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Novel chloride dependent GABA-mediated responses in developing rat hippocampus and in cultured cerebellar granule cells.

Thesis submitted for the Degree of
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Candidate:
Marco Martina

Supervisor:
Prof. Enrico Cherubini

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TRIESTE
Strada Costiera 11

TRIESTE

SUMMARY

INTRODUCTION

Among the great variety of the membrane proteins acting as ion channels two main superfamilies have been found: i) the voltage activated ion channels and, ii) the ligand gated ion channels. In this thesis I will focus on the latter.

LIGAND GATED ION CHANNELS

The ligand gated ion channels are ubiquitous transducers of most inhibitory and excitatory synaptic transmission in the central nervous system (CNS). It is known since many years that all the main neurotransmitters (Ach, glutamate, GABA, glycine) operate through an action on these channels; an increasing body of evidence is now indicating that also many other substances as purines, peptides and cyclic nucleotides can gate some ion channels and exert in this way at least part of their physiological actions.

REVERSAL POTENTAIALS AND ACTION OF MAJOR PHYSIOLOGICAL IONS		
Na^+	+55 mV	depolarizing
K^+	-75 mV	hyperpolarizing
Ca^{2+}	+200 mV*	depolarizing
Cl^-	-70 mV	hyperpolarizing

The main excitatory neurotransmitters, acetylcholine and glutamate, operate channels that are selective for cations (Na^+, K^+, Ca^{++}). As the reversal potential for currents carried by the ensemble of these ions is significantly higher than the membrane resting potential (around -60 mV, see tab.

1), the opening of these channels leads to a depolarization of the cells. The inhibitory neurotransmitters such as GABA and glycine, on the other hand, usually gate anion selective channels whose equilibrium potential is more negative than the resting membrane potential (tab.1) and therfore hyperpolarize (or repolarize) the cell membrane.

The inhibitory neurotransmitter GABA, on the other hand, can also activate a different type of receptor ($GABA_B$) and hyperpolarize the membrane by opening or closing a potassium or a calcium selective conductance respectively. The inhibitory action however does not imply necessarely an hyperpolarization of the cell; the amplitude of the excitatory post-synaptic potential (V_{EPSP}) in fact is given by:

$$V_{EPSP} = \frac{I_{EPSP}}{g_m} \qquad \text{Eq. 1.1}$$

this means that an increase in g_m reduces the amplitude of the post-synaptic potential (shunt effect). Both excitatory and inhibitory channels can be gated by the ligands either directly or indirectly through a second messenger cascade.

The amino acid receptor-integral channels show several functional similarities regardless of their physiological role (excitatory or inhibitory); all of them are ligand gated, their activation is quite fast and have multiple binding sites whereas the second messengers activated channels show slower kinetics.

THE GABA RECEPTORS

GABA is the main inhibitory neurotransmitter in adult mammalian brain. It exerts its action through different receptors. Up to today three distinct GABA receptors have been characterized: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_A$ and $GABA_C$ have also been cloned (Schofield et al. 1987, Cutting

et al. 1991); they are integral receptor channels selectively permeable to small anions. The $GABA_B$ receptor on the other hand is indirectly coupled, via G-proteins, to potassium or calcium selective channels. Electrophysiological studies have shown that $GABA_A$ and $GABA_B$ receptors are widely expressed in the entire mammalian brain; In situ hybridization experiments confirm the wide expression of $GABA_A$ receptor in almost every area of rat brain. On the contrary the $GABA_C$ receptor expression seems to be limited to the rat retina (Polenzani et al., 1991) and bovine cerebellum but is not detectable in rat central nervous system using this tecnique. However Southern blot studies on PCR amplified cDNAs obtained by reverse transcription of total RNA isolated from rat retina as well as from several brain areas confirmed that the expression of one of the subunits of the $GABA_C$ receptor (the ρ_1) is limited to the retina but showed that the other one (the ρ_2) is present also in the CNS and particularly in the hippocampus (Enz et al. 1995).

