3 pyramidal neurones were performed. According to Debanne *et al.* (1995) criteria for monosynaptically connected pairs consisted of short latency responses (4.9 ± 0.9 ms, n=15) with small fluctuations. Seven different pairs of neurones were studied in control conditions and in the presence of low concentration of 4-AP (10 μM). Usually, pairs of presynaptic action potentials (50 ms apart), delivered at the frequency of 0.05 Hz, evoked two sequential EPSCs that fluctuated in amplitude from trial to trial. As shown in the example of Figure 20A and B, a transmitter failure to the first spike was always associated to a success to the second one. On the contrary, a success to the first spike was associated to a synaptic response to the second one whose size was half of the first EPSC. Application of 4-AP (10 μM) produced a broadening of the action potentials and strongly increased the number of successes to the first spike. Moreover, in 4-AP some delayed responses appeared on the top of EPSCs (see arrows in Figure 20A) suggesting either a "delayed" release or the activation of previously silent connections. Overall, 4-AP-induced in all

cells tested spike broadening (from 16 to 53%, with a mean of 29 ± 6%, n=7; P<0.05) and a significant increase in the percentage of successes to the first pulse (from 51 ± 13% to 70 ± 12%; P<0.05; Figure 20C). In line with a presynaptic effect, 4-AP decreased the ratio between the amplitude of the second and the first EPSC (PPR) from 2.5 ± 0.9 to 1.2 ± 0.4 (n=5; Figure 20D) and significantly increased CV^{-2} by 250 ± 100% (n=7; P<0.05). Figure 21 shows an example in which no failures were detected in control conditions. In this case, 4-AP strongly increased EPSC amplitude (Figure 21A-B), indicating an increase of synaptic efficacy by blocking I_D-like current. Interestingly, in 4-AP, the EPSC latency increased significantly as demonstrated by latency distribution histogram (Figure 21C). Overall a change in the mean latency from 5 ± 1 ms to 6 ± 2 ms was observed (n=7; Figure 21D). This was associated with a small increase in scattering of response latencies (on average the standard deviation of EPSC latencies was 0.38 ± 0.8 ms and 0.5 ± 0.2 ms, in control and in 4-AP, respectively; n=4).

According to the voltage dependence of I_D and its role in the modulation of synaptic efficacy, additional double-patch recordings were performed in order to check whether small changes in the resting potential could modulate neurotransmitter release by removing inactivation of I_D in the presynaptic neurone (n=8, Figure 22). As already mentioned, 10 mV hyperpolarisation significantly reduced the action potential duration, with relative changes ranging from 13% to 28% and a mean of 20 ± 2% (n=8, Figure 22D). Surprisingly, only the cell whose action potential width was reduced 28% by hyperpolarisation showed a simultaneous reduction in the percentage of successes (from 97% to 87%, Figure 22 A-C) suggesting a role of I_D in this phenomenon. However, the remaining 7 neurones showed a small increase in the percentage of successes, giving an overall change from 72 ± 9% to 78 ± 9% (Figure 22E). No significant changes were detected in the kinetics or in the amplitude of EPSCs (P>0.05). In five double patches, in which a paired-pulse protocol was applied, the increased percentage of successes to the first and second EPSC was accompanied by a slight, but significant decrease in PPR (from 1.5 ± 0.2 to 1.2 ± 0.2, n=5; P<0.05). These results suggest that different processes involved in neurotransmitter release might be modulated in opposite directions by changing the membrane potential in the presynaptic neurone.

