GABA_A-mediated synaptic activity in rat hippocampal neurones *in vitro* and its modulation by other neurotransmitters and second messengers.

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GABA\(_A\)-mediated synaptic activity in rat hippocampal neurones \textit{in vitro} and its modulation by other neurotransmitters and second messengers.

Thesis Submitted for the degree of

Doctor Philosophy in Neuroscience, PhD

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Part of the results obtained during the project (on which this thesis is based) has been published as indicated below:


I state that the material presented in the thesis has entirely originated from my own research work which was carried out independently during the supervision of Prof. E. Cherubini. The contribution of Hosokawa, Stratta and Fisher to the publications in which I am a co-author was based on experimental techniques and results which are not presented in this thesis. I also state that the experiments concerning single-channel recordings were carried out in collaboration with Dr. M. Martina.
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Abstract

The patch-clamp technique (whole-cell and outside-out configurations) has been used to characterize spontaneous \( \gamma \)-aminobutyric acid A (GABA\(_A\)) receptor mediated currents in pyramidal cells of thin hippocampal slices obtained from neonatal rats. In early postnatal life, GABA is the main excitatory neurotransmitter on hippocampal pyramidal cells. The frequency distribution histogram of spontaneous GABAergic currents could be fitted by a single exponential function revealing the random nature of these events.

The present results demonstrate that in tetrodotoxin (TTX) solution spontaneous GABA\(_A\) receptor mediated miniature postsynaptic currents (mPSCs) were present. At -70 mV the first peak in the current amplitude distribution was 16 ± 6 pA (n = 13). This value was similar to that found for GABAergic currents (14 ± 6 pA) elicited by low intensity extracellular stimulation, suggesting that this effect was due to the release of elementary units of GABA. In outside-out patches, GABA activated single-channel events of 24 and 35 pS conductance. Assuming that a postsynaptic current of 15 pA corresponds to a single quantum of GABA, one could calculate that one quantal current represents the simultaneous opening of 6 to 9 GABA\(_A\) receptor channels in the postsynaptic cell.

The metabotropic Glutamate Receptor (mGluR) agonist, trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD), induced an increase in frequency but not in amplitude of spontaneously occurring GABAergic currents; this potentiating effect was blocked by the Protein Kinase A (PKA) antagonist Rp-adenosine 3’, 5’-cyclic monophosphotioate triethylamine (Rp-cAMPS), suggesting that glutamate, acting on
mGluRs, is able to increase GABA release through the metabolic pathway which involves PKA. The potentiating effect of l-ACPD was not observed in TTX solution indicating that the site of action of the mGluR agonist is probably located at the somatodendritic level and not on the nerve terminals of GABAergic interneurons.

In the presence of forskolin, which increases intracellular cyclic AMP (cAMP) levels, a rise in frequency but not in amplitude of miniature GABA<sub>A</sub> receptor mediated currents was observed, an effect that was prevented by the selective PKA antagonist Rp-cAMPS.

These experiments suggest that presynaptic mGluRs localized on GABAergic interneurones may facilitate the activity of these cells and their release of GABA through cAMP-dependent PKA. Moreover, PKA may interfere directly with the mechanism of GABA release as demonstrated by its action on miniature events.

The present results provide new evidence that the release of a major neurotransmitter such as GABA is up-regulated by another neurotransmitter namely Glutamate, thus demonstrating an important reinforcement of excitatory signals during an early stage of brain development.
Chapter 1

Introduction

1.1 Hippocampal formation

The hippocampus is a region forming the medial margin of the cortical hemisphere and located on the medial wall of the lateral ventricle; its longitudinal axis forms a semicircle around the thalamus. The hippocampus is among the best characterized cortical structures because its layered organization which is suitable for physiological and anatomical investigations. The hippocampus receives inputs from numerous limbic, cortical and subcortical areas, primarily via the entorhinal cortex and subiculum; it is involved in a large number of neuronal plasticity processes like learning and memory, as well as in neurological disorders such as epilepsy, stroke and Alzheimer's disease (for a review see Paxinos, 1995).

Figure 1a shows some of the neuronal elements present in a hippocampal slice cut transversely to its longitudinal axis; much of the intrinsic circuitry of the hippocampus is preserved in slices taken with this orientation (Skrede and Westgaard, 1971). The dentate gyrus, the subiculum and the entorhinal cortex are included. The entorhinal cortex provides a major sensory input to the hippocampus. Among the most important inputs, the entorhinal cortex receives fibers from the association cortices, the olfactory cortex, several thalamic nuclei, the claustrum and the amygdala. The area between the dentate gyrus and the CA3 region is called the hilus. The CA1 and
CA3 regions constitute most of the hippocampus proper. CA3 neurones have many recurrent axon collaterals that project back to their neighbours and are responsible for interictal discharges in disinhibited slices (Miles and Wong, 1986). The CA2 region is so small that is often ignored. The pyramidal cells are vertically oriented with their cell bodies in the *pyramidal layer* and their thick apical dendrites going through the *stratum radiatum* to the *stratum lacunosum-moleculare*.

**Fig. 1a** Neuronal elements of the hippocampal formation (modified from Cajal, 1911). Labeled areas include the subiculum, part of the entorhinal cortex, the dentate gyrus, fornix and regions CA1 and CA3. The hippocampus proper is divided into stratum oriens (a), stratum pyramidale (b), stratum radiatum (c) and stratum lacunosum-moleculare (d).
There are also several basal dendrites of each pyramidal cell which together form *stratum oriens*. The organization of the hippocampus is often called "lamellar" due to the existence of a trisynaptic circuit organized approximately in a plane perpendicular to the long axis of the hippocampus (Andersen et al., 1971): *the perforant path*, originating from enthorinal cortex, passes through the subicular complex and *terminates on the dentate granule cells*, with collaterals to CA1 and CA3 pyramidal cells; *mossy fibers* from granule cells *excite CA3 pyramidal cells* and hilar interneurones. Through the Schaffer collaterals, *CA3 pyramidal cells excite CA1 pyramidal cells* which send their axons to the subiculum and the enthorinal cortex. One major output of pyramidal cells is through the fornix, which crosses the midline of the brain. Thus, the hippocampus projects back to many regions that provide its input. The hippocampus receives three main neuromodulatory inputs: serotonergic fibers from the medial raphe nucleus (Azmitia and Segal, 1979), noradrenergic projections from the locus coeruleus (Lindvall and Bjorkland, 1974), and cholinergic innervation from the septal nuclei (Storm-Mathisen, 1977).

### 1.2 GABAergic interneurones in the hippocampus

Anatomical studies have revealed that GABAergic interneurones in the hippocampus are different in morphology, axonal arborization pattern and localization (Ramon y Cajal, 1911; Lorente de No, 1934; Ribak et al., 1978; Somogyi et al., 1983). They are identified on the basis of their positive staining with antibodies against the GABA-synthesizing enzyme, glutamate decarboxylase (GAD) or the $\text{Ca}^{2+}$ binding proteins calbindin, parvalbumin, calretinin (Kawaguchi and Hama, 1987; Kunkel et al., 1988;
Rocamora et al., 1996). Binding studies with the GABA\textsubscript{A} agonist muscimol and the GABA\textsubscript{A} receptor antagonist bicuculline, confirm the presence of GABA-sensitive sites (MacDonald et al., 1992). Intereurones are activated by recurrent collaterals of the pyramidal cells and by hippocampal excitatory afferents mediating feedback and feedforward inhibition, respectively (Frotscher and Leranth, 1988).

The following classes of GABAergic interneurones are typically found in the hippocampus:

a) **Basket cells** (Knowles and Swartzkroin, 1981; Ashwood et al., 1984; Lacaille, 1991), **bistratified cells** (Buhl et al., 1994) and **axo-axonic cells** are found in stratum pyramidale (Buhl et al., 1994; Kawaguchi and Hama, 1988; Li et al., 1992). An elegant study was done by Buhl et al. (1994) combining electron microscopic analysis of synaptic connections with paired intracellular recording. These authors identified the membrane region of pyramidal cells where different interneurones had synaptic contacts: thus depending on the topography of the distribution of GABAergic synapses each class of interneurones was able to control distinct excitatory inputs. In accordance with Buhl et al. (1994) scheme, basket cells make synaptic contacts predominantly on the somata and proximal dendrites of pyramidal cells. These interneurones not only mediate recurrent inhibition but are also involved in the induction of rebound firing of pyramidal cells and, by virtue of their divergence to hundreds of pyramidal cells, in the synchronizing mechanisms of pyramidal cell activity. Bistratified cells form synapses predominantly on the dendrites of pyramidal cells in stratum radiatum and oriens of the CA1 region. In this case the axons of GABAergic bistratified cells are found to be running in parallel with glutamatergic Schaffer collaterals projecting to CA1 pyramidal cells. Since bistratified cells are
strongly activated by Schaffer collaterals, their release of GABA onto dendrites of pyramidal cells can spatially and temporally overlap with the release of glutamate from Shaffer collaterals (Buhl et al., 1994). It seems therefore likely that bistratified cells have a role in the control of this excitatory input. Axo-axonic cells form synapses predominantly on the axon initial segment of principal cells (Somogyi, 1977). The strategic location of GABA\textsubscript{A} receptors in this region, where the action potential threshold is considered to be the lowest (Eccles, 1964), suggests that their main role is to control firing threshold. Basket cells and axo-axonic cells are suggested to be the source of the prominent IPSPs of pyramidal cells following activation of synapses located at or near the somata of principal cells. (Buhl et al., 1994). Based on anatomical and physiological evidence, both basket and oriens/alveus interneurones (O/A, Lacaille et al., 1987; Lacaille and Williams, 1990; McBain et al., 1994) appear to mediate feedforward and feedback inhibition of CA1 pyramidal cells.

b) Stellate cells are located near the stratum lacunosum-moleculare/radiatum border (Kawaguchi and Hama, 1988; Lacaille and Schwartzkroin, 1988a,b; Williams et al., 1994). They mediate solely feedforward inhibition of pyramidal cells.

c) Interneurones in the Stratum Lacunosum Moleculare (L / M) lack the high frequency spontaneous synaptic activity observed in basket cells and O/A interneurones. They are found to mediate feedforward inhibition only (Lacaille and Schwartzkroin, 1988a).

The hilus has a complex local circuitry, with distinct cell types (Amaral, 1978); one group of GABAergic neurones which make extensive connections with CA3 cells, is located in the hilar region (Muller and Misgeld, 1990). Different interneurones seem to mediate distinct synaptic responses of target cells. Thus, stimulation of st. L / M
interneurones appears to elicit the late, $K^+$-mediated component of the slow inhibitory postsynaptic potential (IPSPs, Lacaille and Schwartzkroin, 1988b), whereas activation of O/A and pyramidal cells layer interneurones may mediate the early phase of the fast IPSP (Knowles and Schwartzkroin, 1981; Miles and Wong, 1984; Lacaille et al., 1987; Miles, 1990).

1.3 GABAergic activity in adult rats

In adult rats the majority of fast excitatory synaptic transmission is mediated by glutamate receptors. Fast excitatory transmission results from the activation of $\alpha$-amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) ionotropic postsynaptic receptors. GABA is the primary inhibitory transmitter released by interneurones on both soma and dendrites of pyramidal and granule neurones. The action of GABA depends on the class of postsynaptic receptors to which it binds. There are two main subtypes of GABA receptors: GABA$_A$ and GABA$_B$. GABA$_A$ receptors mediate fast synaptic responses (early IPSP lasting 50-100 msec) and contain an integral chloride ion permeable channel. GABA$_A$-mediated responses are selectively and competitively blocked by bicuculline. They are also blocked by picrotoxin (a channel blocker) in a non-competitive way (for a review see Sivilotti and Nistri, 1991). Alger and Nicoll (1979) and Andersen et al. (1980) reported that in the adult hippocampus GABA applied to the dendritic layer induces a membrane depolarization of pyramidal cells, whereas when applied to the soma it usually hyperpolarizes pyramidal cells. A difference in chloride equilibrium potential between soma and dendrites could account for these findings (Misgeld et al., 1986).
GABA_B receptors mediate the late phase of the IPSP which can last hundreds of msec (Janigro and Schwartzkroin, 1988; Thalmann, 1988). They are selectively blocked by phaclofen or 2-hydroxy-saclofen (Newberry and Nicoll, 1985; Bormann, 1988): GABA_B receptor activation is associated with an increase in K⁺ conductance (Newberry and Nicoll, 1984, 1985) or a decrease in a voltage-dependent Ca²⁺ conductance (Dunlap, 1981; Dolphin and Scott, 1986) via GTP-binding proteins and second messengers. Pre and post-synaptic GABA_B receptors in the hippocampus have different pharmacological properties. In hippocampal cultures baclofen, which binds to GABA_B receptors, does not induce postsynaptic actions, whereas it depresses GABAergic synaptic transmission acting on phaclofen-insensitive, G protein-independent presynaptic receptors. In the same preparation, phaclofen increases IPSC amplitude (Harrison, 1990). GABA_B autoreceptors, probably localized on GABAergic and glutamatergic nerve terminals, have been identified in a number of preparations, in which they control the release of excitatory and inhibitory amino acids (Calabresi et al., 1991; Otis and Mody, 1992; Thompson and Gähwiler, 1992).

1.4 GABAergic activity in neonatal rats

Previous immunocytochemical studies have shown that GABAergic neurones have an early ontogeny, being generated between E13 and E15 embryonic age (Amaral and Kurz, 1985), two days before pyramidal neurones. In contrast, it is only towards the end of the second postnatal week that the mossy fibres, which represent the main glutamatergic input to pyramidal cells, reach their targets and consolidate their excitatory synaptic contacts with CA3 cells (Amaral and Dent, 1981; Gaiarsa et al.,...
In neonatal hippocampal neurones, GABA depolarizes and hyperpolarizes CA3 pyramidal cells, acting on GABA\textsubscript{A} and GABA\textsubscript{B} receptors, respectively (Ben Ari et al., 1989; Cherubini et al., 1991). The depolarizing effect of GABA in young animals has been attributed to a reversed chloride gradient, resulting from a delayed maturation of the Cl\textsuperscript{-} extrusion system (Zhang et al., 1991). There is also the possibility that GABA\textsubscript{A}-activated currents are carried by HCO\textsubscript{3}\textsuperscript{-}, that has a reversal potential more positive than Cl\textsuperscript{-} (Wu et al., 1992).

A neurotrophic role has been proposed for GABA based on its ability to modulate the differentiation and maturation of neurones both in vitro and in vivo (Hansen et al., 1987; Belhage et al., 1990; Barbin et al., 1993). GABA-induced depolarization could result in Ca\textsuperscript{2+} influx via voltage-sensitive Ca\textsuperscript{2+} channels or NMDA receptor / channels (Yuste and Katz, 1991; Lainekegel et al., 1995). The increase in intracellular Ca\textsuperscript{2+} can elicit trophic effects (Mattson et al., 1988).

Spontaneous synaptic activity recorded in CA3 hippocampal pyramidal cells of neonatal animals is characterized by ongoing background events (Hosokawa et al., 1994) and Giant Depolarizing Potentials (GDP, Ben Ari et al., 1989). Ongoing spontaneous activity occurs randomly; it is generated by transmitter released from repetitively firing nearly cells and is GABA\textsubscript{A} receptor-mediated (Hosokawa et al., 1994). GDPs consist of large depolarizations (> 30 mV lasting 200-600 ms) with superimposed fast action potentials. They occur at more or less regular intervals with a frequency ranging between 0.07 and 0.2 Hz and can be maintained during prolonged intracellular recording without significant run-down. GDPs are mediated by GABA\textsubscript{A} receptors since they reverse at the same potential as responses to GABA (-25 or -51 mV, depending on the use of KCl or potassium methylsulphate-filled electrodes,
respectively) and are blocked by bicuculline. They are also blocked by the broad spectrum ionotropic glutamate receptor antagonist kynurenic acid, suggesting that an excitatory glutamatergic drive is essential for their induction (Ben Ari et al., 1989). GDPs are network-driven events as shown by the following properties: i. their frequency does not change by depolarizing or hyperpolarizing the postsynaptic membrane of pyramidal cells. ii. they can be simultaneously recorded with intracellular and extracellular electrodes. iii. they are synchronous in pairs of intracellularly recorded CA3 neurones (= 300 µm apart). As already mentioned, GDPs are driven by glutamatergic ionotopic receptors of the NMDA or non NMDA type (Ben Ari et al., 1989; Gaiarsa et al., 1991). It has been suggested that GDPs are modulated by Zn$^{2+}$ which can synchronize GABA release (Smart et al., 1994). This hypothesis is supported by the observation that Zn$^{2+}$ can increase GDP frequency or trigger them, probably acting on presynaptic voltage-dependent channels. GDPs have an excitatory role by virtue of the strong membrane depolarization with superimposed action potentials.