THE $GABA_A$ RECEPTOR

kinetics

The different GABA receptors show deeply different biophysical and pharmacological features. The most common receptor, the $GABA_A$, is characterized by very fast macroscopic kinetics. The onset of the current is complete in about one millisecond in the spontaneous synaptic events; the onset of the current due to exogenous application of the neurotransmitter is obviously dependent on the speed of the perfusion system but it is possible to study it using very fast perfusion devices; using such methods Machonochie et al.(1994) and Celentano and Wong (1994) found activation times of as fast as 0.2 and 1 ms respectively. These values are in the same range of the on-rates (0.2 to 1.6 ms) of the synaptic $GABA_A$ currents described in tissue slices (Edwards et al.,Schennenburg and Konnerth) or in cell cultures (Virginio et al.1995). The on-rate of the current is concentration

dependent; the presence of a high concentration asymptote in this dependence (Maconochie et al. 1994, fig.1.1) seems to clash with the accepted theory that binding and not gating is the rate limiting step (Colquhoun and Sakmann 1985, Celentano and Wong 1994). Nevertheless the comparison of the rising phases (and the decaying ones as well) of current evoked by exogenous transmitter application and the spontaneous currents allowed to estimate the GABA concentration in the synaptic cleft to be in the millimolar range.

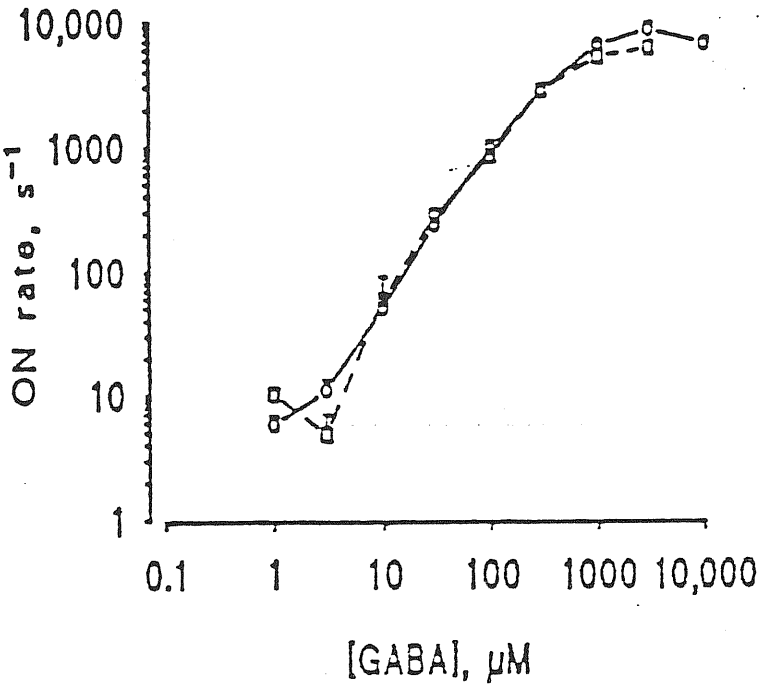

Fig. 1.1 The on rate of the GABA current plotted against the concentration of GABA. Note that the curve saturates for high GABA concentrations. (adapted from Maconochie et al. 1994).

The desensitization of this current is very deep and fast; the time course of the desensitization process has been described by one, two or three exponential functions depending on the different preparations, agonist concentrations and on the speed of the perfusion devices used for the experiments. In all cases the desensitization is a voltage, concentration and temperature dependent phenomenon. The values of the time constants vary enormously in the various experimental conditions. Interestingly however the fastest values (15 ± 4 ms) obtained for exogenous applied GABA at saturating concentrations with very fast perfusion devices (exchange time lower than 1.5 ms,Celentano and Wong 1994) are in good agreement with the data obtained recording the spontaneous GABA activity on hippocampal slices (22-25 ms, Otis and Mody 1992, 2 and 54 ms Edwards et al.). Using the fast perfusion system Celentano and Wong showed that the $GABA_A$ desensitization process induced by 1 mM of the agonist can be described in 70% of cases by the sum of three exponential components of 15, 207 and 1370 ms respectively (V_h= -40 mV) and in the remaining 30% by the sum of two exponentials (= 44 and 725 ms). Interestingly the values of the time constants are not affected by the concentration of the agonist applied to the membrane patch; the concentration dependence of the desensitization process results only from a different contribution of three phases to the process. These data can easily explain the reason why using conventional "slow" perfusion systems the fast desensitization component cannot be seen; in this case in fact the diffusion of the neurotransmitter is rate limiting and the onset of the current is slower than the faster desensitization component. Using fast perfusions however the onset of the current is only limited by the association rates of the transmitter that is function of the concentration; at higher agonist concentrations the peak current is higher because the association rate exceedes the fastest desensitzation rate and an additional component of the current appears. The concentration dependence of the on-rate of the current suggests a possible cooperative scheme in the activation

of the receptor. This item is usually described by the Hill plot that, in fact, for $GABA_A$ currents usually gives a value of the Hill coefficient around 2; in several cases however a Hill number very close to unity has been found.