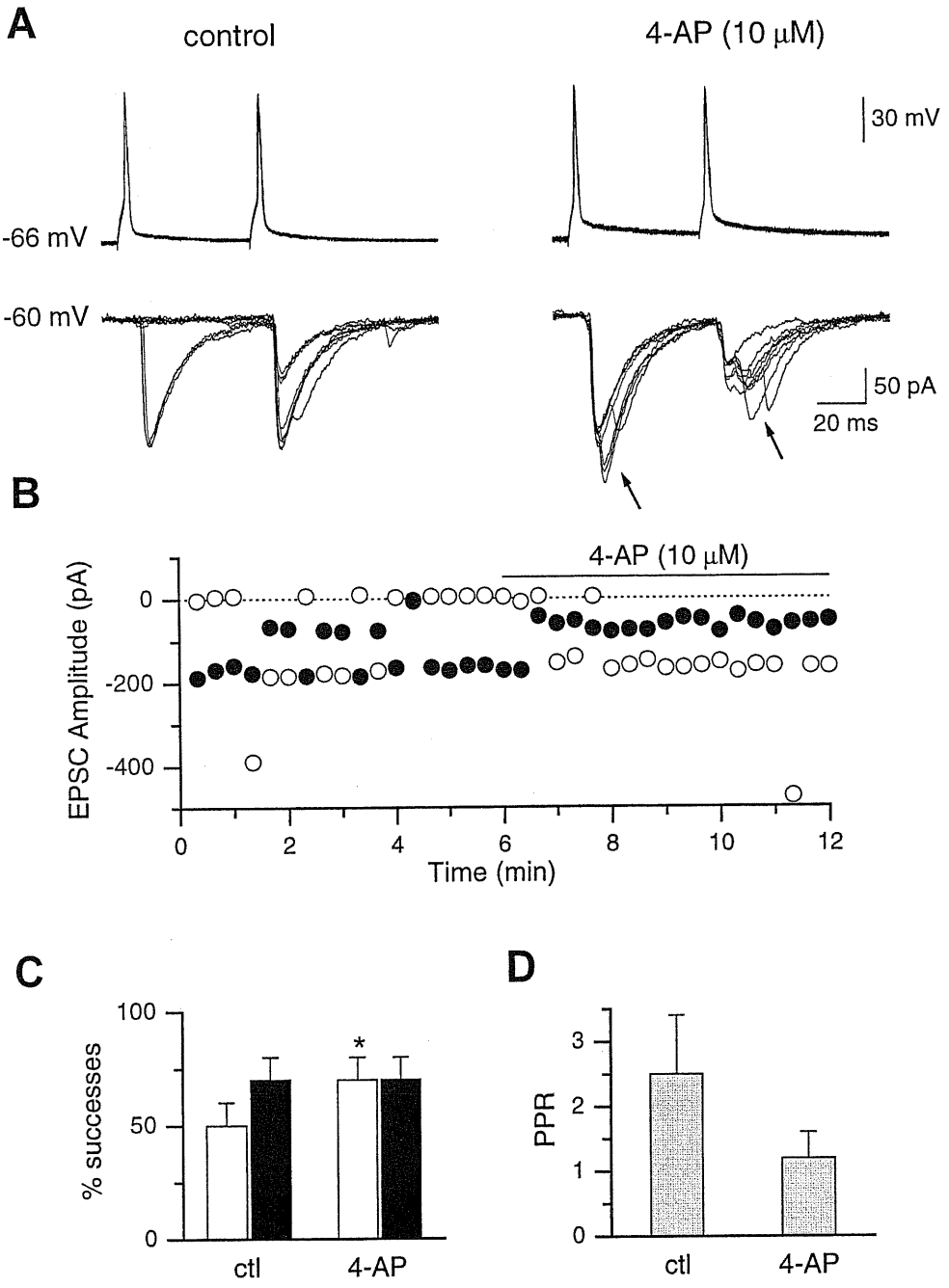

Figure 20. I_D-like current modulates neurotransmitter release at CA3-CA3 connections. A) Pairs of action potentials are generated (50 ms intervals, 0.05 Hz) in the presynaptic cell (top traces) while EPSCs are recorded from the postsynaptic cell (bottom traces) in control (left) and in the presence of 4-AP (right). Eight traces are superimposed. **B)** Time course of the peak amplitude of the first (open circles) and second (filled circles) EPSCs recorded from the cell shown in A. **C)** Percentage of successes to the first (white column) and to the second (black column) spike in control and in the presence of 4-AP (5-7 pairs). **D)** Mean pair-pulse-ratio (PPR) calculated from five different pairs of neurones in the two experimental conditions. (* $P<0.05$)