During the second week of postnatal life, the reversal potential for responses to GABA becomes more negative and GDPs reverse their polarity. Thus, spontaneously occurring hyperpolarizing potentials can be detected from CA3 neurones (Ben Ari et al., 1989). After the second postnatal week GDPs disappear. During the first postnatal week in the presence of kynurenic acid, the GABA uptake blocker nipecotic acid (1mM) induces the appearance of GDPs, suggesting that endogenous GABA depolarizes a population of GABAergic interneurones, thus facilitating their synchronization even in the absence of glutamatergic drive (Cherubini et al., 1996).
It is difficult to distinguish a synaptic GABA<sub>B</sub> receptor-mediated hyperpolarizing response in early postnatal neurones because of the masking effect of the concomitant GABA<sub>A</sub> mediated depolarization (Cherubini et al., 1991). Recently, it has been shown that presynaptic GABA<sub>B</sub>-mediated inhibition has an earlier development than the postsynaptic one. Only after P6 does electrical stimulation of interneurones induce a GABA<sub>A</sub> and a GABA<sub>B</sub>-receptor mediated IPSPs (Gaiarsa et al., 1995). In accordance with these data bath application of low (100-300 nM) baclofen concentrations to CA3 pyramidal cells (between P1 and P10) reduces the GDPs frequency acting presinaptically to the CA3 cell, since no change in membrane potential or input resistance of the postsynaptic cell is observed. Higher concentrations of baclofen (30 μM) induce membrane hyperpolarization with an increase in membrane conductance (Cherubini et al., 1996).

A new type of depolarizing bicuculline and baclofen insensitive GABA response, blocked by picrotoxin, has been recently found in neonatal hippocampal neurones in slices (Strata and Cherubini, 1994). For its pharmacological characteristics this response closely resembles GABA<sub>C</sub> responses obtained from retinal horizontal and bipolar cells (Qian and Dowling, 1993; Feigenspan et al., 1993) and central visual pathway (Nistri and Sivilotti, 1985; Arakawa and Okada, 1988). This GABA receptor mediated response is present only in young rats and disappears towards the end of the first postnatal week. GABA<sub>C</sub> responses are also insensitive to compounds that allosterically modulate GABA<sub>A</sub> receptors, such as benzodiazepine, barbiturates or steroids (Feigenspan and Bormann, 1994). Since in the hippocampus bicuculline and baclofen insensitive responses show only modest desensitization from which they rapidly recover, their persistence would strengthen and prolong the depolarizing
action of GABA, thus favouring the entry of Ca$^{2+}$ through voltage-dependent and NMDA gated channels (Martina et al., 1995).

1.5 GABA$\alpha$ receptors

GABA$\alpha$ receptors belong to the ligand-gated channel superfamily. cDNA cloning studies of GABA$\alpha$ receptors for several mammalian species have shown that these receptors are hetero oligomeric assemblies of polypeptides of two $\alpha$ and one $\beta$, $\delta$ and $\gamma$ subunit classes (Seeburg et al., 1990; Macdonald and Olsen, 1994). Each subunit consists of four membrane-spanning regions (M1-M4) and a cytoplasmatic loop located between M3 and M4. Expression of different combinations of these subunits produces GABA$\alpha$ receptors that vary in their pharmacological and biophysical properties. Different combinations of various subunits are responsible for the wide range of physiological responses found in native receptors. Molecular cloning studies have established that 16 genes encode the subunits assembled in GABA$\alpha$ receptors (Olsen and Tobin, 1990; Burt and Kamatchi, 1991). The GABA$\alpha$ receptors are ubiquitous in the nervous system but their configurations vary with brain regions and cells. A combination of 2 $\alpha$, 1 $\beta$, 2 $\gamma$ subunits is likely to represent the subunit stoichiometry of at least some GABA$\alpha$ receptors. Each of these subunits comprises various isoforms which are usually identified by a subscript, e.g. $\alpha_1$, $\alpha_2$ etc. The combination of one $\alpha$ and the $\gamma_2$ subunit is a prerequisite for the formation of benzodiazepine sites (Pritchett et al., 1989). During development, embryonic receptors containing the $\alpha_2$ subunit are increasingly replaced by receptors containing
the \( \alpha_1 \) subunit, a phenomenon which is particularly prominent in the early postnatal period around the time of synapse formation (Fritschy et al., 1994).

The GABA\(_A\) receptor is characterized by its permeability to anions (Krnjevic and Schwartz, 1967; Barker and Ransom, 1978) and its sensitivity to the competitive antagonist bicuculline and to agonists such as isoguvacine (Macdonald et al., 1992).

In hippocampal neurones, main conductance states of 16 and 23 pS are consistently found (Bormann et al., 1983; Gray and Johnston, 1985; Edwards et al., 1989, 1990), sometimes a subconductance state of 14 pS (Edwards et al., 1990) is also detected.

Several binding sites have been identified on the extracellular domain of the GABA\(_A\) receptors: i. a binding site for channel blocker picrotoxin; ii. binding sites for ligands of therapeutic interest such as benzodiazepines, tranquilizers that can allosterically cause an increase in probability of GABA-induced channel openings; iii. the site for barbiturates, which prolong the lifetime of the GABA-activated channel; iv. a site for steroids, which are also positive modulators of the GABA\(_A\) receptor-complex (Olsen and Venter, 1986). The steroid site may be of particular physiological significance, since the brain is capable of synthesizing steroids that affect GABA\(_A\) receptor function (neurosteroids, Beaulieu and Robel, 1990). The GABA\(_A\) receptor also has binding sites for Zn\(^{2+}\), which is highly concentrated in the brain, especially in synaptic boutons (Perez-Chausell and Danscher, 1985) from which it can be released upon nerve stimulation (Assaf and Chung, 1984; Aniksztejn et al., 1987). Both depressing and potentiating effects of Zn\(^{2+}\) on GABA\(_A\) mediated responses have been reported in hippocampal neurones in culture and in slices (Westbrook and Mayer, 1987; Smart and Constanti, 1990; Legendre and Westbrook, 1991). The intracellular loop of the \( \beta \)
subunit contains sequence sites for phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and tyrosine kinases (Swope and Moss, 1992) suggesting a role for them in the modulation of GABA_A receptor function. Most published work reports that in cortical (Jalilian Tehrani et al., 1989), hippocampal (Harrison and Lambert, 1989) and spinal cord neurones (Porter et al., 1990), activation of PKA depresses the sensitivity of postsynaptic GABA_A receptors to exogenous GABA. On Purkinje cells PKA has a facilitatory action on GABA_A receptor sensitivity (Kano and Konnerth, 1992), an effect shared by an increase in intracellular calcium which upregulates GABA receptors.

1.6 Ionotropic glutamate receptors

Two families of receptors (ionotropic and metabotropic) are known to mediate the actions of glutamate in the mammalian CNS.

Ionotropic glutamate receptors are heteromultimeric proteins that contain an integral ion channel and mediate fast excitatory transmission at various synapses; in contrast, the metabotropic glutamate receptor (mGluR) family is comprised of monomeric proteins that couple to effectors (e.g., ion channels and enzymes) by interacting with G proteins (Nicoletti et al., 1988; Schoepp et al., 1990; Miller, 1991). Ionotropic glutamate receptors can be divided into N-methyl-D-Aspartate (NMDA) and non-NMDA receptors due to their difference in agonist selectivity, gating kinetics, block by extracellular Mg^{2+}, permeability to Ca^{2+} and pharmacological properties. Non-NMDA receptors have been classified as α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (KA) receptors. Quisqualate (QA) is a mixed agonist
acting at metabotropic and ionotropic non-NMDA receptors (Monaghan et al., 1989). NMDA receptors open predominantly large single conductance channels (40-50 pS). Lower conductance states can be also observed but usually with few openings (Jahr and Stevens, 1987; Ascher et al., 1988; CullCandy et al., 1988). AMPA receptor activation opens channels of a relative low conductance (< 20 pS); KA activates primarily low conductance channels of 4 pS (Ascher and Nowak, 1988; Sciancalepore et al., 1990). Differences exist in the time-course of excitatory synaptic currents. Those mediated by non-NMDA receptors last few msec whereas synaptic currents mediated by NMDA receptors last hundreds of msec (Randall and Collingridge, 1992). NMDA and non-NMDA receptors are suggested to be colocalized on the postsynaptic membranes (Bekkers and Stevens, 1989; Monaghan et al., 1989). The NMDA induced current is characterized by a strong negative slope conductance in the potential range close to rest. This is due to the block by magnesium ions of the channel pore near resting potential; depolarization removes this block (Nowak et al., 1984; Mayer et al., 1984; Ascher and Nowak, 1988). The NMDA receptor requires glycine as co-agonist (Johnson and Ascher, 1987). It has an hetero oligomeric configuration always comprising the subunit NMDAR1 which is assembled together with one or more of the NMDAR2 (2A, 2B, 2C, 2D) subunits. An asparagine residue in the M2 transmembrane segment is responsible for the high Ca\(^{2+}\) permeability (Burnashev et al., 1992). NMDA receptor channels are selectively blocked by MK 801 (channel blocker), or by the selective receptor antagonists D(-)2-amino-5-phosphonovalerate (D-AP5), DL-2-amino-7-phosphonoheptanoate (D-AP7) and (±)3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP). The complex receptor structure has different allosteric sites from which the receptor activation can
be modulated (Monaghan et al., 1989). They include: i. a transmitter binding site, which binds L-glutamate, ii. a site within the channel that binds phencyclidine and related compounds, iii. a voltage-dependent Mg$^{2+}$ binding site, iv. a glycine binding site, v. a Zn$^{2+}$ binding site and vi. phosphorylation sites which potentiate NMDA responses.

Ca$^{2+}$ negatively modulates the activity of NMDA receptors; although it permeates the NMDA receptor channel complex, extracellular Ca$^{2+}$ (in the millimolar concentration range) reduces the single-channel conductance (Ascher and Nowak, 1988; Medina et al., 1996). NMDA receptors play a critical role in synaptic plasticity, due to the fact that their open probability depends on the voltage. In the CA1 hippocampal region, for instance, when stimulation of presynaptic fibers coincides with postsynaptic depolarization, long-term potentiation (LTP) occurs. In fact, depolarization removes the Mg$^{2+}$ block and allow Ca$^{2+}$ entering through the NMDA receptor channels and allow Ca$^{2+}$ to enter triggering the cascade of events leading to LTP (Malenka, 1992). Antagonists of NMDA receptors block this form of neuronal plasticity.

Non-NMDA channels are assembled from a family of subunits consisting of several different members (GluR-1 to -4, GluR-5,6,7, KA-1, KA-2, Dingledine, 1991). The channels assembled from GluR-1 to -4 subunits are activated preferentially by AMPA, but also by KA. These AMPA-type glutamate receptors have been shown to exist in two alternative spliced versions designated as flip and flop subunits (Sommer et al., 1990). The rectification properties and the Ca$^{2+}$ permeability of GluR-2 on one hand and GluR -1, -3 and -4 on the other hand are different (Verdoorn et al., 1991). The channels formed by GluR-5 and GluR-7 subunits are preferentially activated by KA which, however, has higher affinity for KA-1 and KA-2 receptor subunits (Werner et
al., 1991). When expressed as homomeric proteins, KA-1 and KA-2 subunits do not form functional channels. Because of the absence of homomeric receptors in vivo, it is possible that pharmacologically “pure” KA receptors do not exist in the brain. On the other hand, no data support the existence of pure AMPA receptors that cannot be activated by KA (Hollmann and Heinemann, 1994). In conclusion, the traditional classification in KA and AMPA receptors has probably to be changed.

In hippocampal and spinal cord cultures, Mayer and Westbrook (1987) estimated that the relative Ca\(^{2+}\) permeability of non-NMDA receptors is about 100-fold lower than that of NMDA receptors. This idea was challenged by Iino and co-workers (1990) who found a subset of cultured hippocampal neurons in which non-NMDA receptors exhibited high Ca\(^{2+}\) permeability. These receptors were subsequently identified as AMPA receptors on the basis of their pharmacological properties (Ozawa and Iino, 1993). The Ca\(^{2+}\) permeability of AMPA receptors appears to be regulated by the positively charged arginine residue which replaces a neutral glutamine in the M2 transmembrane segment of GluR-2 only (Hollmann and Heinemann, 1994). Substituting arginine with glutamine increases Ca\(^{2+}\) permeability. The majority of native AMPA receptors appear to contain a GluR-2 subunit and exhibit a low Ca\(^{2+}\) permeability. Recently, GABAergic interneurones have been found to express Ca\(^{2+}\) permeable AMPA receptors (Koh et al., 1995; Jonas et al., 1994). The discovery that, in addition to NMDA receptors, some of the cloned non-NMDA receptors are quite permeable to Ca\(^{2+}\), raises the question of their role in determining intracellular calcium levels during plastic phenomena as LTP or Long-Term Depression (LTD) as well as their role in many degenerative brain diseases, which may involve Ca\(^{2+}\)-induced excitotoxicity and cell death. Non-NMDA receptors are selectively antagonized by
quinoxalinediones such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX).

1.7 Metabotropic glutamate receptors

In comparison with synaptic responses mediated by ionotropic glutamate receptors, those elicited by mGluRs are slower and result from blockade of K+ conductances (Charpak and Gähwiler, 1991). The mGluR is a single polypeptide chain which includes seven hydrophobic domains (Masu et al., 1991). At present, eight distinct mGluR subtypes (mGluR 1-8) have been cloned. *In vitro* expression experiments have shown that mGluRs differ not only in their sequence but also in their signal transduction pathway. They can be arranged into three groups based on agonist pharmacology, coupling to specific signal transduction pathways and sequence homology (Hollman and Heinemann, 1994; Nakanishi, 1992; Pin and Duvoisin, 1995), see Table I. The cloned receptors mGluR1 and GluR5 (Group I) are coupled to phosphoinositide hydrolysis and are more sensitive to QA than to the selective metabotropic agonist *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD). Activation of mGluR1 also stimulates production of cyclic-AMP (cAMP) and arachidonic acid release (Dumuis et al., 1990). The cloned receptors mGluR2 and mGluR3 (Group II) are more sensitive to *t*-ACPD than QA and inhibit forskolin-stimulated cAMP formation. *In situ* hybridization of mRNAs coding for mGluR has suggested that mGluRs are differently expressed throughout the brain. For example, mGluR1 mRNA is concentrated in almost all neuronal cells of the dentate gyrus and areas CA2-CA4 of the rat hippocampus (Shigemoto et al., 1992), while mGluR5
mRNA is localized in CA1-CA4 areas of the hippocampus and granule cell area of dentate gyrus (Abe et al., 1992). mGluR6 is only found in the retina (Nakajima et al., 1993). In the cerebellum mRNA encoding mGluR1 is found in Purkinje cells while mGluR5 is localized to a small population of Golgi cells (Schoepp and Conn, 1993). Moreover, the same type of neuron may contain mRNAs coding for more than one mGluR subtype. mGluRs can be functionally distinguished on the basis of agonist pharmacology.

Table I. Pharmacology and transduction mechanisms of cloned mGluR subtypes.