The desensitization process, as well as the activation, seems to require some phosphorylation reactions.

modulation

There is a fine modulation of the channel activity that depends on a phosphorilation-dephosphorilation cycle (Steltzer 1992) that acts mainly through calcineurin, a calcium/calmodulin dependent phosphatase. On the other hand internal calcium seems to down regulate $GABA_A$ currents also decreasing the affinity of the receptor for GABA itself (Inoue et al. 1986, Martina et al. 1994). The modulation of the $GABA_A$ receptor depends upon many other pharmacological agents acting either at the external or at the intracellular side of the membrane. From the outer side the channel can be operated by GABA and some structural analogues such as muscimol, isoguvacine and DAVA (δ-aminovaleric acid) all acting on the same binding site even if with deeply different affinity and potency. On the same site binds also bicuculline, an alkaloid that is the most important competitive antagonist specific for the $GABA_A$ receptor. However many allosteric binding sites have been described to exist on the outer site of $GABA_A$ receptor (Sieghart 1992); they are presented in figure 1.2. Between these agents zinc seems to play a physiologically particularly important role.

This transition metal is in fact particularly abundant in several CNS areas and particularly in the hippocampus; furthermore in this area the zinc concentration changes during development in parallel with changes in the physiological role of GABA that switches from an excitatory ,limited to the early postnatal period (for a review see Cherubini et al.1991) to the common inhibitory role. An important physiological role for zinc in the hippocampus has already been shown

by Xie and Smart (1991) who described a role of endogenous zinc in the induction of the giant depolarizing potentials (GDPs) in young hippocampal neurones. Almost all the data reported so far refer to post-synaptic $GABA_A$ receptors. It is known however that GABA mediates also a presynaptic inhibition and participates to the regulation of transmitter release.

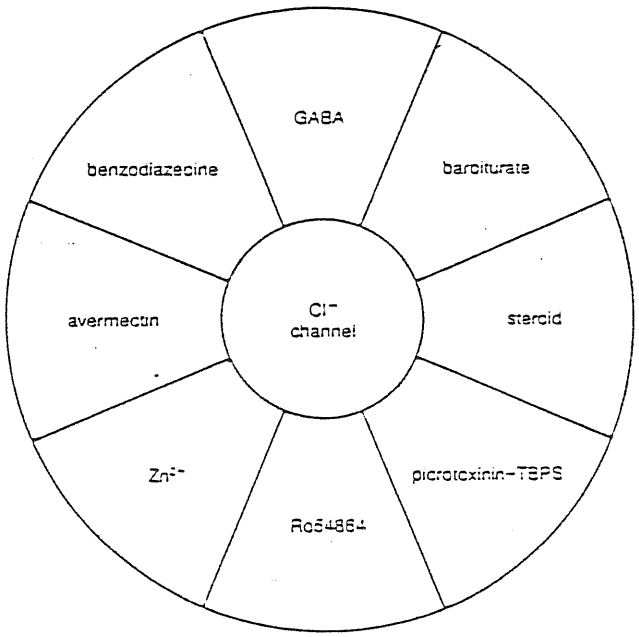

Figure 1.2

Allosteric binding sites of $GABA_A$ receptors. Additional binding sites might exist for propofol and ethanol. TBPS t-butylbicyclophosphorothionate

(From Sieghart 1992).

Although $GABA_B$ receptor is generally regarded as the more prevalent presynaptic GABA receptor (Bowery 1989), many systems posses presynaptic $GABA_A$ receptors (Rudomin 1990) and less is known on the nature of these receptors. It has recently been found that these channels,

while having a "classic" pharmacology, show some differences at single channel level (Zhang & Jackson 1993).

molecular biology

Since 1987 when the first $GABA_A$ receptor channel subunits were cloned, our knowledge on the molecular biology of this structure has improved enormously helping to give an explanation to the existence of so many different biophysical and pharmacological features of this receptor.