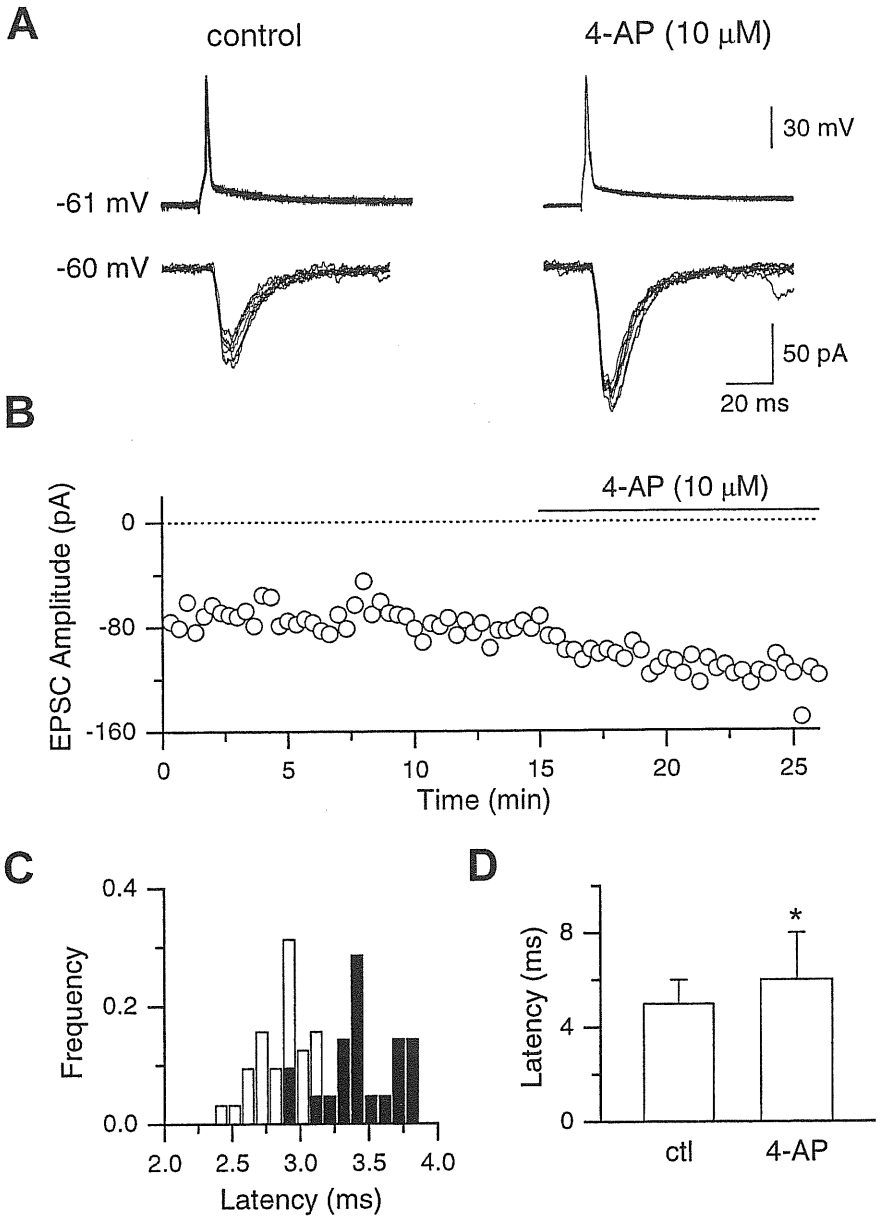

Figure 21. I_D-like current increases the latency of postsynaptic responses. **A)** Eight superimposed action potentials generated (0.05 Hz) in the presynaptic cell (top traces) while EPSCs are recorded from the postsynaptic cell (bottom traces) in control (left) and in the presence of 4-AP (right). **B)** Time course of EPSC amplitude in control condition and during the application of 4-AP (bar) for the pair shown in A. **C)** Normalised latency distributions in control (white columns) and in 4-AP (black columns) for the same neurone shown in A-B (bin size = 0.08 ms). The latency was calculated as the time interval between the onset of the response and the peak of the presynaptic spike. In this case the average latency increased from 2.9 to 3.4 ms. **D)** Mean latency of postsynaptic responses obtained from 7 different pairs. For each experiment, the latency was calculated after averaging all the successes. (* $P<0.05$)