<table>
<thead>
<tr>
<th>mGluR subtype</th>
<th>Order of agonist potency</th>
<th>Antagonist</th>
<th>Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR1, mGluR5</td>
<td>QA &gt; glutamate &gt; (1S,3R)-ACPD</td>
<td>MCPG</td>
<td>↑PI</td>
</tr>
<tr>
<td></td>
<td>4CPG</td>
<td>4C3HPG</td>
<td>↑cAMP♦</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR2, mGluR3</td>
<td>DCG-IV &gt; L-CCG-I &gt; (1S,3R)-ACPD &gt; QA</td>
<td>MCPG</td>
<td>↓cAMP</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR4, mGluR6</td>
<td>L-AP4 &gt; L-SOP &gt; QA &gt; (1S,3R)-ACPD</td>
<td>MCPG*</td>
<td>↓cAMP</td>
</tr>
<tr>
<td>mGluR7, mGluR8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MCPG does not antagonize mGluR4

♦ mGluR5 not coupled to increased cAMP synthesis or AA release

PI, phosphoinositide; AA, arachidonic acid.
They can be selectively activated by \( t \)-ACPD or by its active isomer \( 1S,3R \)-ACPD (Schoepp et al., 1990; Desai and Conn, 1990). (2S, 1'R,2'R, 3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) and (2S, 1'S, 2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) appear to be relatively specific agonists for Group II mGluRs (Hayashi et al., 1993). mGluR4 and mGluR6 (Group III) are sensitive to AP4 (2-amino-4-phosphonobutyrate) and L-serine-O-phosphate (L-SOP, Tanabe et al., 1993; Nakajima et al., 1993) and inhibit adenylyl cyclase activity. In order to identify the physiological role of these receptors, it is fundamental the discovery of more specific and potent antagonists than those currently available is fundamental. MCPG is a competitive antagonist at group I and II mGluRs (Hayashi et al., 1994; Thomsen et al., 1994). Although inactive on mGluR4, MCPG may have some blocking action on other members of group III mGluRs. Interestingly, the mGluR1 antagonists 4-carboxy-3-hydroxyphenylglycine (4C3HPG) and 4-carboxyphenylglycine (4CPG) are agonists with high and low affinity at mGluR2, respectively. At mGluR5, 4C3HPG is a partial agonist and 4CPG is inactive (for review see Pin and Bockaert, 1995).

1.7.1 mGluR activation modulates voltage- and ligand gated channels

Electrophysiological studies of hippocampal neurones have established that stimulation of postsynaptic mGluRs by racemic \( t \)-ACPD leads to a membrane depolarization or (in voltage clamp experiments) to an inward current (Mayer and Miller, 1990). On central neurones a wide variety of actions of \( t \)-ACPD has been
found to be due to modulation of voltage-gated ion channels. In hippocampal pyramidal cells activation of mGluRs by t-ACPD increases cell excitability by reducing at least three potassium conductances. In particular, mGluR activation induces a depolarization with an increase in input resistance mediated by inhibition of a leak potassium conductance (Charpak et al., 1990; Desai and Conn, 1991; Liu et al., 1993). mGluR activation also inhibits a slow calcium-dependent after-hyperpolarizing current, I_{AHP} (Charpak et al., 1990; Stratton et al., 1990), thus blocking spike frequency adaptation. Modulation of the muscarine-sensitive potassium current (I_M) may also participate in this response (Charpak et al., 1990; Storm, 1990).

Gerber et al. (1992), using staurosporine as blocker of PKA and PKC, found that these two kinases are not involved in I_{AHP} reduction mediated by mGluR activation. Similar results, relative to the possible PKA involvement, were found by Pedarzani and Storm (1993). On the contrary, Baskys et al. (1990) found that 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), suggested to act by a mechanism similar to staurosporine (block of the ATP-binding site of various kinases), suppresses the inhibition of I_{AHP} by mGluR activation.

Initial experiments were carried out in order to see if mGluRs are involved in modulation of voltage-dependent \( \text{Ca}^{2+} \) currents without distinguishing various subtypes of mGluRs. In hippocampal neurones mGluR activation was reported to inhibit the voltage-dependent high-threshold \( \text{Ca}^{2+} \) channels (Lester and Jahr, 1990). The transduction pathway was however unidentified. The effect of mGluR activation is not inhibited by non-specific protein kinase inhibitors or by buffering intracellular \( \text{Ca}^{2+} \); it is not blocked by calmodulin inhibitors or occluded by cAMP, cGMP or IP3 (Lester and Jahr, 1990). A G protein is claimed to be involved. According to one study
this G protein is pertussis toxin (PTX) sensitive (Sahara and Westbrook, 1993) while in another study it is PTX insensitive (Lester and Jahr, 1990).

Recently, the reduction of Ca\(^{2+}\) currents by mGluR has been better investigated. There is evidence that in hippocampal neurones, Group I mGluRs inhibit N-type Ca\(^{2+}\) channels (Swartz and Bean, 1992). In olfactory bulb neurons Group III mGluRs inhibit an uncharacterized high-voltage activated Ca\(^{2+}\) channel (Trombley and Westbrook, 1992). In cerebellar granule cells Group II mGluRs are known to inhibit the voltage-dependent L-type Ca\(^{2+}\) channels via a PTX sensitive G protein (Chavis et al., 1994); conversely, in the same cells, Group I mGluRs are found to potentiate the same channels (Chavis et al., 1995).

More recently, an elegant study was done by Takahashi et al. (1996) who simultaneously recorded presynaptic Ca\(^{2+}\) currents and the excitatory postsynaptic currents (EPSCs) in brainstem slices. Agonists of Group III mGluRs suppressed high voltage-activated P/Q-type calcium conductance in presynaptic terminals, thereby inhibiting transmitter release at glutamatergic synapses. Some of the responses mediated by mGluRs involve intracellular Ca\(^{2+}\) mobilization as a consequence of an altered PI metabolism (Schoepp and Conn, 1993).

Recent experiments have revealed an additional excitatory effect due to mGluRs activation; this consists of an afterdepolarization following a series of action potentials in neocortical neurones (Greene et al., 1992), in hippocampal pyramidal cells (Caeser et al., 1993) and olfactory cortex neurones (Constanti and Libri, 1992; Sciancalepore and Constanti, 1995).

In addition to their action on voltage-dependent conductances, mGluRs can also modulate ligand gated channels. This is exemplified by potentiation of NMDA...
responses of CA1 neurones (Aniksztein et al., 1992), an effect mediated by PKC activated by the phosphoinositide hydrolysis. In accordance with these data, activation of mGluR leads to a PKC-mediated increase in NMDA currents in *Xenopus* oocytes injected with rat brain RNA (Kelso et al., 1992).

Metabotropic glutamate receptors are known to participate in the modulation of synaptic transmission in different brain regions. Activation of presynaptic mGluRs is found to reduce transmitter release at both inhibitory (Desai and Conn, 1991; Pacelli and Kelso, 1991; Hayashi et al., 1993; Gereau and Conn, 1995) and excitatory synapses (Lanthorn T.H. et al., 1984; Manzoni et al., 1985; Forsythe and Clements, 1990; Baskys and Malenka, 1991; Sladeczek et al., 1993). The effect of mGluR activation on voltage-dependent Ca^{2+} channels may account for the presynaptic depressant action on many glutamatergic and GABAergic synapses.

It is however uncertain whether the activation of mGluRs depresses synaptic activity by a direct effect on nerve terminals or indirectly via a network mechanism. In support of the latter possibility, facilitation of GABA release by mGluR agonists has been found in hippocampal slices by Miles and Poncer (1993), probably due to an increase in GABAergic interneuron firing following depolarization and reduction of after-hyperpolarizing potentials. McBain et al. (1994) found that ACPD enhances excitatory transmission onto st. oriens GABAergic interneurones, thus causing an increase in inhibition.

mGluR activation has been shown to upregulate glutamate release in several part of the brain. Repetitive stimulation of mGluRs in immature CA3 neurones induces periodic inward currents (PICs) mediated by glutamate acting on ionotrophic glutamate receptors when synaptic transmission is blocked and potassium conductances are
minimized by extracellular TEA and Cs⁺. PIC induction is independent of NMDA receptor activation because these responses still develop in the presence of NMDA blockers although intracellular calcium stores are necessary for their generation and expression (Aniksztejn et al., 1995).

The role of mGluRs in LTP is still controversial and incomplete. Some authors have found that at the Schaffer collateral synapses LTP induced by high frequency stimuli is potentiated by t-ACPD (McGuinnes et al., 1991). Using MCPG as antagonist of mGluRs, NMDA receptor dependent and NMDA-receptor independent forms of LTP induction in the CA1 and the CA3 region, respectively, have been found. mGluR activation is considered as a necessary mechanism for LTP induction (Bashir et al., 1993). These results obtained using MCPG have not been reproduced by others (Chinestra et al., 1993; Manzoni et al., 1994). Furthermore, t-ACPD was shown to cause no long-lasting phenomena at the Schaffer-collateral pathway (Baskys and Malenka, 1991; Chinestra et al., 1993) and at the mossy fiber synapse (Ito and Sugiyama, 1991).

Recently, the involvement of the mGluR family in LTD has been studied. mGluRs have been reported to be essential for LTD induction in the CA1 region (Selig et al., 1995; Kobayashi et al., 1996), visual cortex (Haruta et al., 1994) and cerebellum (Linden and Connor, 1995). LTD of the mossy fibers-CA3 synapses is significantly impaired in mutant mice deficient in the mGluR2 subunit whereas NMDA-independent LTP is normally induced (Yokoi et al., 1996): mGluR2 is therefore a predominant and selective receptor for induction of mossy fiber LTD. In cerebellar Purkinje cells, LTD seems to require the functional cooperation of both AMPA-kainate receptors and mGluRs (Linden et al., 1991).
1.8 Aim of the present study

While in the neonatal hippocampus GDPs have been well studied in terms of their origin, propagation and modulation by neurotransmitters, spontaneous GABAergic synaptic activity has not been fully characterized. Thus, a first set of electrophysiological experiments aimed at elucidating the properties of spontaneous GABA release onto pyramidal cells during the first week of postnatal life when glutamatergic inputs are still poorly developed.

In a second set of experiments the role of neurotransmitters in regulating such a GABA release has been studied. In the hippocampus, most transmitters produce presynaptic inhibition (Thompson et al., 1993); few agents are known to mediate presynaptic facilitation of GABAergic transmission (Roper and Guy, 1991; Behrends and Bruggencate, 1993). In my opinion, it is of great interest to know if GABA release can be positively modulated by neurotransmitters or second messengers.

mGluR activation was first tested as a possible candidate for modulation of GABA receptor mediated activity, because of the typical developmental profile of phosphoinositol (PI) metabolism and cAMP pathway associated with activation of mGluRs. In fact, IP$_3$ formation and cAMP synthesis are high during the first two postnatal weeks and then decrease (Nicoletti et al., 1986; Schoepp and Johnson, 1993).

These aims were pursued with electrophysiological recordings of synaptic activity as they provide numerous advantages for the study of neurotransmitter modulation: millisecond time resolution, high sensitivity to pharmacological agonists and the possibility to study release with relatively physiological stimuli. A technique such as
patch-clamp employed in the present study provides high resolution in terms of signal to noise ratio due to the giga-seal recording conditions: in this way it is possible to identify the presynaptic or postsynaptic origin of synaptic modulation through analysis of miniature current amplitude and frequency. In fact, a change in frequency can supply information about the presynaptic release process, while changes in amplitude can originate from a combination of post and presynaptic effects (Manabe et al., 1992). The experimental preparation used for the present project was the hippocampal slice because it allows visualization of single neurones and contains well-characterized local circuits. In this preparation I studied indirectly the modulation of the interneurone properties by recording their synaptic activity from their physiological target (pyramidal cells) without interfering with the interneurone membrane properties or metabolism.
Chapter 2

Methods

Whole-cell recordings

2.1 Thin-slice preparation

Transverse slices were prepared from hippocampi of 6-12 day old Wistar rats according to the methods described by Edwards et al. (1989). Rats were decapitated under anaesthesia (5% urethane i.p.) and their brains were rapidly removed and placed in ice-cold salt solution. After bisecting the brain, the tissue was immersed in low temperature (2-4 °C), oxygenated (95% O₂, 5% CO₂) salt solution; the cerebellum was removed and a half hemisphere was then placed on its medial surface and further cut to partially remove the parietal region of the cortex. The surface of this cut was used to glue the tissue to the vibrating microslicer (Vibracut, FTB, Weinheim, Germany) chamber. Slices (250-300 μm thick) were transferred to an incubating chamber containing salt solution bubbled continuously with 95% O₂ - 5% CO₂. Slices were incubated at 32 °C for up to 8 h before use. They were transferred into and out of the recording chamber (schematically shown in Fig. 1b) using the large tip of a Pasteur pipette.
**Fig.1b** Schematic representation of recording chamber (top view). The shaded area indicates the part of the bath containing the slice in the superfusing solution.
2.2 Electrophysiological recordings

Whole-cell patch-clamp methods were used to record synaptic currents from pyramidal neurones, directly visualized through a high-numerical-aperture water immersion 40x lens, 0.75 numerical aperture, 1.9 mm working distance) of an upright fixed-stage microscope (Axioscope FS, Zeiss, Germany) which provided differential interference contrast (DIC) optics. Pyramidal cells were visualized using infrared differential interference contrast (IR-DIC) videomicroscopy (Stuart et al., 1993), utilizing an infrared filter (RG-9; Schott, Germany) and a Newvicon camera (C2400; Hamamatsu, Japan). Images were visualized and stored on computer using a frame grabber (SE100 Video Blaster, Creative Labs., CA). Pipettes were moved with a piezoelectric micro-manipulator (Physik Instrumente, Germany) connected to a coarse micromanipulator (Micro Control) fixed to an aluminum tower. An antivibration table ensured minimal sensitivity to mechanical movements. A Faraday cage isolated the recording chamber from surrounding noise sources (see Fig. 2).

Tight-seal whole-cell recordings were made with patch-pipettes pulled from glass capillaries (Hildelberg, Germany; 2.0 mm o.d., 0.3 mm wall thickness) having resistance 2-4 MΩ when filled with intracellular solution. The seal resistance was measured by applying 5 mV, 20 ms voltage pulses and observing the reduction in the amplitude of the resulting current response. For all cells used in the present study series resistance was always greater than 2 GΩ. Patch-pipettes were not fire-polished. Positive pressure was continuously applied to the patch pipette as it was advanced through the slice under visual control. The desired amount of pressure was maintained by closing a valve switch. Once the pipette tip touched the membrane of the cell, the
Fig. 2 Patch-clamp set up. The two panels show the patch-clamp workstation inside a Faraday cage (upper panel) and the instrument rack (lower panel). The set up is equipped with the following instruments: (1) antivibration-table; (2) up-right microscope (Zeiss Axioscope); (3) video camera (C2400, Hamamatsu) and (7) video monitor (Sony); (4) hydraulic micromanipulator for stimulating pipette (Narishige WR88); (5) piezoelectric micromanipulator for patch pipette (Physik Intrumente); (6) gravity perfusion system; (8) tape recorder (Philips); (9) filter (Frequency Devices 902); (10) oscilloscope (Hewlett Packard); (11) patch-clamp amplifier (List, EPC-7); (12) stimulator (Grass S88); (13) computer (IBM compatible PC); (14) chart recorder (Gould).
pressure was released and slight negative pressure was applied by mouth until a high resistance seal was established.

Further negative pressure broke the patched membrane to establish a whole-cell configuration. In all experiments, compensation of the series resistance was performed using the standard procedure of the EPC-7 amplifier (List Electronics, Darmstadt, Germany). The input resistance ($R_{in}$) was estimated in voltage-clamp recordings by analyzing current transients associated with 5-10 mV, 100 msec long voltage pulses. Cells were voltage clamped at -70 mV unless otherwise stated in the text.