There is a certain degree of homology between the primary sequences of the subunits of many different amino acid receptor channels cloned so far. This homology ranges from more than 75% when matching the different $GABA_A$ α subunits (α_1 to α_6) to an homology degree about 20% when comparing the $GABA_A$ and glycine subunits to the nicotinic acetylcholine α subunits. There seems to be no homology however between these subunits and the kainate receptor clone. These data could argue for the classification of the amino acid receptor channels into two different superfamilies deriving from two distinct ancestors.

The $GABA_A$ receptor is thus considered a member of the gene superfamily whose paradigmatic member is the nicotinic acetilcholine receptor. Up to today 14 different subunits of the $GABA_A$ receptor have been cloned: 6 alpha, 4 beta, 3 gamma and 1 delta. All of these polypeptides when expressed in heterologous cells produce GABA activated chloride channels even if bearing different biophysical and pharmacological properties. Thus a different subunit composition produces different features; this fact, toghether with anatomical studies demonstrating the different expression of the various subunits in the CNS, can explain the wide differences in the properties of the receptor above described.

As previously stated, four different families of subunits of the $GABA_A$ receptor have been cloned; there is no definitive proof, however, about the way in which the different subunits are

assembled nor about their number in the native receptor.

Figure 1.3 is a model of the hetero-oligomeric $GABA_A$ receptor structure, reconstructed by analogy with the nicotinic acetylcholine receptor, the best studied component of the ligand gated ion channels superfamily. The number of subunits in the native receptor is probably five, as results also from target size (Nielsen et al. 1985) and hydrodynamic measurments (Siegel et al. 1983). Each subunit showes four putative membrane spanning domains (M1 to M4) containing 22-23 α-helical residues with predominantly hydropathic character in the COOH-terminal half of the protein. The long hydrophilic NH_2 terminal half of the protein contains asparigine glycosylation consensus sequences.

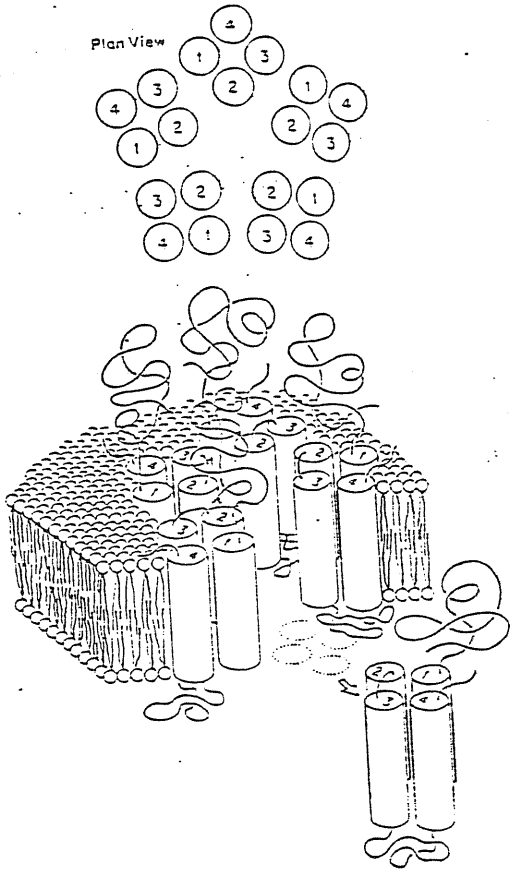

Figure 1.3

Model of the $GABA_A$ receptor chloride channel protein complex. The channel complex is proposed to be a hetero-oligomer composed of 5 subunits eachone having 4 membrane-spanning domains (cylinders numbered 1-4).

(Adapted from Olsen and Tobin 1992).

These proteins are glycoproteins actually, as proved by the fact that their size on SDS gel electrophoresis is larger than the deduced sequences. Between M3 and M4 are long intracellular domains that are belived to be involved in the intracellular modulation of the channel.
All the different subunits are likely to have a binding site for GABA, even if with deeply different affinities (Bureau and Olsen 1989, Olsen et al. 1990), barbiturates and picrotoxin.