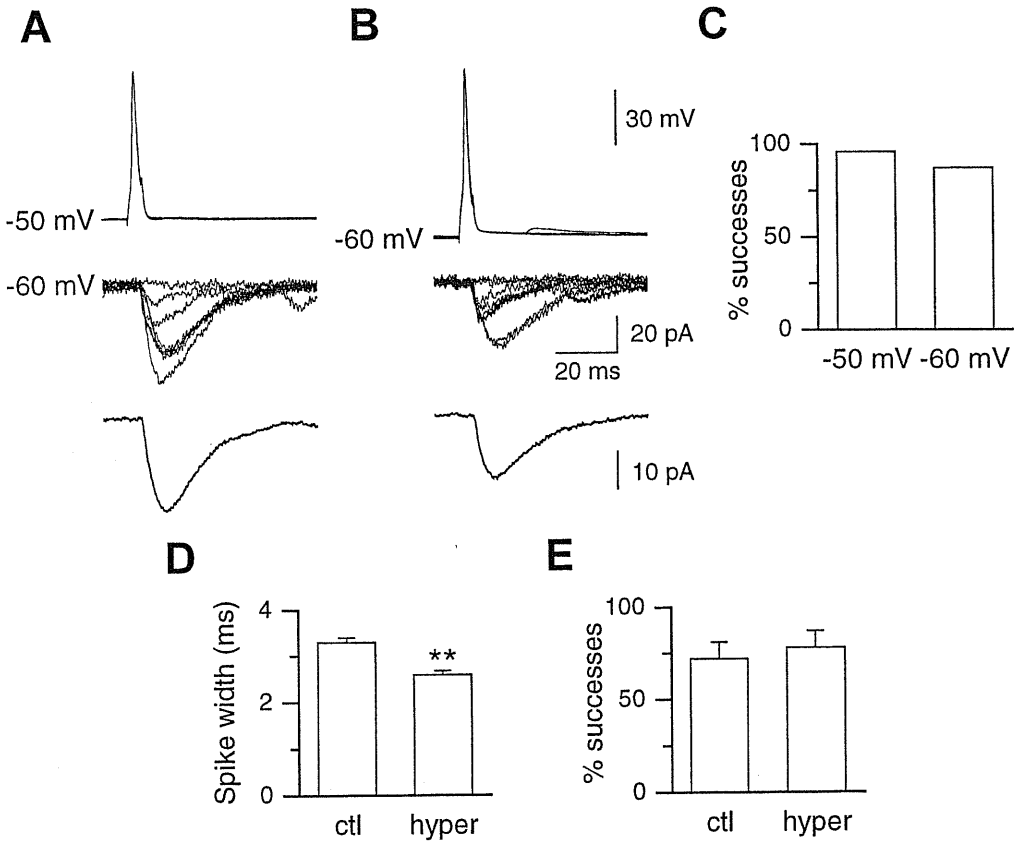

Figure 22. Modulation of neurotransmitter release by changes in presynaptic membrane potential. A-B) Eight action potentials (top traces) are generated from -50 mV (A) and -60 mV (B). Corresponding EPSCs are recorded from the postsynaptic cell (middle traces, A-B) and their means over the whole recording period are shown for each conditions (bottom traces, A-B). **C)** Percentage of successes for the neurones showed in A-B at -50 and -60 mV, respectively. Note that the percentage of successes decreases at more hyperpolarised membrane potential. **D-E)** Mean action potential width (D) and mean percentage of successes (E) calculated in 8 pairs at resting membrane potential (ctl, -52 ± 1 mV) and at more hyperpolarised level (hyper, -62 ± 1 mV). (** P<0.001)

3.5 Discussion

Paired recordings from interconnected cells have revealed the presence of a voltage and calcium-dependent potassium current with biophysical and pharmacological properties similar to I_D that plays a crucial role in regulating the firing properties of neurones, spike repolarisation and transmitter release at CA3-CA3 connections in the rat hippocampus. This current, named I_D-like, strongly contributes to the "ensemble" current previously described by Bossu *et al.* (1996) as the dominant voltage-gated potassium current seen in somatic recordings from pyramidal cells in hippocampal slice cultures, named $I_{K(AT)}$. As

the "ensemble" current, it showed fast activation and slow inactivation kinetics and a pharmacological profile conformed to I_D described in hippocampal pyramidal cells by Storm (1988). In particular, the higher sensitivity of I_D to 4-AP is commonly used to separate it from the fast inactivating voltage-dependent outward potassium current I_A (Rudy, 1988). Similarly to I_D and to the "ensemble" current, activation and inactivation curves overlapped and revealed a steady activated "window" current that contributes to the resting membrane potential. Moreover, small fluctuations of membrane potential induced by local changes in extracellular ions or neurotransmitters would activate or inactivate the I_D-like current, leading to changes in firing properties and in spike width with consequent modifications in the coding properties of the neurone and transmitter release.

3.5.1 Down-regulation of the I_D-like current by intracellular calcium

Unlike I_D (Storm, 1988; Storm, 1990), the fast activating slowly inactivating potassium current described here was down-regulated by a rise of intracellular calcium possibly through voltage-dependent calcium channels, as demonstrated by cadmium-induced increase in the delay of the first spike evoked by a sustained membrane depolarisation. Suppression of a rapidly inactivating, voltage-gated potassium current similar to I_A by intracellular calcium has been reported (Chen & Wong, 1991). In the case of I_D, the calcium sensitivity was inferred on the basis of the observation that the delay current could be suppressed by metabotropic glutamate receptor agonists of type I and II whose activation is known to trigger calcium-dependent mechanisms (Wu & Barish, 1999). Indeed, in low calcium I_D amplitude was found to be significantly larger (Wu and Barish, 2000). Functional voltage-gated potassium channels are formed by homo or heteromeric tetramers of principal α subunits, each of them consisting of six transmembrane helices, often supplementd by accessory β subunits (Jan & Jan, 1997). Several genes, grouped into four main subfamilies named Kv1, Kv2, Kv3 and Kv4 have been so far cloned (Hille, 2001). The genes coding for the pore forming α subunits polypeptides mediating I_D are still uncertain. According to Bossu & Gähwiler (1996), the channels mediating $I_{K(AT)}$ could result from the heteromultimeric assembly of a non-inactivating Kv3.1 with a fast inactivating Kv3.3 or by the association of non-inactivating Kv3.1 or Kv3.2 with the β subunit. On the contrary, it is widely accepted that the channel proteins responsible for I_A belong to the Kv4 family (Serôdio *et al.*, 1994; Serôdio *et al.*, 1996; Serôdio & Rudy,