2.3 Elicited currents

A second pipette was used as a stimulating electrode to elicit synaptic GABAergic events in the presence of kynurenic acid. The stimulating pipette was placed about 100 µm away from the recorded pyramidal cell either in stratum radiatum or stratum oriens. When recording minimal elicited currents the stimulation intensity was adjusted above threshold for eliciting PSCs with a failure rate of 10-30 % and was kept constant throughout the experiment. The number of failures was obtained by counting the number of responses that fell into the 0 pA bin in the amplitude distribution histogram with SD identical to that of the background noise. Stimuli of intensity 4-10 V and duration 40-50 µs were delivered at 0.1 Hz from an isolated pulse generator (Grass stimulator). In such conditions, stimulation no longer elicited synaptic currents when the pipette was moved 5-10 µm from its original position.
2.4 Solutions and drugs

During recording, slices were continuously superfused at 3ml/min at room temperature (22-24 °C) with artificial cerebrospinal fluid (ACSF) of the following composition (mM): 126 NaCl, 3.5 KCl, 2 CaCl₂, 1.2 NaH₂PO₄, 1.3 MgCl₂, 25 NaHCO₃ and 11 glucose; pH 7.4, bubbled with 95% O₂- 5% CO₂. A home made multibarrel perfusion system driven by gravity was used. The flow of the different solutions was controlled by electrovalves. The pipette solution (intracellular solution) contained (mM): 135 CsCl, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 ethylen-glycole-tetra-acetic acid (EGTA) and 1 MgCl₂. In some experiments, 3-tetracesium bis (o-aminophenoxy) ethane-N,N,N,N'-tetraacetatic acid (BAPTA, 10mM) was added instead of EGTA in order to chelate intracellular Ca²⁺ more rapidly and to avoid its involvement in the activation of mGluRs. CaCl₂ (1mM) was also added to this solution. Na₂ATP (1.5mM) was routinely added to minimize the rundown of spontaneous GABAergic currents (Chen et al. 1990). In order to block glutamatergic synaptic currents and to eliminate polysynaptic events, kynurenic acid (1 mM) was always added to the saline. Stock solutions of forskolin and 1,9 dideoxy-forskolin were prepared in dimetyl sulphoxide (DMSO) at a concentration of 10 mM. Control experiments were done in the presence of DMSO; no differences in amplitude or frequencies of spontaneous events were found. All other substances were diluted in water.
2.5 Data acquisition and analysis

Continuous data of spontaneous activity were stored on magnetic tape and transferred to a computer (ATARI 1040ST or Personal Computer) after digitization with a A/D converter (ITC-16, Instrutech or Digidata 1200). Currents were sampled every 0.1 ms and filtered at 2 kHz using an 8-pole low-pass Bessel filter. The amplitude and frequency of spontaneous GABAergic currents were analyzed with the use of a peak detector program with an adjustable threshold, set at 6-10 pA, and kept constant for a given experiment. Single spontaneous events were selected also on the basis of their rise time (< 3 ms) in order to sample from synapses located close to the soma. Continuous recordings for at least 60 s were used for the analysis of the mean amplitude and the mean frequency of GABAergic currents either in the absence or in the presence of drugs. Decay time constants of spontaneous currents were calculated by performing a least-squares fitting of experimental records with a single exponential function. Rise times were measured as the time from 10 to 90 % of the peak current. Rise time values and decay time constants were derived by single events. Statistical analysis (Student's t-test) of mean data was performed accepting as level of significance P ≤ 0.05. Even if data points for each cell sample were not necessarily distributed in Gaussian fashion, the Student's t-test remains valid for the mean values from a population of cells: in fact, according to the central limit theorem, the means of random samples from any distribution will themselves have a normal distribution (Altman and Bland, 1995). Further validation of the statistical assessment of data was obtained by using a non parametric test (Kolomogorov Smirnov test) on a random number of samples which yielded the same level of significance found with the t-test.
Data are presented as mean ± SD (n is the number of cells). Due to the large variability observed from cell to cell, the values of amplitude and frequency of the currents are expressed as \( I_t / I_c \) and \( F_t / F_c \), where \( I_t \) and \( F_t \) are the amplitude and frequency in test condition and \( I_c \) and \( F_c \) are the amplitude and frequency in control condition, respectively (Llano and Gerschenfeld, 1993). Mean ratios were calculated from individual test / control ratios in each cell.

**Single channel events**

### 2.6 Acutely dissociated hippocampal neurones

CA3 hippocampal neurones were acutely dissociated following the method described by Kay and Wong (1986). P0-P8 old Wistar rats were anaesthetized and decapitated. The brain was quickly removed from the skull and put into a Petri dish containing ice cold standard salt solution continuously bubbled with 95% O\(_2\), 5% CO\(_2\). Hippocampi were then sliced (300 \( \mu \)m) with a McIlwain tissue chopper. Slices were stored for at least 30 min at room temperature in continuously oxygenated salt solution in which they could be maintained for up to 8 hours. A single slice was then incubated for 35-60 min at 30 °C in 10 ml of oxygenated, low chloride (10 mM, SO\(_4^–\) substituted for Cl\(–\)) salt solution containing 1mg/ml pronase E and then washed at room temperature for at least 30 min with the same solution without enzyme. Cells were then mechanically dissociated (in ice cold low chloride solution) using sequentially three fire polished Pasteur pipettes of decreasing tip diameters. The cell suspension was plated to the
recording chamber containing the bath solution. Cells were usually viable for up to 90 min.

2.7 Solution and drugs

Bath solution (control solution) was continuously applied with a multibarrel perfusion system with gravity-fed inputs whose openings were controlled by electrovalves connected to an home made electronic selector and a timer. Solutions were continuously removed by suction. The average flow rate was 3 ml/min. The bath solution contained (in mM): 137 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES-NaOH, 20 D-glucose. The pipette was filled with a solution containing (in mM): 137 CsCl, 2 MgCl₂, 11 EGTA, 2 ATP-Na₃, 1 CaCl₂, 10 HEPES-KOH. The pH of both solutions was adjusted at 7.3.

2.8 Data acquisition and analysis

GABA-elicited single-channel currents were studied at room temperature (22-24 °C) using a standard patch-clamp amplifier (EPC-7, List Medical Instruments, Germany). The current signal was recorded on a video tape recorder. An ATARI microcomputer and a digital to analogue converter (ITC-16, Instrutech) were used to control the potential in the pipette. Stored data were filtered with a Butterworth filter (Krohn-Hite 3202, USA, cut-off frequency of 1-2.5 kHz) and then transferred to the microcomputer at a sampling interval of 100 μs according to the sampling theorem which states that the sampling rate should be faster than twice the highest frequency
component within the signal (Nyquist, 1928). Single-channel currents were analysed with the TAC program (Instrutech) which uses a 50 % threshold criterion for the detection of channel opening.
Chapter 3

Results

3.1 Electrophysiological properties of pyramidal cells

All the recordings were performed on visually identified pyramidal cells in the CA1 and CA3 layers of hippocampal slices obtained from postnatal (P) days P6-P12 old rats.

A frame grabber imaging (Fig. 3 A) shows an overview of hippocampal layers through a 10x objective. In order to visualize single cells, a 40x immersion objective was used (Fig. 3 B). In slices from young rats (< P10), pyramidal cells appeared clearly; nerve cells in slices from older rats were usually covered by non-neuronal cells which scattered the light and prevented good visibility.

Because of their similar responses to the agents used in the present research, data from CA1 and CA3 cells were pooled, unless otherwise reported. The experiments comprise a total of 170 pyramidal cells which had an initial resting potential more negative than -55 mV; dialysis of the neuron with Cs+ usually resulted in stabilization of cell membrane potential close to 0 mV. Throughout the experiment, the membrane potential was held at -70 mV in order to avoid cell firing. In control condition, series resistance value was 8 ± 2 MΩ and input resistance was 255 ± 23 MΩ (n =30). On
Fig. 3 Photomicrographs of a transverse hippocampal slice at P9. (A) Frame grabber imaging of hippocampal region through a 10x objective. CA3, CA1 and dentate gyrus (DG) regions are indicated by arrows. (B) CA3 pyramidal cells are viewed in the same slice through a 40x immersion objective. Slice is obtained from a 9 days old rat.
average, neurones lasted in the whole-cell configuration for about one hour without
deterioration.

3.2 Properties of spontaneous GABA-mediated currents

Under the recording conditions used (symmetrical chloride solution and -70 mV
holding potential), spontaneous firing of GABAergic interneurones induced GABA_A
receptor mediated inwardly directed currents; the amplitude of which decreased as the
cell was held at less negative potentials and the direction reversed around 0 mV, close
to the Cl' equilibrium potential calculated with the Nernst equation (Fig. 4 A). At P6-
P12, spontaneous activity was totally mediated by GABA_A receptors, since it was
fully blocked by bicuculline methiodide (10 μM), the specific GABA_A receptor
antagonist. As shown in Fig. 5, bicuculline suppressed all spontaneous events
(recovery was obtained after 30 min wash; not shown). Addition of bicuculline
induced a slight steady outward current (6 ± 2 pA, n = 4) probably due to a block of
inward currents caused by tonic release of GABA. Even if spontaneous glutamatergic
events on pyramidal neurones are not fully developed before P12 (Hosokawa et al.,
1994), all the experiments were performed in the presence of kynurenic acid (1mM), a
broad spectrum ionotropic glutamate receptor blocker. Under these experimental
conditions GABA_A receptor mediated currents were thus pharmacologically isolated.
The recorded currents ranged in size from 6 to hundreds of pA depending on the cell,
showing a large variability from cell to cell. In a random sample of 20 neurones the
mean amplitude was 36 ± 10 pA. Amplitude histograms were differently shaped,
depending on the cell activity; a common property was that they were skewed
towards the lower values (Fig. 4 B, 7 B). In most experiments only one peak could be clearly detected in the amplitude distribution histogram. Frequencies varied from 0.2 to 6.6 Hz, depending on the experiment. On average, frequencies were typically higher in CA3 than in CA1 cells but the difference did not reach significance in this sample. This was presumably due to the activity and number of GABAergic terminals which impinge on the recorded cell.

Histograms of the time interval between spontaneous GABAergic currents were fitted by a single exponential curve revealing the random nature of these events. (Fig. 6 B). The kinetics properties of GABAergic spontaneous events were further examined and appeared to be similar for CA1 and CA3 pyramidal neurones, suggesting similarity of postsynaptic GABA<sub>A</sub> receptors. When measured at -70mV, the decay time was fitted with a single exponential curve in most cells examined. In CA3 and CA1 cells the time constants of decay ranged between 25 and 40 ms; in CA1 the mean was 32 ± 4 ms (n = 8); in CA3 30 ± 6 ms (n=10). The rise time varied between 0.7 and 3 ms, with a mean of 2.3 ± 0.1 ms in CA1 and 2.5 ± 0.2 in CA3.
Fig. 4 Properties of spontaneous GABA-mediated currents. (A) Samples of continuous recording of spontaneous PSCs at different holding potential ranging from -20 to 20 mV. Reversal potential is around 0 mV. (B) Amplitude histogram of spontaneous GABA-mediated currents recorded from a CA1 cell at P7 in kynurenic acid during a 160 s period. Binwidth 3 pA.
Fig. 5 Spontaneous synaptic currents were completely blocked by bicuculline, a GABA<sub>A</sub> receptor antagonist. Samples of continuous recording of spontaneous PSCs recorded in control condition and in the presence of bicuculline methiodide (10 μM) in a CA1 cell at P 7. Bicuculline completely blocked spontaneous synaptic currents (holding potential -70 mV).
Fig. 6 Effect of TTX application on GABAergic PSC frequency. (A) Representative trace of spontaneous GABAergic PSCs before and after application of TTX (1μM) is shown at a slow timebase (CA3 cell at P6). Time interval distribution histograms of the spontaneous events are shown before (B) and after (C) addition of TTX. The distribution is fitted with a single exponential; time constant values (τ) are reported on the plots. Binwidth is 50 ms.
3.3 Miniature GABAergic events

Spontaneous GABA\textsubscript{A} receptor mediated miniature postsynaptic currents (mPSCs) were recorded in TTX solution in order to provide an estimate of the conductance change induced by one quantum of GABA during the first postnatal days. After addition of TTX miniature GABAergic currents were hardly detected in CA1 cells; when measurable, their frequency was really low (0.2 ± 0.05 Hz, n = 4). Therefore, miniature events were mainly obtained from CA3 pyramidal cells. As shown in Fig. 6 A (with a slow timebase), following TTX application, a clear decrease in frequency and amplitude of spontaneous currents occurred.

In two cells in which spontaneous activity was particularly high, TTX induced a shift of the baseline current (from 8 to 20 pA) in the outward direction. This was probably due to the block of inward currents induced by activity-dependent release of GABA from GABAergic interneurones. It is worth noting that such an effect resembled the one elicited by bicuculline application. The histograms B and C of Fig. 6 represent the time intervals of events recorded in the absence or in the presence of TTX. In TTX solution the time interval between events increased. Fast timebase samples of spontaneous GABAergic currents from the same cell before and after TTX application are shown in Fig. 7 A. The amplitude distribution histogram of GABA\textsubscript{A} receptor mediated miniature events was narrower, when compared with that of action potential dependent events (Fig. 7 B) and ranged from 6 to 60 pA (Hosokawa et al., 1994). The average modal peak was 16 ± 6 pA (n = 13). The largest events were blocked by TTX but the first peak of the amplitude histograms remained in the same
position or was shifted by only a few pA. Variability in the size of TTX-resistant currents was also observed. The variation in the current amplitude of mPSCs expressed as coefficient of variation \((CV = SD / \text{mean})\) after subtraction of the background noise component was \(0.3 \pm 0.09 (n = 13)\). In Fig. 8 plots relating the time course of frequency (A) and amplitude (B) of mPSCs calculated in 32 s-epochs for 45 min are shown. In 5 recordings of more than 45 min duration the mean amplitude of miniature currents remained stable suggesting that there was no run-down. Although electrotonic filtering of synaptic events originated along the dendrites at different distances from the soma was in general considered as a possible cause for such a variability in mPSCs amplitude, it did not seem to affect significantly the spontaneous currents recorded in the present study. In fact, no correlation between the peak amplitude of individual events and their rise or decay time constants \((r \leq 0.1, n = 5, \text{Fig. 9 B, C})\) was found. Similarly, the rise and decay time constants were found to be unrelated \((r \leq 0.1)\). In most cases, the decay phase of mPSCs was fitted with a single exponential having a time constant of \(29 \pm 4\) ms and the rise time was \(2.5 \pm 0.2\) ms \((n = 5, \text{see example in Fig. 9 A})\). These values did not differ from those observed in the absence of TTX. In order to exclude that the variability of mPSCs amplitude was due to the variability in the recording noise, analysis of the amplitude of baseline noise was performed during kynurenic acid and TTX application. Narrower amplitude histograms were found for background noise (see Fig. 10, left). In comparison with mPSCs, the amplitude histogram of background noise always followed a normal distribution. The standard deviation of mPSCs amplitude distribution varied between 2.5 and 8 pA whereas the standard deviation of the
background noise varied between 0.8 and 2.8 pA (n = 7). The amplitude of spontaneous miniature currents, rather than being distributed normally, was skewed towards that of the baseline noise.