The α subunits.

Early binding studies showed that the α subunits have high affinity for GABA; Heterologous expression studies indicated that the different α subunits are responsible for the different affinity, cooperativity and maybe desensitization of the channel. The most likely explanation for the observed pharmacological differences among $GABA_A$ receptor subtypes in vivo is that they express different α subunits. Levitan et al. (1988) used Xenopus laevis oocytes to express different α subunits clones associated with one β subunit and found different affinity for GABA; the maximal response was also different the response to α3 being about six folds larger than to α1 but the Hill number was close to unity for every alpha subunit tested, in contrast with the vast majority of the native receptors that show a certain degree of cooperativity. Those channels were blocked in a competitive way by bicuculline and potentiated by pentobarbital but were not sensitive to benzodiazepines. Further proofs about the importance of α subunits for the binding of GABA (and bicuculline, that binds to the same site) was obtained in 1992 by Sigel et al. who showed that a point mutation in position 64 of the rat brain α1 subunit strongly decreased the affinity of the receptor to both GABA (EC_{50} shifted from 6 to 1260 μM) and bicuculline (60 to 200 fold).

The same authors noticed that the affinities remained pratically unchanged when an homologous

mutation was introduced either in β2 or in γ2 subunits.

The β subunits

Researchers are still in the dark with regard to the different β subunits function. It is known that these polypeptides are essential for the correct assembly and expression of the receptor and that they should play a major role in agonist binding (Amin & weiss 1993). Verdoorn et al. (1990) showed that the β2 subunit may control channel gating properties. It was also showed that subunits undergoes a PKA-dependent (Kirkness et al. 1989) and PKC-dependent (Browning et al. 1990) phosphorylation that could be important for the modulation of the receptor channel function, nevertheless pharmacological distinctions between different β subtypes were not described yet.

The γ subunits.

The γ subunits, particularly the γ2, are essential to confer benzodiazepine sensitivity to αβ complexes (Pritchett et al. 1989). These subunits are also important in determining the single channel conductance and desensitization rate. In addition to this the presence of either the γ1 or the γ2 subunit dramaticaly reduces the sensitivity of GABA currents to the non competitive block by Zn^{2+} (Draughn et al. 1990). The γ subunits however are not the only ones involved in the Zn^{2+} binding; very recently it has been shown that zinc blocks GABA currents elicited in Xenopus oocytes through the activation of receptors containing the γ2 subunit with different EC_{50} and efficacy depending on the α subunit composition of the receptor (White and Gurley 1995).

The δ subunit.

Less is known about the δ subunit. An important study of the distribution of γ and δ polypep-

tide types in rat brain showed that these two subunits should be part of distinct receptors subtypes (Shivers et al. 1989). The δ subunit was correlated with high affinity muscimol binding areas without benzodiazepine sensitivity (Olsen et al. 1990).

The ρ subunits.

In 1991 Cutting et al. identified in the retina a new type of subunit using the polymerase chain reaction. These subunits (named ρ subunits) were shown to form homo-oligomeric GABA gated chloride permeable channels with peculiar kinetics and pharmacology when expressed in Xenopus laevis oocytes. For this reason they were considered to constitute a new GABA receptor, called $GABA_C$, instead of new $GABA_A$ subunits; it cannot be excluded however that these subunits could be assembled together with $GABA_A$ subunits in certain native GABA receptor showing peculiar pharmacological and biophysical features.

Accepting the hypothesis that the $GABA_A$ channels are pentameric structures and considering the fact that 14 different subunits have been already discovered a huge number of pentameric combinations could be present in the native receptors; it seems however that some constraints exist limiting the number of possible combinations: γ and δ subunits for instance appear to be never expressed toghether. Several approaches have been used to sort out the combinatorial problem in "real life" and to correlate it with the biophysical properties of native receptors; the most important are in situ hybridization, immunocytochemistry and immunoprecipitation studies. It came out that each of the six α subunits has a peculiar transcription pattern in mammalian brain; as each of these subunits confers a unique functional profile to the receptor, at least six functionally different channels must be present. It must be added that in many places of CNS α subunit gene expression overlaps suggesting that different subunits could coexist in the same receptor complex thus further increasing the possible number of combinations. Moreover this matter is complicated by the fact that a developmental change in the subunit gene expression in the same

areas has been found in the rat brain where a developmental shift from α2 to α1 occurs. The information flux between expression and in vivo studies is thus bidirectional; on one side the comparison of the functional properties of cloned subunits to anatomical studies can explain the differences found when recording from native receptors; on the other side the knowlege of single subunit properties can help to envisage the possible composition of GABA receptors with peculiar features that have not been cloned yet.