1998). These subunits have been shown to bind proteins, called Kv channel-interacting proteins, that act as calcium sensors (An *et al.*, 2000). By coupling the A-current to activity-dependent fluctuations of intracellular calcium, these proteins may dynamically regulate cell excitability. Interestingly, when transfected in heterologous systems together with Kv4.2 subunits these proteins yielded I_A currents with more negative voltage activation, with slower inactivation and faster recovery from inactivation (An *et al.*, 2000). This kind of modulation might account for the proposed switch of the native $I_{K(AT)}$ channel between two different gating modes expressing either sustained or transient inactivation kinetics (Bossu & Gähwiler, 1996). As already mentioned, according to Bossu & Gähwiler the switch would be controlled by intracellular factors such as calcium-dependent kinases or phosphatases as reported for a voltage-gated cation channel in Aplysia bag cells (Wilson & Kaczmarek, 1993). In the present experiments, the lack of effect of the calcium calmodulin-dependent phosaphatase 2B inhibitor FK 506 allowed the involvement of this enzyme in regulation of I_D like current to be ruled out. However other kinases or phosphatases may be responsible for the observed calcium-dependent modulation of I_D.

While cadmium strongly produced a retard or abolished spikes evoked by a sustained membrane depolarisation, it did not affect spike width. This apparent discrepancy can be explained by the fact that calcium produces opposite effects on voltage-dependent potassium currents: it suppresses I_D and activates calcium-dependent potassium currents, involved in spike repolarisation (Storm, 1989). Thus, the expected decrease in spike width due to the up regulation of I_D (following block of calcium flux) would be counterbalanced by its increase due to the lack of activation of I_C. The relative contribution of these two opposite effects would depend on the relative affinity of different potassium channels for calcium, the time course of calcium rise, the location of voltage-dependent calcium channels relative to potassium ones and spatial distribution of calcium microdomains. That I_D was full operative in cadmium was confirmed by the experiments in which 4-AP was added to cadmium. This drug, at low concentration was still able to block I_D, slowing down action potential repolarisation and reducing the delay of the first spike evoked by a sustained membrane depolarisation.

3.5.2 *A presynaptic I_D-like current regulates synaptic efficacy in target neurones*

Changes in spike repolarisation should affect transmitter release at nerve endings. Indeed, paired recordings from interconnected neurones clearly demonstrate that an I_D-like current regulates synaptic efficacy at CA3-CA3 connections. The observed effect was presynaptic in origin as demonstrated by the decrease in transmitter failures and paired-pulse ratio and by the increase in CV^{-2}, which are considered traditional indexes of presynaptic modifications (Katz, 1969; Zucker, 1989). In particular, in a paired-pulse protocol, the PPR is inversely related to the initial release probability (Dobrunz & Stevens, 1997). Thus, an enhanced probability of release to the first pulse would be associated to a reduction in PPR, as observed in the present experiments following 4-AP application.

The presence of I_D channels on axon terminals could be inferred by the finding that 4-AP in the low micromolar range was able to reduce the number of transmitter failures, to increase latency, amplitude and jitter of synaptic currents. Light and electron microscopy techniques have revealed that synaptic terminals in different brain areas apparently display distinct set of potassium channel subunits (Roeper & Pongs, 1996). In particular, the 4-AP sensitive Kv1.1 subunits have been found on CA3 axon terminals (Grissmer *et al.*, 1994; Wang *et al.*, 1994) where they regulate excitability of the CA3 recurrent axon collateral system (Smart *et al.*, 1998). When co-assembled with other α or β subunits Kv1.1 forms heteromultimeric channels with different kinetic properties (Robertson, 1997) which may mediate the I_D-like current.

In the present experiments, very low concentrations of 4-AP were able to broaden presynaptic spike and to increase synaptic efficacy. This suggests that similar changes in spike width may occur at nerve terminals where most of the calcium enters into during spike repolarisation. Thus, broadening of presynaptic action potential increases calcium influx primarily by prolonging the duration of presynaptic calcium current (Sabatini & Regher, 1997). The 4-AP-induced increase in amplitude of EPSCs may reflect, in analogy to the neuromuscular junction, increased number of quanta delivered simultaneously by a single nerve pulse due to enhanced synaptic vesicles recycling (Heuser & Reese, 1981). In line with an increased probability of release following blockade of an I_D-like current, the appearance of delayed responses with multi peaks, could be due to the activation by 4-AP of previously presynaptically "silent" connections.