Three experiments were performed in TTX and nominally Ca\(^{2+}\)-free solution, in order to test if miniature GABA\(_A\) receptor mediated events were, in some way, affected by extracellular Ca\(^{2+}\) concentration. Ca\(^{2+}\)-free media slightly affected their amplitude and frequency (Fig. 11 A, B) while elicited currents were completely blocked. In control conditions the mean amplitude of mPSCs recorded in TTX solution was 35 ± 5 pA whereas in Ca\(^{2+}\)-free solution was 29 ± 6 pA. Mean frequency of mPSCs was 1 ± 1.2 Hz in TTX solution and 0.8 ± 0.9 Hz after omitting Ca\(^{2+}\). The difference in frequency and amplitude was not significant.
Fig. 7 Effects of TTX application on GABAergic PSC amplitude. (A) Continuous recording of spontaneous GABA-mediated PSCs from the cell of Fig. 7 shown with higher gain and faster timebase. (B) Amplitude histograms of spontaneous GABA-mediated currents recorded in normal solution and in TTX solution. In each condition, spontaneous currents were measured during 160 s period. The cross-hatched columns represent the amplitude of the currents recorded in TTX. Binwidth is 3 pA.
Fig. 8 Frequency and amplitude time course of miniature PSCs. (A) Plot of the number of events versus time; each symbol represents the number of events detected in a 32-s interval. (B) Plot of mean amplitudes versus time; each symbol represents the mean amplitude of the events detected in a 32-s interval. CA3 cell at P9.
Fig. 9 Kinetics of GABAergic mPSCs. (A) Average of 20 GABA-mediated mPSCs recorded from a P6 CA3 cell at room temperature. The synaptic currents were normalized and aligned with their peaks. The continuous line superimposed on the decay time course of the averaged trace represents a single exponential (time constant = 30 msec). Amplitude is plotted against the rise time (10-90 %, B) and the decay time (C) derived from the fitting analysis (r=0.1). The average amplitude was 41 pA; the average rise time was 2.2 ms and the average decay time was 30 ms.
Fig. 10 Standard deviation (SD) of amplitude distribution histogram of GABAergic mPSCs is larger than SD of background noise. Amplitude histogram of mPSCs recorded from a CA3 cell at P10. On the left, amplitude distribution of background noise is shown (binwidth = 0.5 pA, SD = 0.8). On the right amplitude distribution of mPSCs is plotted (binwidth = 3 pA, SD = 7.2).
A

Control

Ca$^{2+}$-free

wash

for figure legend see opposite page
Fig. 11 Frequency and amplitude of miniature GABAergic events are slightly affected by Ca\textsuperscript{2+}-free solutions. (A) Representative recording traces of mPSCs are shown at -70 mV before, during and after application of nominally Ca\textsuperscript{2+}-free solution. (B) Amplitude histograms of mPSCs recorded during 448 s period. CA3 cell at P8, binwidth = 3 pA.
3.4 Stimulus-elicited GABAergic currents

GABAergic currents were elicited in pyramidal cells at P8 in the presence of kynurenic acid by focal stimulation once every 10 s (Fig. 12). The stimulating pipette was located about 100 μm from the recording pipette. The location of the pipette was critical for evoking a postsynaptic current. When the stimulating electrode was moved away, no response was observed. Like the spontaneously occurring PSCs, stimulus-elicited currents, in the presence of kynurenic acid, were completely blocked by bicuculline methiodide (10 μM) and reversed around 0 mV. When TTX was added, elicited currents were not observed. At the frequency used, no appreciable fatigue was observed. The amplitude of elicited events was found to increase by increasing the stimulus strength, which implied recruitment of additional fibers of GABAergic interneurones. To activate a small number of fibers the intensity of stimulation was adjusted in order to get a relative highly percentage of failures (10-30 %). The mean amplitude of elicited currents ranged from 6 to 80 pA with a mean of 40 ± 10 pA (n = 6); a modal peak was found of 14 ± 6 pA (Fig. 12). Since the latency of the response (interval between the stimulus artefact and the onset of the response) was constant and was less than 3 ms (2 ± 0.5 ms), and the mean rise time was 3.2 ± 0.6 ms (n = 4), it is suggested that elicited GABAergic currents were mono-synaptic.
Fig. 12 Stimulus-evoked GABAergic currents, induced by low intensity stimulation, are in the same range of amplitudes as mPSCs. In the upper panel representative stimulus-evoked currents are shown at -70 mV recorded from a CA3 at P9 (stimulating electrode was in the stratum oriens of the CA3 region). In the lower panel amplitude distribution histogram of evoked currents from the same cell is shown. Binwidth = 3 pA.
3.5 Single channel events

GABA-elicited single channels events were detected with the outside-out configuration of isolated patch recording from acutely dissociated CA3 hippocampal cells at P3 (n = 3, see Fig. 13 A). In the absence of the agonist no single channel openings were detected. Application of GABA (50 µM, at a holding potential of -60 mV) activated single channel currents which were analysed at different holding potentials. Since GABA was continuously superfused via the bathing solution and single channels were detected under steady-state condition, most channels were presumably in the desensitized state. A linear I-V relationship was found in the range of -50 / 50 mV with current inversion close to 0 mV (Fig. 13 B), as expected for a Cl⁻ mediated event in symmetrical Cl⁻ solutions. From the slope of the I-V relationships (based on measurements of approximately 4,000 channel openings) two main single channel conductances were obtained (24 ± 3.4 and 35 ± 3.6 pS). The most frequently occurring conductance state was 24 pS. Since channel current levels were not incrementing as multiples of the smallest level, the most likely interpretation for distinct channel activity is the existence of two open states, each one with a characteristic conductance (Hawkes and Colquhon, 1995). An alternative possibility is that a same GABA activated channel possesses subconductance states although no obvious transitions were found. A representative amplitude histogram of single channel amplitudes is shown in C. The mean open time of GABA activated channels measured at -50 mV was 0.8 ± 0.04 ms. In agreement with previous studies (Weiss, 1988), the open probability (Np) was voltage-dependent, varying from 0.025 ± 0.005 at -60 mV to 0.06 ± 0.03 at -30 mV. The low Np reflects the possible desensitization
state of GABA receptor channels. Np was calculated as the ratio between the sum of all open times and the total observation time.
Fig. 13 GABA-evoked single-channel currents in outside-out patches from CA3 hippocampal neurones. (A) Example of activity induced by GABA (50 μM) at different membrane potentials. Note that in these tracings only one channel conductance (36 pS) is apparent. Band width 1.5 kHz. (B) Current-voltage relationship of single-channel events recorded for 80 s during which two distinct channel activities were detected. Current values were fitted by least-squares linear regressions, yielding slope conductances of 36 pS (circles) and 22 pS (triangles) marked on the right of the panel as \( g_1 \) and \( g_2 \). (C) Amplitude histogram of GABA-activated single-channel currents at a holding potential of -60 mV. Lines represent Gaussians fitted separately for background noise (close state), 22 and 36 pS channels; binwidth = 0.25. Notice that the 22 pS conductance state is the most frequent. All data are from the same patch recording.
Modulation of GABAergic activity

3.6 Modulation of spontaneous GABAergic activity by mGluR activation

Modulation of spontaneous GABAergic PSCs by mGluR activation was tested by superfusing cells with trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) which acts as a mixed agonist on mGluR subtypes. As shown in the example of Fig. 14 A, superfusion with t-ACPD (3-10 μM) for 10-15 min markedly enhanced the frequency of GABA\textsubscript{A} receptor mediated spontaneous events in all neurones tested (n = 30). The effect of t-ACPD began 1-1.5 min after the onset of drug application and reached a steady-state value after 3-4 min; no significant changes were observed in membrane input resistance or baseline membrane current during and immediately after t-ACPD application. When t-ACPD was applied for 2 min, a full recovery was obtained 10 min after wash out. Figure 14 B shows that the mean interval between spontaneous events decreased significantly (P < 0.05) during t-ACPD application (the frequency increased from 2.7 Hz in control solution to 6.6 Hz in 3 μM t-ACPD). The increased frequency of spontaneous synaptic currents by t-ACPD (10 μM) was measured at steady-state level. In a sample of 10 neurones mean frequency ratio (t-ACPD over control) was 2.8 ± 0.6.

Similar values of rise or decay time of spontaneous GABAergic currents were observed before and during t-ACPD application (values of rise or decay time were 2.3
± 0.1 ms, or 32 ± 4 ms in control conditions and 2.3 ± 0.1 or 32 ± 1.7 ms in the presence of t-ACPD, respectively, when measured in detail in 4 cells.

The effect of t-ACPD on the amplitude of spontaneous GABAergic currents was rather variable. In ten neurones no change in amplitude distribution of spontaneous PSCs was found (see Fig.15 A and B) since the mean amplitude ratio of spontaneous events recorded in t-ACPD (10 μM) over control was close to 1 (1.1 ± 0.4). In two cases increases in amplitude of GABA\textsubscript{A} receptor mediated PSCs were observed after t-ACPD application (mean amplitude ratios were 1.2 and 1.3). This suggests that in these two cells a quiescent set of GABAergic interneurones has been recruited.

Four cells were patched with pipettes containing the calcium chelator BAPTA in order to block the postsynaptic effect of mGluRs on Ca\textsuperscript{2+}-dependent potassium currents (Charpak et al., 1990). Also in these cases, t-ACPD increased the mean frequency of spontaneous currents (from 1.7 ± 0.4 to 3.8 ± 0.8 Hz). The mean frequency ratio (t-ACPD over control) was 2.2 ± 0.2. The effect of t-ACPD on the frequency of spontaneous GABAergic currents was concentration dependent in the range of 3-30 μM (Fig. 14 C). As shown in this Figure, the dose-response curve was quite steep, the maximal effect being already present at a concentration of 10 μM t-ACPD. Higher ( > 30 μM) doses of t-ACPD failed to produce further increases in current frequency probably because they largely depolarized GABAergic interneurones beyond the activation threshold of voltage-dependent Na\textsuperscript{+} currents (Miles and Poncer, 1993). In three cells the competitive mGluR antagonist MCPG (Bashir et al., 1993) was tested. Superfusion of MCPG (bath-applied at a concentration of 1 mM for 10 min) did not affect spontaneous activity but partially
prevented the effect of 10 μM \(\alpha\)-ACPD (Fig. 14 D). In the presence of MCPG, the potentiating effect of \(\alpha\)-ACPD on spontaneous GABA\(\text{A}\) receptor mediated currents was reduced by 65 ± 15% (n = 3). A partial recovery was obtained 10 min after washout. To exclude a possible postsynaptic action of \(\alpha\)-ACPD on GABA receptors, on four acutely dissociated pyramidal cells this compound was tested in whole-cell configuration on the currents activated by exogenous application of GABA in the presence of TTX (see example in Fig. 16). Superfusion of GABA (100 μM) for 20 s at -70 mV induced inward currents that after an initial peak declined to a steady state value in the continuous presence of the agonist. The peak amplitude of GABA-elicited current was 616 ± 202 pA (n = 4). No significant changes in peak amplitude were observed in \(\alpha\)-ACPD solution (10 μM, 603 ± 160 pA).

### 3.7 Modulation of elicited GABAergic activity by mGluR activation

Electrically elicited GABA\(\text{A}\) receptor mediated currents were induced in pyramidal cells (at -70 mV) by stimulating the st. radiatum or st. oriens in the presence of kynurenic acid. Stimulation in the pyramidal layer was often unsuccessful. Stimulus intensity was adjusted to give sufficiently large GABA\(\text{A}\) receptor mediated response. The amplitude of elicited currents ranged from 20 to 60 pA. In the same cell in which \(\alpha\)-ACPD (10 μM) induced an increase in frequency (at least 2-fold, Fig. 17 A) of spontaneous GABAergic PSCs, a clear decrease in the average elicited currents was observed (Fig. 17 B) without change in cell input resistance. In the presence of \(\alpha\)-
ACPD the mean amplitude ratio (t-ACPD over control and excluding failures) was 0.5 ± 0.1. (n = 4); in t-ACPD solution the number of failures increased from 33 to 58%. A partial recovery was obtained more than 20 min after t-ACPD washout (Fig. 17 B).
for figure legend see opposite page
Fig. 14 t-ACPD enhances spontaneous GABA_A receptor mediated synaptic activity in neonatal CA1 pyramidal cell (P8) and is blocked by the mGluR antagonist MCPG. (A) Samples of continuous recordings of spontaneous GABAergic currents in control and during bath application of t-ACPD (3 μM). (B) Interval distribution histogram of the currents shown in A before and after addition of t-ACPD. The distribution was fitted with a single exponential; time constant values (τ) are reported in the plots. The holding potential was set at -70 mV; binwidth was 50 ms. (C) Plot relating the frequency of GABAergic events to different t-ACPD concentrations. Each symbol represents the mean of three experiments. Bars represent the standard error of the mean. (D) Plot of the frequency of spontaneous GABAergic currents, calculated for 16-s epochs (o) as a function of time. t-ACPD (10 μM) application is represented by horizontal bars. Bath application of MCPG (1 mM, open bar), drastically reduced the potentiating effect of t-ACPD. A partial wash was obtained after return to control solution.
**Fig. 15** 
$i$-ACPD increases the frequency but not the amplitude of spontaneous GABAergic events.

Amplitude distribution histograms of spontaneous GABA-mediated currents recorded during 210 s period before (A) and after (B) $i$-ACPD application. CA3 cells at P9, binwidth = 3 pA
Fig. 16 Currents induced by exogenously applied GABA are not affected by t-ACPD application. Currents induced by pressure ejection of exogenous GABA (50 μM, 20 s) before, during and after t-ACPD (10 μM) application in the presence of TTX. Acutely dissociated pyramidal cell at P4. GABA application is represented by horizontal bars.
Fig 17 t-ACPD depresses stimulus-evoked GABAergic currents recorded from pyramidal cells. (A) Samples of continuous recording of spontaneous GABA-mediated PSCs before, during and after application of t-ACPD (10 μM). Upward deflections correspond to electric stimuli applied at 0.1 Hz with an electrode placed in the stratum oriens 100 μm from the recorded CA3 pyramidal cell (P8). t-ACPD induced a clear increase in the frequency of spontaneous PSCs. (B) Each current is the average of 20 consecutive responses obtained before, during and after t-ACPD (10 μM). Failures were not counted.
3.8 Enhancement of spontaneous GABAergic activity by mGluR involves cAMP-dependent PKA

Among the cloned mGluRs, it was noticed that mGluR1 is positively coupled with cAMP (Aramori and Nakanishi, 1992). Because in neonatal rat hippocampus activation of mGluRs stimulates cAMP synthesis (Schoepp and Johnson, 1993), the following experiments were undertaken to explore the possibility that cAMP, as a second messenger in the intracellular signal transduction pathways, could be responsible for the enhancement of GABA release by mGluRs activation.

Cyclic AMP is formed from ATP by the action of adenylyl cyclase, an integral membrane protein that requires Mg\(^{2+}\) for its action.

\[
\text{ATP} + \text{Mg}^{2+} \xrightarrow{\text{cyclic AMP phosphodiesterase}} \text{cyclic AMP + PP}_i + \text{H}^+
\]

Cyclic AMP is degraded by a specific phosphodiesterase, which hydrolyzes it to AMP.

\[
\text{Cyclic AMP + H}_2\text{O} \xrightarrow{\text{PP}_i} \text{AMP + H}^+ + \text{Mg}^{2+}
\]

In order to activate membrane adenylyl cyclase, the diterpene forskolin (Seamon et al., 1983) was perfused via the bathing solution at a concentration of 30 \(\mu\)M. This compound markedly increased the frequency of spontaneous activity from 1.9 \(\pm\) 0.8 to 3.4 \(\pm\) 1.2 Hz (Fig. 18 A and B); no clear change in amplitude was observed. In ten cells the mean frequency ratio (forskolin over control) was 1.8 \(\pm\) 0.2, whereas the mean amplitude ratio was 1.0 \(\pm\) 0.1. No further increase in frequency was observed with a higher (50 \(\mu\)M) concentration of forskolin.
Fig. 18 Forskolin or IBMX enhance the frequency of spontaneous GABAergic events. Samples of continuous recordings of spontaneous GABAergic currents before and after superfusion of forskolin (30 μM, A) or IBMX (200 μM, C). (B) and (D) Cumulative distributions of inter-event intervals of spontaneous GABAergic events (shown in A and C) before and after application of forskolin or IBMX. The sampling time was the same for both experimental conditions (180 s).
Unlike forskolin, 1,9-dideoxyforskolin, which is an analogue inactive on adenylyl cyclase (Hoshi et al., 1988; Seamon and Daly, 1986), had no effect on the frequency of spontaneous GABAergic events (mean amplitude ratio was 0.9 ± 0.2). The effects of IBMX, which inhibits phosphodiesterase activity and increases the endogenous levels of cAMP (Butcher and Sutherland, 1962) were also tested. As shown in Fig. 18, C and D, IBMX (200 μM) consistently increased the frequency of spontaneous GABAergic currents (from 1.4 ± 0.5 to 2.7 ± 1.3 Hz); no changes were observed in the mean amplitude of spontaneous GABA_A receptor mediated events before and after IBMX application. In four cells the mean frequency ratio (IBMX over control) was 1.8 ± 0.2, whereas the mean amplitude ratio was 1.0 ± 0.1. In Fig. 19 B a summary of the effects of t-ACPD (10 μM), forskolin, or IBMX on the amplitude and frequency of spontaneous GABAergic currents is presented. All these drugs significantly increased the frequency but not the amplitude of spontaneous synaptic events, a phenomenon consistent with a presynaptic site of action. Moreover, no changes were observed in baseline membrane current and leak conductance during and after their application. To exclude a possible postsynaptic action of forskolin on GABA_A receptor mediated spontaneous PSCs, in four cells this compound was also tested on the currents activated at -70 mV by exogenous application of GABA (100 μM) in the presence of TTX. Forskolin (30 μM) did not modify (n = 2) or slightly reduced (15 and 23 %, respectively) the peak amplitude of GABA currents (Fig. 19 A).
Fig. 19 Effect of forskolin on GABA-mediated responses and its comparison with other pharmacological treatments. (A) Currents induced by pressure ejection of GABA (100 μM, 20 s) in the presence of TTX, before and after addition of forskolin. GABA application is represented by horizontal bars. (B) Pooled data of the effects of t-ACPD (10 μM, n = 10), forskolin (30 μM, n = 7) and IBMX (200 μM, n = 4) on the frequency and amplitude of spontaneous PSCs. Each column in the bar chart represents the mean ratio (test over control) of event frequency and amplitude. Bars represent SD. *P < 0.001 versus control when statistical significance was assessed with Student t-test applied to raw data.
Because the effect of forskolin and IBMX on spontaneous GABAergic currents were similar to that of t-ACPD, subsequent experiments examined whether the potentiation of GABAergic events by t-ACPD involved the activation of cAMP-dependent protein kinases. Most of the effects of cAMP results from the activation of PKA. Protein kinase consists of two subunits: a regulatory subunit (R), which can bind cAMP, and a catalytic subunit (C). In the absence of cAMP, the R and C subunits form a tetrameric complex (R\textsubscript{2}C\textsubscript{2}) that is enzymatically inactive. cAMP acts as an allosteric effector; the binding of two molecules of cAMP to each of the R subunits leads to a change in the conformation of R subunits and to its dissociation from the R\textsubscript{2}C\textsubscript{2} complex. The free catalytic subunits are then enzymatically active and free to phosphorylate specific proteins to allow a functional response (Kandel et al., 1991).

In order to see if PKA was involved in the modulatory effect of t-ACPD, we used Rp-adenosine 3', 5'-cyclic monophosphothioate triethylamine (Rp-cAMPS), a cell permeant competitive antagonist at the cAMP binding site of PKA (Wang et al., 1991). The experiments were performed according to the protocol shown in Fig. 20 C. Representative examples are shown in Fig. 20 A and B. In Fig. 20 B the effects of t-ACPD (10 µM) on spontaneous GABAergic currents are shown in control condition (a), in the presence of Rp-cAMPS (b) and after Rp-cAMPS wash out (c). In the presence of Rp-cAMPS, the effects of t-ACPD were clearly depressed. After 10 min of Rp-cAMPS wash out, a partial recovery was obtained and t-ACPD, superfused for the third time, increased the frequency of spontaneous events.
Fig. 20 Rp-cAMPS prevents the potentiating effect of mGluR activation. (A) Samples of continuous recording of spontaneous GABAergic currents in control (a) and in the presence of Rp-cAMPS (b, 30 μM). Rp-cAMPS clearly reduces the frequency of spontaneous PSCs. (B) Representative traces of spontaneous GABA_A receptor mediated currents in the presence of t-ACPD (10 μM) before (a), during (b) and after (c) application of Rp-cAMPS. (C) Plot of the frequency of spontaneous GABAergic currents vs. time before, during and after bath application of Rp-cAMPS (30 μM, open bar). Each symbol represents the mean frequency of events detected in a 16-s epoch. t-ACPD application (10 μM) is represented by closed bars. (D) Pooled data of the effects of t-ACPD and t-ACPD plus Rp-cAMPS on the frequency of spontaneous GABAergic events. Data are expressed as frequency ratio of t-ACPD over control (left column) and t-ACPD plus Rp-cAMPS over Rp-cAMPS alone (right column). Bars represent SD. Data are from 5 cells.
In five experiments the mean frequency ratio of \( t\)-ACPD (10 \( \mu \)M) over control and \( t\)-ACPD plus Rp-cAMPS over Rp-cAMPS alone was 3.2 ± 0.7 and 1.3 ± 0.5, respectively (Fig. 20 D). It is interesting to note that in the presence of Rp-cAMPS, superfused at the concentration of 30 \( \mu \)M for about 10 min (see Goh and Ballyk, 1993), the frequency of spontaneous GABAergic currents was reduced from 1.93 ± 0.2 to 1.36 ± 0.6 Hz (\( n = 4 \), see Fig. 20 A).

Table II. Effects of \( t\)-ACPD, forskolin, IBMX and Rp-cAMPS on GABAergic spontaneous currents in CA1 and CA3 pyramidal cells.

<table>
<thead>
<tr>
<th></th>
<th>Mean frequency ratio</th>
<th>Mean amplitude ratio</th>
</tr>
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<tbody>
<tr>
<td>( t)-ACPD (10 ( \mu )M) / control</td>
<td>2.8 ± 0.6 (( n = 10 ))</td>
<td>1.1 ± 0.4 (( n = 10 ))</td>
</tr>
<tr>
<td>Forskolin (30 ( \mu )M) / control</td>
<td>1.8 ± 0.2 (( n = 10 ))</td>
<td>1.0 ± 0.1 (( n = 10 ))</td>
</tr>
<tr>
<td>IBMX (200 ( \mu )M) / control</td>
<td>1.8 ± 0.2 (( n = 4 ))</td>
<td>1.0 ± 0.1 (( n = 4 ))</td>
</tr>
<tr>
<td>( t)-ACPD + Rp-cAMPS (30 ( \mu )M) / Rp-cAMPS (30 ( \mu )M)</td>
<td>1.3 ± 0.5 (( n = 5 ))</td>
<td>-</td>
</tr>
<tr>
<td>Rp-cAMPS (30 ( \mu )M) / control</td>
<td>0.7 ± 0.2 (( n = 5 ))</td>
<td>-</td>
</tr>
</tbody>
</table>

\( n = \) number of cells
3.9 mGluR activation does not potentiate miniature events

frequency

In order to see whether activation of mGluRs by t-ACPD affected also miniature GABAergic events, the mGluR agonist t-ACPD (10-20 μM) was also tested in the presence of kynurenic acid and TTX. Samples of miniature events before, during and after bath application of t-ACPD (20 μM) are shown in Fig. 21 A. Even if applied for brief (5 min) or long periods (10 min), did not significantly (P > 0.1) modify the frequency (Fig. 21 B) and amplitude (Fig. 21 C) of miniature events. In three CA1 cells, the mean frequency and amplitude ratio (t-ACPD over control), were 0.7 ± 0.3 and 0.9 ± 0.1, respectively; in four CA3 cells the mean frequency and amplitude ratio were 1 ± 0.2 and 1 ± 0.4, respectively.
Control (TTX)

\[ f-ACPD \ (20 \ \mu M) \]

wash

for figure legend see opposite page
Fig. 21 Miniature GABA-mediated currents are not affected by $t$-ACP D. (A) Samples of continuous recording of mPSCs before, during and after application of $t$-ACP D (20 $\mu$M) to the same neurone. (B) Plot of the number of mPSCs versus time before, during and after application of $t$-ACP D. Each symbol represents the number of events detected in a 64-s time interval. (C) Plot of the mean amplitude of mPSCs versus time before, during and after application of $t$-ACP D. Each symbol represents the mean of the mPSCs amplitude detected in a 64-s time interval. Drug application is represented by horizontal bars.
3.10 Other neurotransmitters can positively modulate spontaneous GABA-mediated currents

In order to test whether activation of cholinergic and noradrenergic receptors may regulate GABA release, the effect of carbachol or noradrenaline (NA) on spontaneous GABA<sub>A</sub>-receptor mediated activity was tested. Carbachol and NA were superfused at a concentration of 10 and 20 µM, respectively (Fig. 22). Both drugs increased the frequency of spontaneous GABA<sub>A</sub> receptor mediated currents, without any apparent effects on their amplitude. Mean frequency and amplitude ratio for carbachol were 1.7 ± 0.2 and 1.1 ± 0.2, respectively (n = 3); mean frequency and amplitude ratio for noradrenaline were 2.1 ± 0.2 and 1 ± 0.1, respectively (n = 3).

3.11 Miniature GABA-mediated currents are potentiated by PKA

Phosphorylation of ion channels and/or receptor proteins by a cAMP-dependent protein kinase plays an important role in regulation of neuronal function (Nestler and Greengard, 1983). Protein kinases localized on presynaptic sites modulate transmitter release (Greengard et al., 1993). Thus, an increase in transmitter release by a direct activation of adenylyl cyclase is known to be involved in synaptic plasticity processes such as long-term facilitation at the crustacean neuromuscular junction (Dixon and Atwood, 1989) or LTP at the mossy fiber-CA3 synapse in the hippocampus (Weisskopf et al., 1994). Activation of PKA induced a potentiation of spontaneous
and elicited release of glutamate at excitatory synapses in the hippocampus (Chavez-Noriega and Stevens, 1994).

In the present study it was observed that in immature rat CA3 hippocampal neurones, the adenylyl cyclase activator forskolin greatly enhanced the spontaneous release of GABA from GABAergic interneurones. Miniature GABAergic currents, obtained from CA3 pyramidal neurones, were recorded at -70 mV, in TTX (1 μM) and kynurenic acid (1mM) solution. Continuous superfusion with forskolin (30 μM, for 3-8 min) induced in all neurones tested (n = 7) a marked increase in the frequency, but not in the amplitude of mPSCs, suggesting a presynaptic site of action (Fig. 23 A and B). This effect started within 2-3 min from the onset of forskolin superfusion and lasted for 30-40 min after the drug was washed out. A recovery was never obtained (Fig. 23 C). In four cells the mean amplitude ratio of mPSCs in forskolin (30 μM) over that in its absence was 1.2 ± 0.2. On the contrary, the mean frequency ratio (forkolin over control) was 2 ± 0.5 (Fig. 23 D). No changes in baseline membrane current or leak conductance were observed during forskolin application. The kinetics of the synaptic currents were the same before and after forskolin application (values of rise and decay times were 2.3 ± 0.1 ms and 32 ± 4.0 ms in control condition and 2.4 ± 0.2 and 32 ± 1.8 ms in the presence of forskolin, respectively). In contrast to forskolin, its isomer, 1,9-dideoxyforskolin (30 μM), which does not activate adenylyl cyclase, had no effect or slightly reduced the frequency of mPSCs (Fig. 24 A). The time course of the effects of 1,9-dideoxyforskolin and forskolin in one representative experiment is shown in Fig. 24 B. The mean frequency ratio (1,9-dideoxyforskolin over control) was 0.8 ± 0.06 (n = 3). In order to examine whether the effects of forskolin were mediated via PKA, Rp-cAMPS was used. In three cells, Rp-cAMPS
(30 μM), applied for 10 min, did not change the amplitude or the frequency of mPSCs (Fig. 25 A). Concomitant application of forskolin (30 μM) only slightly, but not significantly (P > 0.5), increased the frequency of mPSCs (the mean frequency ratio of Rp-cAMPS plus forskolin over forskolin was 1.3 ± 0.1). A summary bar chart of the effects of forskolin, dideoxyforskolin and Rp-cAMPS + forskolin is shown in Fig. 25 B.

**Table III.** Effects of t-ACPD, forskolin, 1,9-dideoxyforskolin and Rp-cAMPS on miniature GABAergic currents recorded in CA3 pyramidal cells.

<table>
<thead>
<tr>
<th></th>
<th>Mean frequency ratio</th>
<th>Mean amplitude ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>t</em>-ACPD (20 μM)</td>
<td>1 ± 0.2 (n = 4)</td>
<td>1 ± 0.4 (n = 4)</td>
</tr>
<tr>
<td>Forskolin (30 μM)</td>
<td>2 ± 0.5 (n = 4)</td>
<td>1.2 ± 0.2 (n = 4)</td>
</tr>
<tr>
<td>1,9-dideoxyforskolin</td>
<td>0.8 ± 0.06 (n = 3)</td>
<td></td>
</tr>
<tr>
<td>Rp-cAMPS+forskolin / forskolin</td>
<td>1.3 ± 0.1 (n = 3)</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 22 Carbachol and noradrenaline potentiate GABAergic activity. (A) Samples of continuous recording of spontaneous GABAergic PSCs are shown before and after 10 μM carbachol application to a CA1 cell (P8). (B) Samples of continuous recording of spontaneous GABAergic PSCs are shown before and after 20 μM noradrenaline application to a CA1 cell (P10).
A - Control

B - Forskolin

C and D: Graphs showing changes in frequency and amplitude over time. For figure legend see opposite page.
Fig. 23 forskolin enhanced the frequency of GABA-mediated mPSCs. (A) Samples of continuous recordings of mPSCs before and after application of forskolin (30 μM). (B) Plot of the frequency of mPSCs versus time before and after application of forskolin. Each symbol represents the frequency of events detected in a 16-s epoch. (C) Pooled data of the effects of forskolin on the frequency and amplitude of mPSCs. Each column in the bar chart represents the mean ratio (test over control) of event frequency and amplitude. Bars represent the SD. The number of cells studied was 7. *P < 0.001 versus control when statistical significance was assessed with Student t-test applied to raw data.
Fig. 24 Forskolin but not 1,9-dideoxyforskolin potentiated the frequency of mIPSCs. (A) Samples of continuous recordings of mIPSCs before and after application on the same neuron. of 1,9-dideoxyforskolin (30 μM) or forskolin (30 μM). (B) Plot of the frequency of mPSCs versus time before, during and after application of 1,9-dideoxyforskolin (30 μM) and forskolin (30 μM). Each symbol represents the frequency of events detected in a 16 s time interval. Drug application is represented by horizontal bars.
Fig. 25 Rp-cAMPS prevents the effects of forskolin on mPSCs. (A) Samples of continuous recording of mPSCs in the presence of Rp-cAMPS (30 μM) and Rp-cAMPS plus forskolin (30 μM). (B) Each column in the histogram represents the mean ratio (test over control) of event frequency of mPSCs in different experimental conditions. Bars represent the SD. The number of cells studied was 7 in forskolin, 3 in 1,9-dideoxyforskolin and 3 in forskolin + Rp-cAMPS. *P < 0.01 versus control when statistical significance was assessed with Student t-test applied to raw data.
4.1 Whole cell versus intracellular recordings: advantages and disadvantages

The advantage of using the patch-clamp technique, when compared with conventional sharp electrode recording, is the high signal-to-noise ratio. This is particularly due to the low pipette resistance which reduces the series resistance (pipette resistance plus access resistance) between the amplifier and the cell. This is important when recording small synaptic currents as miniatures which, with sharp electrode recordings, are often unresolved from background noise. The major limitation of the patch-clamp technique can be the dialysis of the cell cytoplasm which can induce a “rundown” of the recorded signals due to a loss of molecules involved in signal-transduction pathways. To prevent washout of cytosolic constituents the perforated patch technique has been introduced by Horn and Marty (1988). This technique consists of adding to the pipette solution substances (such as nystatin) that form channels which allow passage of small ions only. In the present experiments, this technique was not used because it requires a long time to obtain low recording resistance and traces often remain noisy. In most experiments of the present study, when ATP was present in the recording pipette, no rundown of GABA-mediated currents was observed within the first 45 min of recording.
Another requirement of patch-clamp recording is to maintain a stable series resistance as the latter can change during the course of the experiment introducing errors of space clamp and resulting in alterations in apparent membrane potential and in time course of single events. When a change in series resistance was observed, a gentle positive pressure was applied in order to clear the pipette tip. Sometimes there was a drastic change in series resistance value due to a complete resealing of the membrane. In these cases the experiments had to be discarded.

4.2 Mean electrophysiological properties of spontaneous GABA-mediated events

In accordance with previous results with sharp microelectrodes (Hosokawa et al., 1994), GABA was found to be the main neurotransmitter on hippocampal pyramidal cells of neonatal animals: spontaneous glutamatergic events on these neurones were not detected until P12. Spontaneous synaptic currents were mediated by GABA, acting on GABA_A receptors since they were blocked by bicuculline, a specific GABA_A receptor antagonist. GABA_A-mediated currents were carried by Cl^- as indicated by their reversal potential close to the predicted equilibrium potential for this ion. They were also blocked by the channel blockers picrotoxin or penicillin G (M. Atzori, personal communication). Moreover, glycine, which is known to permeate Cl^- channels and mediate inhibitory synaptic transmission in other brain areas (Kelly and Krnjevic, 1968), is not involved in spontaneous neurotransmission in the neonatal hippocampus (see also Edwards et al., 1990).
Patch-clamp conditions (whole-cell configuration) which use an artificial pipette solution, often interfere with the reversal potential of Cl\(^-\) and, thus, with the physiological direction of Cl\(^-\) flux. In our recording conditions, with symmetrical Cl\(^-\) solutions at -70 mV, the Cl\(^-\) flux was outwardly directed and thus generated a current which would depolarize the cell in current clamp mode. In hippocampal slices from neonatal rats, using conventional microelectrodes filled with K-methylsulphate, GABA is actually found to depolarize and excite pyramidal cells through an outward flux of Cl\(^-\) (Ben Ari et al., 1989; Cherubini et al., 1991).