In the same preparation, an I_A-like potassium conductance has been reported to modulate

synaptic transmission by gating action potential propagation at axonal branches (Debanne *et al.*, 1997). However, unlike the present results, in Debanne *et al.* (1997) experiments, propagation failures was prevented by very high concentrations of 4-AP (40 mM into the patch pipette) or when presynaptic action potentials were preceded by a brief or tonic hyperpolarisation. In the present experiments, a tonic hyperpolarisation produced a reduction in spike width, as expected by removal of inactivation of I_D but this effect was not associated with changes in failure rate. In fact a tendency towards an increase in the number of successes was commonly observed. This can be explained by the fact that an increased driving force for calcium leading to an increased transmitter release would in these cases counterbalance the I_D-induced hastening of spike repolarisation and reduced synaptic efficacy (Katz & Miledi, 1967a). Only in one pair, exhibiting a strong reduction in spike width, an increase in failure rate was detected upon membrane hyperpolarisation. However, this procedure was able to significantly delay the appearance of the first spike, suggesting that even small modifications in resting membrane potential may affect temporal coding of information.

Moreover, in the present experiments a clear increase in latency of synaptic responses upon 4-AP application was observed. According to Katz & Miledi (1967a,b) an inward movement of calcium through the axon would facilitate release by raising permeability to calcium but at the same time would prevent an inward movement of positive ions and this will account for the delayed action of the presynaptic spike. While the broadening of action potential may account for changes in latency of synaptic currents, the enhanced scattering of EPSCs observed in some experiments with 4-AP could be attributed to the lack of synchronicity in vesicle exocytosis (Heuser & Reese, 1981).

3.5.3 Switching off I_D-like current may facilitate seizure activity in the hippocampus

Low concentrations of 4-AP have been shown to induce spontaneously occurring interictal discharges in the hippocampus (Rutecki *et al.*, 1987; Perreault & Avoli, 1989). As recently demonstrated in the intact hippocampal formation, the 4-AP-induced interictal bursts, which can give rise to ictal discharges, originate in the CA3 hippocampal area and then propagate to the CA1 and entorhinal cortex (Luhmann *et al.*, 2000). The CA3 hippocampal region would act as the pacemaker for the generation of synchronised activity (Miles & Wong, 1983; Ben-Ari & Cossart, 2000). This may largely depend on the dense network of collateral of axons interconnecting pyramidal neurones (Miles & Wong,

1986). It is therefore conceivable that an enhancement of intracellular calcium following activity-dependent processes (Heinemann & Hamon, 1986) may switch off I_D at CA3-CA3 excitatory connections, generating a positive feedback mechanism. In fact, the higher is the firing rate, the more depolarisation and, consequently, the more calcium influx would occur. Thus, a stronger inactivation of I_D would further increase neurone excitability, inducing longer depolarisation, bigger calcium influx and so on. This mechanism may lead to network synchronisation, by facilitating the cross talk between neurones, and to a boosting in the release of excitatory transmitter and seizure activity. This may be relevant for human temporal lobe epilepsy.

GENERAL DISCUSSION

Electrophysiological recordings have been used to study different kinds of modulation of synaptic efficacy that may be important for the refinement of the neural circuitry, information processing and storage. In particular, a critical role is thought to be played by silent synapses, both during development and activity-dependent processes. In the first part of this study, it has been shown that both MF-CA3 and SC-CA1 synapses express functional AMPA receptors since the first postnatal week, but they may appear silent because of a very low probability of glutamate release. In fact, tools known to increase release probability, such as paired-pulse stimulation, raising the temperature or CTZ application, succeeded in activating silent synapses. These results are in agreement with recent findings suggesting that synapses expressing functional AMPA receptors may appear silent because of a low concentration of glutamate released in the cleft (Choi *et al.* 2000; Renger *et al.*, 2001; Zakharenko *et al.*, 2001). Indeed, the presence of functional AMPA receptors on the subsynaptic membrane could be detected by iontophoretic glutamate applications or when enhancing their affinity for glutamate by CTZ application (Choi *et al.*, 2000; Renger *et al.*, 2001). In line with the idea of presynaptically silent synapses, an increase in glutamate concentration and its rate of rise were described following LTP (Choi *et al.*, 2000). Moreover, an enhanced presynaptic function has been detected by endocytotic markers, both during development (Renger *et al.*, 2001) and during long-term synaptic plasticity (Zakharenko *et al.*, 2001), suggesting the involvement of modification in the process of vesicular fusion during synaptic transmission. The low concentration of glutamate in the cleft could be explained with a vesicular-fusion pore that in control conditions operates in a non-expanding mode (Choi *et al.*, 2000). Indeed a "kiss and run" mode of exocytosis has been revealed at synapses of cultured hippocampal neurones and it has been estimated that about 20% of vesicles normally use this kind of pathway (Stevens & Williams, 2000). According to this model, establishment of LTP would be accompanied by a conversion of the fusion pore from a non-expanding to a rapidly expanding mode (Choi *et al.*, 2000). Although these results strengthen the role of presynaptic mechanisms in controlling synaptic efficacy, the possibility that functional AMPA receptors may be recruited at postsynaptic sites during LTP cannot be ruled out (Isaac *et al.*, 1995; Liao *et al.*, 1995; Liao *et al.*, 1999; Shi *et al.*, 1999). Very recently, the existence of postsynaptically silent synapses have been described at CA3-CA3 connections of hippocampal slice cultures, where increasing