Spontaneous GABAergic PSCs were found to occur randomly in the presence of kynurenic acid which blocked the excitatory drive to GABAergic interneurones. How can GABAergic interneurones be spontaneously active in the presence of kynurenic acid? One possibility could be the existence of a population of hippocampal interneurones with endogenous pacemaker properties. In stratum oriens interneurones are found to possess an I\(_h\) current which could generate pacemaker activity (Maccaferri and McBain, 1996). It seems unlikely that pacemaker neurones were localized to the hilus since surgical separation of the pyramidal layers from the hilus does not prevent spontaneous GABAergic activity (Strata et al., 1995). Another possibility is that a network of interneurones relied on GABA as the excitatory signal to support intercellular signalling within the same circuit. Spontaneous activity would then depend on the wiring properties of the network rather than on some specialized neurones. In accordance with this notion, GABA synchronizes the firing of an in vitro population of hilar interneurones in the absence of fast glutamatergic transmission following application of 4AP (Michelson and Wong, 1991). Furthermore GABA\(_A\) receptor-mediated synaptic transmission can synchronize a sparsely connected
network of fast-spiking interneurones as indicated by electrophysiological and modelling investigation (Wang and Buzsaki, 1996). However, GABA is not the exclusive agent to synchronize the network since in the presence of the GABA antagonist picrotoxin, a subset of GABAergic interneurones retain spontaneously active firing (Michelson and Wong, 1994) possibly due to their electrical coupling.

**Analysis of frequency of spontaneous GABAergic currents**

In the absence of TTX spontaneous GABAergic currents occurred randomly. The frequency distribution histogram could be well fitted with the theoretical curve expected for random occurrence given by the equation \( n = \frac{N}{T} \exp\left(-\frac{t}{T}\right) \), where \( N \) is the total number of events observed, \( T \) is the mean interval between events and \( n \) is the expected number of events occurring at interval \( t \) (Fatt and Katz, 1952).

**Analysis of amplitude of spontaneous GABAergic currents**

Amplitude distribution histograms were found to be skewed towards lower values as reported previously for EPSCs (McBain and Dingledine, 1992) and IPSPs of CA3 pyramidal neurones (Miles, 1990). In most of the present experiments only one clear modal peak was identified. This single peak persisted after TTX application suggesting that it corresponded to the current underlying one single quantum. The present findings accord with other studies in which plots of spontaneous GABAergic activity never displayed multiple peaks (Miles, 1990; Randall and Collingridge, 1992; Borst et al., 1994) since a large number of GABAergic interneurones with different release probability project to a single pyramidal cell, thus blurring differences between release sites. Conversely, multiple peaks in amplitude histograms of elicited synaptic events can be present and are the result of statistical superposition of quantal events of rather uniform size (for a review see Redman, 1990). Numerous statistical models
have been proposed to describe transmitter release at central synapses after stimulation of presynaptic fibers (Walmsley et al., 1988; Redman, 1990; Korn and Faber, 1991). In the present experiments amplitude histograms of elicited GABAergic currents did not show multiple peaks. Due to the absence of multiple peaks, a release mechanism based on the properties of binomial or Poisson statistics could not be applied to interpret the data relating to evoked currents presented in this thesis. In agreement with data obtained at the neuromuscular junction (del Castillo and Katz, 1954) amplitude histograms of elicited EPSCs at the mossy fiber-CA3 synapse present equidistant peaks although this is by no means a constant phenomenon since in 4 of 9 cells a single broad peak is reported (Jonas et al., 1993). Amplitude distribution histograms of elicited inhibitory currents recorded from granule cells of dentate gyrus can be fitted with multimodal functions whereas only one modal peak (18 pA) is found when spontaneous synaptic currents are measured (Edwards et al., 1990). It cannot be excluded that for the evoked GABAergic currents described in the present thesis the difficulty of finding multiple peaks might be due to the limited number of events detected in these experiments while maintaining stable recording conditions.

4.3 Miniature GABA-mediated events

Miniature GABA-mediated currents can be resolved with the patch-clamp technique in the presence of TTX (Edwards et al., 1990; Otis et al., 1991). In the present study these events were distinguishable from background noise, the amplitude distribution of which was always fitted with a Gaussian having SD lower than the one found for
the recorded events. SD of noise amplitude was found to range from 0.8 to 2.8 pA (in the same range found by Edwards et al. 1990, 1.9-3.2 pA). TTX application, at a concentration of 1 μM, blocks all action potential driven events and reduces amplitude and frequency of spontaneous postsynaptic currents (Hosokawa et al., 1994). In those cells in which spontaneous activity was particularly high, TTX acting presynaptically to block fast sodium spikes, induced a shift of the baseline current in the outward direction. This was probably due to the block of inward current induced by activity-dependent release of GABA from GABAergic interneurones. Consistent with previous reports on either mEPSC (Scholz and Miller, 1992; Thompson et al., 1993) or mIPSCs (Otis and Mody, 1992; Thompson et al., 1993), it was found that the amplitude and frequency of miniature GABA-mediated events were not significantly affected by extracellular calcium as indicated by the experiments performed in a nominally Ca\(^2+\)-free solution. Resting Ca\(^2+\) level in interneurones or in their terminals can be sufficient to induce spontaneous GABA release. Nominally Ca\(^2+\)-free solutions cannot reduce the intracellular free Ca\(^2+\) concentration (Otis and Mody, 1992).

Miniature GABA-mediated postsynaptic currents were found to have an exponential decay. Time course of GABAergic current decay is due to: i. the closure of ion channels opened as a consequence of postsynaptic receptor occupancy by the synaptic transmitter (Magleby and Stevens, 1972), ii. the removal of neurotransmitter from the synaptic cleft by diffusion and uptake (Dingledine and Korn, 1985), iii. receptor desensitization. After brief GABA application, channels enter a long closed state and subsequently reopen. These “sojourns” in the desensitized state would buffer the channel in a bound conformation and would underly the expression of long-lasting
components of the synaptic current (Jones and Westbrook, 1996). In the present study almost all GABAergic spontaneous currents recorded under voltage-clamp decayed monoexponentially. This observation is consistent with previous recordings made with intracellular microelectrodes (Collingridge et al., 1984; Segal and Barker, 1984) and patch-clamp pipettes (McBain et al., 1992; Capogna et al., 1994: Hosokawa et al., 1994). The values of decay time constants are comparable with those found by other authors (Ropert et al., 1990; Otis and Mody, 1992).

4.4 Variability in amplitude distribution of miniature events

Within the same experiment amplitude distribution of miniature GABA-mediated currents was found skewed towards lower values and could not be described by a simple Gaussian function (see also Jonas et al., 1993 for miniature excitatory postsynaptic currents (mEPSCs) and Edwards et al., 1990; Ropert et al., 1990 for miniature GABAergic currents). Amplitude histograms were narrower than those observed studying spontaneous PSCs. Larger events ( > 60 pA) disappeared. The variability in the amplitude of miniature events in a single cell is common to others studies on different brain areas (Raastad et al., 1992; Takahashi, 1992; Llano and Gerschenfeld, 1993). The values of the CV of GABA-mediated mPSCs was too large to be explained solely by the contribution of background noise and it was in the range measured for GABAergic IPSCs (Ropert et al., 1990) or glutamatergic EPSCs (Bekkers et al., 1990). The amplitude of the first peak of the histogram, which is suggested to underlie the quantal event, did not greatly vary between experiments.
However, the variance in the amplitude distribution is large (see also Edwards et al., 1990; Stevens, 1993). Fluctuation in amplitude of miniature GABAergic events can be due to pre or postsynaptic factors. Presynaptic factors include: i. variation in vesicle content within one or more nerve terminals (Bekkers et al., 1990), ii. multivesicular release. Postsynaptic factors include: i. electrotonic filtering (Rall, 1967), ii. stochastic behaviour of channels (Faber et al., 1992), iii. variation in the total number of receptors at different synapses (reviewed in Van der Kloot, 1992; Stevens, 1993). Since it is known from anatomical studies that each GABAergic interneurone makes a minimum of six and a maximum of twelve synaptic contacts with pyramidal cells (Buhl et al., 1994) the most likely explanations for the variability of mPSCs presented in this thesis are that spontaneous release from many different terminals was recorded and that responses were different for each synapse. Electrotonic filtering along the dendritic tree does not seem to be the major cause of amplitude variability because of the weak correlation ($r \leq 0.1$) between amplitude and decay or rise time in the range of amplitudes which was considered. A similar conclusion has been reached by other studies (Bekker et al., 1990; Edwards et al., 1990; Ropert et al., 1990). This view is also confirmed by anatomical data that show a dense cluster of GABAergic terminals around cell somata (Ribak et al., 1978; Rozenberg et al., 1989). Nevertheless, we cannot exclude that, at an immature stage of development, cells like those used for the present study can give ample variance of data since their synapses are in a process of maturation.
4.5 First peak of the histogram underlies quantal event

Miniature postsynaptic currents are proposed to represent the signals generated in response to the release of a quantal packet of transmitter (del Castillo and Katz, 1954). Only one modal peak (16 pA) can be clearly identified in the amplitude distribution histogram. A similar value (14 pA) has been found for GABA-mediated currents elicited by minimal stimulus intensity (Manabe et al., 1992; Raastad et al., 1992). Minimal stimulation is suggested to activate only few afferent fibers at level of st. oriens or radiatum (where some GABAergic interneurones are localized), resulting in 10-30% failures. The latency values of elicited events (less than 3 ms) and their rise time values (around 3 ms) are also compatible with the fact that only a single population of interneurones was stimulated (see Edwards et al., 1990). A quantal size of 15 pA is found for GABAergic events recorded near resting potential under conditions of high Cl' concentrations (Schneeggenburger and Konnerth, 1992; Vincent and Marty, 1996). Because the membrane potential of pyramidal cells (E_m) was held at -70 mV, the E_r values were close to 0 mV and the current underlying the quantum (I_q) was around 15 pA, from the equation I_q = g (E_m-E_r) we estimated that a single GABA quantum was associated with a mean conductance (g) of 220 pS. The latter value is very close to that calculated on the basis of previous current clamp experiments using sharp microelectrodes (Hosokawa et al., 1994). The relatively small conductance change observed was presumably due to the opening of a small number of postsynaptic GABA_A receptor channels. In fact, in outside-out patches from acutely dissociated pyramidal neurones, we found GABA-activated single-channel events, having conductances of 24 and 35 pS, values which are in good agreement
with previously reported ones. In hippocampal neurones, a main conductance state of 16-23 pS is consistently found (Bormann et al., 1983; Gray and Johnston, 1985; Edwards et al., 1989, 1990), and sometimes a subconductance state of 14 pS is present (Edwards et al., 1990). Hence, because we found that a single GABA quantum was associated with a mean conductance of 220 pS, it appears that a single GABA quantum opens relatively few Cl channels (from six to nine) on the postsynaptic cell. A similar estimate has been obtained for miniature inhibitory postsynaptic currents (mIPSCs) of dentate granule cells (Edwards et al., 1990), CA1 pyramidal neurones (Ropert et al., 1990) and cultured colliculus neurones (Kraszewski and Grantyn, 1992). The low number of GABA channels underlying a single quantum is in contrast with 1000 channels participating in miniature endplate currents (Gage and Armstrong, 1968; Anderson and Stevens, 1973; Edwards et al., 1991). The cleft GABA concentration reaches at least 500 μM (Maconochie et al., 1994) and the number of GABA receptors is high (Sivilotti and Nistri, 1991), yet few channels underly a single quantal event. What factors determine the size of quantal conductance? At central GABAergic synapses, modelling studies (Edwards et al., 1990; Faber et al., 1992) as well as pharmacological investigations with benzodiazepines (De Koninck and Mody, 1994) indicate that it is the small number of synaptic channels rather than the small amount of vesicular transmitter responsible for the small size of elementary synaptic currents. Data presented here are not incompatible with the view that single quanta saturated postsynaptic receptors generating a quantum of fixed amplitude, as suggested by other studies (Redman, 1990; Edwards, 1991; Stevens, 1993). A constant quantal size, as well as the large variance in the total amplitude distribution of miniature currents can be also explained
by the presence of clusters of GABA receptors aggregating the postsynaptic membrane opposite a bouton as suggested by Edwards and Stern (1991). This notion implies that a large number of GABA receptors detected with a variety of biochemical or pharmacological methods (see Sivilotti and Nistri, 1991) are probably "extrasynaptic".

4.6 Modulation of spontaneous GABAergic activity by mGluRs

Glutamate is the main excitatory neurotransmitter at most CNS synapses where it acts on pre- and postsynaptic receptors. Presynaptic glutamate receptors have been shown to influence GABA release from neonatal hippocampal neurones (Ben Ari et al., 1989; Gaiarsa et al., 1991); this effect is due to glutamate acting on ionotropic NMDA and non-NMDA receptors. The present experiments showed that presynaptic mGluRs also affected GABA release. The effects of mGluR activation was in fact observed in the presence of kynurenic acid which blocks ionotropic glutamate receptors. This study shows that stimulation of mGluRs, using low doses of t-ACPD, enhanced the discharge frequency of spontaneous GABA-mediated currents recorded from CA1 and CA3 pyramidal cells without changing their amplitude values. The potentiating effect was presynaptic to the recorded cell because: i. Cs\(^+\) in the pipette was presumed to block most potassium conductances in the recorded pyramidal cell. Potassium conductances are suggested to be the main "target" of mGluR activation (Charpak et al., 1990), ii. t-ACPD did not affect the holding current or the input conductance of the postsynaptic cell, iii. t-ACPD did not affect the response to exogenously applied
GABA, iv. t-ACPD did not modify the kinetics (rise and decay time) of GABAergic currents. In keeping with this proposal presynaptic mGluRs have been found in the cerebral cortex (Herrero et al., 1992; Sladeczek et al., 1993), or in the hippocampus of neonatal rats (Aniksztejn et al., 1995).

A clear effect of mGluR activation on GABA release is also observed in superfused cortical synaptosomes (Diaz-Amesto et al., 1992). The activation of mGluRs can therefore facilitate GABA release at presynaptic level probably through an increase in release probability or in the number of releasing sites.

Frequency distribution of spontaneous GABAergic currents

In view of the block of ionotropic glutamate receptors by kynurenic acid, the increase in frequency of spontaneous events by t-ACPD was likely to be due to an enhancement of the activity of interneurons. As previously reported, in kynurenic acid solutions interneurones can still signal through GABA release (Cherubini et al., 1996). The effect of t-ACPD on mGluRs is suggested to be specific because it was blocked by MCPG, known to be an antagonist selective for mGluRs (Bashir et al., 1993). The increase in frequency of spontaneous GABA-mediated currents by t-ACPD was dose-dependent in the 3-30 μM range. The largest effect of mGluR activation on GABAergic transmission has been found with a dose of 10 μM. Higher doses are known to depolarize interneurones to potentials at which Na⁺ currents are inactivated so that fast action potential no longer occur leading to a reduction in transmitter release (Miles and Poncer, 1993).

Amplitude distribution of spontaneous GABAergic currents

In spite of the increase in frequency of spontaneous GABAₐ-mediated currents, no change in amplitude distribution of spontaneous PSCs after mGluR activation was
found suggesting that the action of r-ACPD was to increase the firing frequency of GABAergic interneurones that were already active in control condition. mGluR activation did not modify the amplitude distribution of spontaneous events which fell within the first peak of the histogram, corresponding to the mPSCs (Ropert et al., 1990): this result suggests that mGluR activation did not induce changes in quantal events. When an increase in mean amplitude was occasionally observed (n=2), it was due to events larger than those corresponding to the quantal current.

Different hypotheses can be put forward in order to explain the mechanism by which mGluR activation increased the frequency of synaptic GABA- mediated activity:

i. increase in spike duration as a consequence of the reduction in voltage-dependent K^+ currents such as I_M and / or the Ca^{2+}-dependent K^+ current I_{AHP} (Charpak et al., 1990; Desai and Conn, 1991) may be expected to prolong the size of presynaptic terminal depolarization and to facilitate calcium entry, ii. rise in intracellular calcium concentration released from internal stores (Murphy and Miller, 1988), iii. modulation of presynaptic Ca^{2+} channels. None of these hypotheses could be resolved with the present technique.

Activation of mGluRs stimulates IP_3 formation through the activation of phospholipase C and induces Ca^{2+} release from intracellular IP_3-sensitive Ca^{2+} stores. Intracellular calcium oscillations are known to be induced by mGluR activation in hippocampal cells (Murphy and Miller, 1988; McBain et al., 1994; Aniksztein et al., 1995). Rise in intracellular Ca^{2+} concentrations can increase the probability that synaptic vesicles will fuse with the presynaptic membrane and release their content into the synaptic cleft.
A direct effect of the mGluR agonist on calcium channels localized on GABAergic interneurones cannot be ruled out. However, at least on neuronal somata, mGluR activation is known to inhibit calcium currents (Lester and Jahr, 1990; Swartz and Bean, 1992; Trombley and Westbrook, 1992; Sahara and Westbrook, 1993; Chavis et al., 1994). It has to be noted that in most cases mGluR activation affects L-type Ca$^{2+}$ channels, which are not generally associated with transmitter release (Lester and Jahr, 1990; Chavis et al., 1994).

4.7 Modulation of elicited GABA-mediated activity by mGluR activation

In contrast to spontaneous GABAergic synaptic currents that were found to be up regulated by mGluR activation, GABA-mediated currents elicited by low stimulus intensity were down modulated by mGluR activation. The reduction in amplitude was associated with an increase in the number of failures suggesting a presynaptic site of action of mGluR agonists. A differential modulation of spontaneous and elicited GABAergic currents by other neurotransmitters has already been reported. In CA1 pyramidal cells activation of muscarinic receptors by carbachol induces an increase in frequency of spontaneous IPSCs but a depression of the elicited ones (Pitler and Alger, 1992; Behrends and Bruggencate, 1993). Noradrenaline reduces stimulus elicited IPSPs (Madison and Nicoll, 1988) but increases the frequency of spontaneous IPSCs (Doze et al., 1991). Also on CA1 cells, the neuropeptide thyrotropin-releasing hormone (TRH) increases the frequency of spontaneous GABAergic currents and depresses the electrically elicited GABA-mediated PSCs (Atzori and Nistri, 1996). At
least two hypotheses can been advanced to explain the differential modulation of spontaneous and elicited synaptic activity in hippocampal neurones: i. spontaneous and elicited currents do not involve the same population of GABAergic interneurones. The elicited currents may arise from the activation (by focal stimulation) of a more homogeneous population of interneurones. Two different classes of interneurone, both in st. oriens, respond in a different way to mGluR agonist application (McBain et al., 1994). ii. Spontaneous activity can interfere with the elicited one. It is possible that mGluR activation depolarizes GABAergic interneurones so that they increase their firing and release transmitter but become refractory to additional stimulation.

4.8 mGluR modulates spontaneous GABAergic activity through cAMP and PKA

The present study indicates that an intracellular transduction pathway involving increase in cAMP (probably through the activation of adenylyl cyclase) and PKA mediated the potentiating effect of mGluR activation on GABAergic transmission. Enhancement of intracellular levels of cAMP by mGluR activation has a developmental profile, being high in the neonatal period and then progressively declining (Schoepp and Johnson, 1993). In the adult hippocampus (≥ 2 weeks), mGluR activation does not appreciably increase basal cAMP but inhibits forskolin-stimulated cAMP formation (Casabona et al., 1992). Forskolin and IBMX are known to increase the intracellular levels of cAMP: forskolin stimulates cAMP synthesis through activation of adenylyl cyclase whereas IBMX prevents cAMP degradation by inhibiting phosphodiesterases. Both forskolin and IBMX mimicked the effects of t-
ACPD as they induced an increase in frequency but not in amplitude of spontaneous GABA-mediated currents, suggesting a presynaptic site of action. Moreover, forskolin did not modify (in a few cases reduced) the pyramidal cell response to exogenous application of GABA, suggesting that its site of action was on GABAergic interneurones.

cAMP can mediate intracellular processes through two main mechanisms: i. activation of PKA or ii. direct effect on cyclic nucleotide-gated or -modulated ion channels (Fesenko et al., 1985). The role of PKA is to transfer highly charged phosphate groups of ATP to a serine, threonine or tyrosine residue of a substrate protein (Nestler and Greengard, 1983). PKA was proposed to be involved in the potentiating effect of GABAergic neurotransmission by forskolin or mGluR activation because the effect was consistently depressed by Rp-cAMPS, a specific antagonist of PKA which binds to the regulatory site of PKA and counteracts cAMP effects (Botelho et al., 1988). In line with these results, it has recently been reported that mGluR activation potentiates GABA release responsible for GDPs in immature CA3 pyramidal neurones, an effect which is mediated through PKA (Strata et al., 1995). The target of PKA activity is still unknown although it might be the AHP potassium channels. In accordance with this view, Baskys et al. (1990) have found that H-7, suggested to block PKA, suppresses the inhibition of IAHP by mGluR activation. In CA1 neurones, reduction of AHP and spike frequency adaptation induced by coapplication of mGluR and isoprenaline is also mediated by protein kinase activation (Gereau and Conn, 1994). Conversely, on pyramidal cells the effect of t-ACP D on the slow AHP seems to be mediated by a mechanism that is independent of PKA (Gerber et al., 1992; Pedarzani and Storm, 1993).
Multiple subtypes of mGluRs exist with different distribution and effectors. Among them, mGluR 1 (which belong to group I) stimulates both phosphoinositide hydrolysis and cAMP formation (Aramori and Nakanishi, 1992; Schoepp and Conn, 1993; Nakanishi, 1992; Pin and Bockaert, 1995). The subunit mGluR1 has been found to be mainly localized on the somatodendritic region of GABAergic interneurones (Martin et al., 1992; Baude et al., 1993) suggesting a role in the regulation of GABAergic neurotransmission. In addition, it has been reported that high levels of mRNA for mGluR1 are present in interneurones localized in CA1 st. oriens. These cells can be also immunostained with an antibody against mGluR1α (Masu et al., 1991; Baude et al., 1993; Fotuhi et al., 1993). In the adult, the horizontally oriented O/A interneurones that contain particularly high levels of mGluRα, project to L/M and elicit no recordable IPSP at the pyramidal CA1 soma. The vertically oriented O/A interneurones innervate more proximal regions of CA1 pyramidal cells but are less dramatically sensitive to mGluR agonists (Mc Bain et al., 1994).

MCPG, which was found to depress the mGluR-induced responses, is a competitive antagonist at group I and II mGluRs (Hayashi et al., 1994; Thomsen et al., 1994). While studying the pharmacological effect of different mGluR agonists on GABA-mediated activity in neonatal CA3 pyramidal neurones, Poncer et al. (1995), have hypothesized that hippocampal inhibitory cells express mGluR1 or mGluR5 on the somatodendritic membrane. Thus, QA (50 nM), which has higher efficacy than t-ACPD on mGluR1 and mGluR5 subtypes (Schoepp and Conn, 1993), increases spontaneous GABAergic currents, whereas DCG-IV, suggested to be a specific
agonist of mGluR2 and 3 subtypes (Ishida et al., 1993; Hayashi et al., 1994), is ineffective.

It is possible that different populations of interneurones express different types of mGluRs. Dual recording from interconnected GABAergic interneurones and pyramidal cells, combined with the polymerase chain reaction technique on the single cell (Lambolez et al., 1992), would help to clarify which mGluR subunits are involved in the regulation of GABAergic neurotransmission.

Various neurotransmitters are known to modulate the effects of other neurotransmitters through changes in membrane potential, action potential duration, firing frequency or firing pattern (Nicol, 1988; Nicoll et al., 1990). L/M interneurones have been shown to receive cholinergic input from the septum and serotonergic input from the raphe nucleus (Freund and Antal, 1988). Serotonin is known to excite GABAergic interneurones in hippocampal slices (Ropert and Guy, 1991). As already mentioned, carbachol, like mGluR agonists, enhances interneurone excitability (Pitler and Alger, 1992; Behrends and Ten Bruggencate, 1993) and was found to increase the frequency of spontaneous IPSCs. Similar effects were observed when NA was applied (see also Madison and Nicoll, 1988). Glutamate (through metabotropic receptors), acetylcholine (through muscarinic receptors), NA, serotonin, histamine, dopamine are known to share a common action: depression of Ca\(^{2+}\)-dependent potassium channels, increasing neuronal excitability and decreasing spike-frequency adaptation. (Madison and Nicoll, 1982; Haas and Konnerth, 1983; Malenka and Nicoll, 1986; Andrade and Nicoll, 1987; Madison et al., 1987; Charpak et al., 1990). All of them act via G protein-coupled receptors (Hille, 1992; Strader et al., 1994) and activate adenylyl cyclase dependent metabolic pathways which in turn can
modulate ionic channels. Different receptors could be coupled to the same G-protein or perhaps to distinct types with significant cross-reactivity (Simmons and Mather, 1991). If an increase in intracellular cAMP elicited a potentiation of GABAergic interneurone activity, other drugs which induced similar increases via receptor coupled adenylyl cyclase can all enhance GABAergic synaptic transmission.

4.9 Miniature events are not affected by mGluR activation

In contrast with spontaneous GABA-mediated synaptic currents, the frequency of miniature events was not changed by mGluR activation with t-ACPD (3-30 μM). This suggests that mGluRs are localized on the somato-dendritic membrane and not on the terminals of GABAergic interneurones. A recent study by Poncer et al. (1995) has reported that t-ACPD in concentration higher than those used in the present study, reduced the frequency of miniature GABA-mediated currents. This phenomenon is mimicked by DCG-IV, which is a specific agonist of mGluR2 and 3 or by L-AP4 (which has high affinity for mGluR4, 6 and 7 subtypes, Tanabe et al., 1993; Nakajima et al., 1993). Since these agents apparently activate a distinct subclass of mGluRs which does not lead to cAMP formation, it seems likely that glutamate acting via metabotropic receptors can have two opposite actions on GABA release: i. facilitation via mGluRs located on cell bodies and positively coupled to adenylyl cyclase activation, ii. inhibition via mGluRs at the level of nerve endings through a mechanism independent of cAMP synthesis. In the light of point i. any cAMP-dependent PKA activity at somatic level could not be propagated to the relatively remote compartment of nerve terminals and was thus unable to potentiate miniature GABAergic events.
This suggestion implies that the same excitatory transmitter might facilitate or inhibit GABA release depending on network activity and local concentrations achieved during repetitive firing.

**4.10 Forskolin increases miniature frequency through PKA**

Spontaneous miniature currents were not affected by mGluR activation. However, their frequency but not their amplitude was potentiated by activation of adenylyl cyclase by forskolin through a presynaptic mechanism. The involvement of a cAMP-dependent PKA pathway in the forskolin action on miniature GABAergic currents is supported by the following points: i. the effects of forskolin were not mimicked by the forskolin analog 1,9-dideoxyforskolin, which shares many of the actions of forskolin including those on potassium conductances (Hoshi et al., 1988) but has no effect on adenylyl cyclase. ii. Rp-cAMPS, a specific blocker of PKA, greatly reduced the effects of forskolin.

The mechanisms by which PKA activated by forskolin enhanced spontaneous transmitter release are not clear. It is unlikely that these effects were mediated by direct (Chetkovich et al., 1991) or indirect (Madison and Nicoll, 1986) modulation of calcium entry since it is well established that mPSCs frequency is independent from extracellular calcium (del Castillo and Katz, 1954; Scanziani et al., 1992; Thompson et al., 1993). Probably a direct effect on the releasing processes such as phosphorylation of vesicle-associated proteins might have accounted for the present results (Greengard et al., 1993). Such a potentiating effect of PKA is not restricted to GABA release since analogous data have been obtained in the case of the release of
glutamate from mossy fiber (Hopkins and Johnston, 1988; Weisskopf et al., 1993) or Schaffer collateral/commissural terminals (Chavez-Noriega and Stevens, 1994). These observations thus suggest that the role of PKA in transmitter release is a rather widespread phenomenon. Far more difficult is to dissect out the molecular mechanism through which PKA can enhance transmitter release. First of all, it seems that this enzyme is not essential for basal release of GABA under resting conditions in view of the fact that pharmacological inhibition of PKA by Rp-cAMPS did not change the frequency of miniature GABA-mediated currents. The PKA pathway can be therefore considered as a permissive process to amplify transmitter release when extra requirements are necessary during repetitive neuronal activity. In this case one might hypothesize that the facilitating action of cAMP-dependent PKA on transmitter release involves mechanisms regulating intraterminal free calcium availability and/or the sequence of events leading to exocytosis. Phosphorylation of synapsin I (a vesicle associated protein) by PKA increases the ability of synaptic vesicles to move to the plasma membrane and enhances transmitter release at the squid giant synapse (Llinas et al., 1985); a cAMP mediated increase in phosphorylation of synapsin I has been reported in the rodent hippocampus (Browning et al., 1990). Another potential target is a synaptic vesicle-associated phosphoprotein (SVAPP-120), which is also phosphorylated by PKA (Greengard et al., 1993).

The present study can not identify the transmitter or metabolic signal physiologically used to activate PKA at nerve terminal level: it can only eliminate glutamate as a candidate in this process.
Microphysiology of mGluR activity

A crosstalk exists between glutamatergic and GABAergic system in the developing hippocampus. GABA, released from GABAergic interneurones (through a feedforward and feedback loop) would activate GABA\(_A\) receptors in pyramidal cells and would depolarize and excite these cells. In turn, glutamate, released from pyramidal cells, would activate both ionotropic and metabotropic glutamate receptors localized on the somato dendritic level of GABAergic interneurones. mGluR activation would enhance PI turnover, intracellular cAMP or calcium; these effect would potentiate cell firing, therefore creating a positive feedback loop reinforcing and prolonging the action of GABA. GABA, through GABA\(_A\) receptors, may synchronize GABAergic interneurones. Furthermore, a different regulatory system directly involved in the release mechanism might act at the level of interneurone terminals; it would also includecAMP increase and PKA activation (Fig. 26).
Adenylyl cyclase

L-GLU

AHP potassium channel

Control

mGluR activation

PKA * cAMP mGluR activation

PKA

Unknown signal

Adenylyl cyclase

Enhanced transmitter release

GABA
Fig. 26 A schematic diagram to account for the experimental observation of the present study. The scheme depicts an idealized GABAergic interneurone which is suggested to release GABA to activate GABA receptors generating spontaneous currents of pyramidal cell. L-Glutamate (L-Glu) is released by the pyramidal cell and acts on ionotropic (I) and metabotropic (M) receptors. In the present scheme, due to their blockade by kynurenic acid, ionotropic glutamate receptors are not operational. mGluRs are localized in the somato-dendritic region of GABAergic interneurones and their activation induces block of cell accommodation and all increase in cell firing (see right panel) through a reduction in AHP potassium channel activity. The transduction pathway induced by mGluR activation, involves a G protein, the activation of adenylyl cyclase with cAMP production which, in turn, activates PKA to PKA*, which can possibly act by phosphorylating the AHP potassium channel. Phosphorylation at the level of interneurone terminals, induced by forskolin, involves PKA and enhances GABA release. However the identity of the endogenous element capable of activating this system is currently unknown.
Conclusions

The present data have provided novel information concerning the basic properties of GABAergic transmission in hippocampus and its modulation. In particular, using as experimental model recording from neonatal hippocampal pyramidal cells (at P6-P12) most spontaneous synaptic activity has been found to be mediated by GABA_A receptors. From cell to cell GABAergic activity has been found to be variable in amplitude and frequency. Amplitude distribution of spontaneous events has always shown a broad skewed peak with a relative fixed modal peak ranging around 16 pA. The broad amplitude distribution could be due to a variability in the probability of release among the different synaptic input to the pyramidal cell. Similar modal peak value has been found when minimal elicited GABAergic currents were detected, reflecting the activation of a fixed number of GABAergic receptors on the postsynaptic membrane.

In hippocampal pyramidal cells electrophysiologically monitored GABA release has been found to be up-regulated by the mGluR agonist t-ACPD, NA and carbachol as well as by agents that enhance the endogenous levels of cAMP. The action of the mGluR agonist has been studied in detail and found to be localized on the presynaptic cells, presumably at somato-dendritic level since this effect was abolished by TTX. The underlying metabolic pathway involved increase in intracellular cAMP with consequent activation of PKA, the target of which is still unknown. Direct activation by forskolin of adenylyl cyclase enhanced miniature GABAergic activity, via an effector system involving PKA.
References


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