release probability by paired-pulse, raising the temperature or CTZ application was not effective in activating silent synapses. These observations may be due to the specific properties of this kind of connections, where PPR is typically <1 (Pavlidis & Madison, 1999) and are in line with the observed dependence of LTP magnitude on release probability and PPR (Larkman *et al.*, 1992; Sokolov *et al.*, 1998). Thus, strong postsynaptic modifications might be involved when the initial probability is large enough to preclude any further increase. Moreover, immunocytochemical studies in dissociated hippocampal cultures have shown that LTP, induced by brief application of glutamate in the absence of Mg^{2+}, is associated with an increase in the number of clusters of the presynaptic protein synaptophysin and in the number of sites at which synaptophysin and GluR1 are co-localised (Antonova *et al.*, 2001). These observations are in agreement with the idea that potentiation involves co-ordinated pre and postsynaptic modifications that, depending on the time of observation and the exact conditions of the experiment, may be differently interpreted by classical quantal analysis (Edwards, 1995b). In line with this hypothesis, the present results could be described by a model of a synapse with low probability releasing sites, some of them lacking AMPA receptor clusters on the subsynaptic membrane.

Indeed, a discrepancy exists on the number of apparently silent synapses detected by both electrophysiological and ultrastructural studies. In particular, it has been shown that the percentage of silent synapses is large at birth and decrease during development (Durand *et al.*, 1996; Liao & Malinow, 1996). However, a recent study has shown that the AMPA/NMDA ratio of spontaneous synaptic event at SC-CA1 synapses is equal to 1 and does not change during the first postnatal week. This indicates that newly formed glutamatergic synapses express both AMPA and NMDA receptors (Groc *et al.*, 2002). Moreover, in the present experiments the percentage of SC-CA1 synapses silent at rest was smaller than in previously reported data (Durand *et al.*, 1996; Liao & Malinow, 1996) obtained at higher frequency of stimulation (0.1-0.3 Hz). In agreement with this, the number of silent synapses at 0.5 Hz was higher that at 0.05 Hz, suggesting that silent synapses may be induced when using a too high frequency of stimulation, thus leading to an overestimation of their number. In line with this hypothesis, at 1 Hz all active synapses tested became completely silent at –60 mV, even if the persistence of NMDA-mediated responses at depolarised potentials was not tested. Indeed, not only activation of silent synapses, but also switching off functional synapses may be relevant at physiological

level, as repetitive firing is known to play a crucial role in information processing (Lisman, 1997). The enhancement in failure rate observed by increasing the frequency of stimulation suggests that this form of synaptic depression could be attributed to changes in presynaptic release mechanisms. This is in agreement with the observation that interfering with the protein machinery underlying vesicle fusion process, by exposing neurones to tetanus toxin, may convert functional synapses into silent (Renger *et al.*, 2001). These results strengthen the importance of presynaptic mechanisms in switching between functional and non-functional synapses. However, a deeper investigation of these processes has been hampered by the use of minimal stimulation of afferent inputs, that does not permit the ascertainment of whether the same presynaptic fibre is stimulated trial after trial. Paired recordings between monosynaptically interconnected neurones allow this problem to be overcome. Thus, double patch-clamp recordings from CA3 neurones in organotypic hippocampal slices allowed the confirmation of the presynaptic origin of LFD and synaptic silencing. Raising the frequency of stimulation significantly increased the failure rate as already observed in acute slices, making 75% of synapses almost completely silent at rest, and significantly changed the paired-pulse ratio. Although different presynaptic mechanisms may be involved in short-term depression, the depletion of the RRP of vesicles is often supposed to mainly account for it (Rosenmund & Stevens, 1996; Silver *et al.*, 1998; Wang & Kaczmarek, 1998; Oleskevich *et al.*, 2000; Meyer *et al.*, 2001). In the present experiments, the use of a paired-pulse protocol came out to be crucial for shedding light on the mechanisms involved. In particular, although a reduction in the probability of release during LFD predicts an increase in the PPR, PPF was converted into PPD by increasing the frequency of stimulation. These results could be explained by expressing release probability as the balance between two processes, one related to the residual calcium and the other to vesicle availability (Zucker, 1973; Quastel, 1997; Scheuss & Neher, 2001). As shown by the simple model developed, PPR decreases at high frequencies when the size of the pool is reduced to such a critical level that the "loss" of vesicles following the first pulse would make the probability of release to the second spike close to zero. Therefore, a critical role is played by the size of the RRP of vesicles that, being proportional to the area of the active zone, may change considerably from synapse to synapse (Schikorski & Stevens, 1997). At synapses of hippocampal neurones, the size of the RRP has been estimated by counting the number of quanta released before the probability decays to zero, using either application of hypertonic

solution or high frequency stimulation (Rosemund & Stevens, 1996; Dobrunz & Stevens, 1997). The pool was found to consist of around ten vesicles immediately available for release. Similar estimates have been obtained also by staining cells with the presynaptic functional marker FM1-43 (Murthy & Stevens, 1999) and by electron microscopy (Schikorski & Stevens, 1997). The properties of LFD also depend on the dynamics of depletion and refilling of the RRP. Although it is accepted that the rate of depletion depends on the stimulation frequency, the possibility of a frequency-dependence of vesicles recruitment is still discussed (Sun *et al.*, 2002). Indeed, the RRP is the most important pool for neurotransmitter release, but it constitutes only a small part of the total vesicle pool (around 200 vesicles according to Schikorski & Stevens, 1997). Thus, both the distribution of vesicles in functionally different pools and the existence of different pathways in synaptic vesicle cycle play a critical role in determining vesicles availability (Sudhof, 2000).

Although a postsynaptic effect, such as receptor desensitisation, cannot be completely ruled out, it appears that a decrease in release probability, due to a reduction in the pool of docked vesicles (Dobrunz & Stevens, 1997), accounts for switching off functional synapses. This phenomenon may vary enormously between different synapses, as it strongly depends on the size of the RRP and on the dynamics of vesicle depletion and recruitment. These results, as well as those on silent synapses, demonstrate the high modifiability of synaptic strength in the hippocampus and are in line with the critical role of this brain structure in information processing and storage. Moreover, the switch between functional and non-functional synapses may be relevant for controlling network activity. For example, it has been shown that silent synapses are essential for corticothalamic activity during early postnatal development (Golshani & Jones, 1999) and it is likely to think that synchronised activity itself may contribute to the activation of silent synapses, in particular during postnatal development.

Network activity may also characterise certain kind of pathological conditions such as seizure activity. In this respect, the recurrent connections between pyramidal neurones of the CA3 region play a critical role in the generation of epileptiform activity (Miles & Wong, 1986; Traub & Miles, 1991), which typically results from a disturbance in the physiological balance between excitation and inhibition. Synchronous neuronal hyperactivity may be induced by the application of GABA antagonists, excitatory neurotransmitters or their agonists. Ictal discharges and/or interictal bursts, which

originate in the CA3 hippocampal area and then propagate to CA1 and entorhinal cortex, can be also induced by low concentrations of 4-AP, suggesting a critical role for a particular type of potassium conductance (Luhmann *et al.*, 2000). Indeed, patch-clamp recordings from CA3 neurones have revealed the existence of a voltage-dependent potassium current, with biophysical and pharmacological properties similar to I_D, that plays a crucial role in regulating temporal coding and synaptic efficacy. Block of the I_D-like current by very low concentrations of 4-AP significantly reduced the delay in the generation of the first spike upon prolonged depolarisation and increased the action potential width. Spike broadening was associated with an increase in synaptic efficacy, suggesting an increase in the probability of release. In line with this, delayed responses appeared, in some cases, following 4-AP application, thus suggesting the existence of presynaptically silent connections that can be activated by increasing release probability. The recruitment of new functional synapses during 4-AP application could further modify the balance between excitation and inhibition, playing a critical role in seizure generation. The particular down-regulation of the I_D-like current by intracellular calcium described in the present study suggests a critical role of this conductance in processes that are accompanied by increase in intracellular calcium. In particular, a moderate inhibition of I_D during activity-dependent processes could enhance cell excitability in a less catastrophic manner than by 4-AP applications, suggesting an involvement in LTP induction (Wu & Barish, 1999). Alternatively, switching off I_D at CA3-CA3 excitatory connections by a massive increase in intracellular calcium may generate a positive feedback mechanism that might be crucial for the generation of epileptiform activity (Heinemann & Hamon, 1986). These topics could be further investigated in organotypic hippocampal slice cultures, where the high degree of connectivity between neurones offers a unique opportunity to study mechanisms involved in the modulation of synaptic efficacy and supports the generation of network activity.

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