As shown by Strata and Cherubini (1994), the period of time during which GABA is excitatory in neonatal hippocampus coincides with the presence of a GABA response that is bicuculline insensitive. Experiments on acutely dissociated hippocampal neurons were undertaken to understand the nature of this response.

Cultured cerebellar granule cells, on the other hand, have been shown to express both high and low affinity $GABA_A$ binding sites when cultured in the presence of ambient GABA (Meyer et al. 1984). Moreover membranes from cerebellum exhibit a bicuculline and baclofen insensitive GABA binding (Drew and Johnstone 1992). Nevertheless electrophysiological studies never showed any heterogeneity in $GABA_A$ receptors of these cells. As low potassium culture media were shown to promote the development of functional GABA synapses (Virginio et al. 1995), it was possible that this condition could lead to the expression of heterogeneous $GABA_A$ receptors in these cells; This hypothesis was tested in the second part of this thesis.

METHODS AND MATERIALS

ACUTELY DISSOCIATED HIPPOCAMPAL CELLS

The method for preparing acutely dissociated cells was derived from that of Kay and Wong (1986). Postnatal (P) day P0-P8 old wistar rats were anaesthetized via intraperitoneal injection of urethane (2g/Kg) and decapitated. The brain was quickly removed from the skull and put into a Petri dish containing ice cold standard Krebs solution continuously bubbled with 95% O_2 5% CO_2 gas mixture. where hippocampi were dissected free. Hippocampi were then cut into 350-500 μM (depending on the age of the rat) slices with a McIlwain tissue chopper. Slices were then stored for at least 30 minutes at room temperature in continuously oxygenated Krebs solution in which they could be mantained for up to 8 hours. A single slice at the time was then incubated for 35-60 minutes (depending on the age of the rat) at 30°C in 10 ml of oxygenated low (10 mM) chloride Krebs solution (SO_4^- substituted for Cl^-) containing 1 mg/ml pronase E and then washed at room temperature for at least 30 minutes in 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 obtained was then plated into the recording chamber bathed with working solution. Cells were usually viable for up to 90 minutes.

CEREBLLAR GRANULE CELL CULTURES.

Cells for cultures were obtained from seven days old wistar rats. Cerebella were removed under a sterile hood immediatly after killing the rats and placed in a Petri dish containing sterile solution 1.Meninges were peeled off under a dissection microscope and the tissue minced with a sterile razor blade. The minced tissue was then spun in an ALC 4227 centrifuge with a 0.186 m radius rotor at 1000 RPM for 1 minute.The pellet was resuspended in trypsin containing solution 2 and digested for 10 minutes at 37 degrees under continous shaking.Digestion was stopped by adding trypsin inhibitor (solution 3) and centrifugating again for 1 minute.DNase was put into the solution to prevent "packaging" of cells during centrifugation.

The pellet was resuspended in solution 4 and the tissue mechanically dissociated using fire polished Pasteur pipettes of different tip diameters.After that,solution 5 was added and cells were centrifugated for 5 minutes at 1000 RPM. The cell pellet was resuspended in Eagle's culture medium added by 10% heat inactivated foetal calf serum and diluted to 10^6 cells per milliliter.

Cells were then plated on 35 mm Petri dishes (2ml each) wich had been previously covered with poly-L-lysine (5μg/ml,Sigma).Neurons were stored in an incubator (Heareus) 5% CO_2 in air at 37^0 C.

The day after plating, a mitotic inhibitor (cytosine arabinoside furanoside 10μM, Sigma) was added to inhibit the growth of non neuronal cells. At the same time the conventional 25 mM potassium culture medium was substituted with a low (5 mM) potassium medium that was shown to allow the cells to develop spontaneous GABAergic activity (Virginio et al. 1995). After one week glucose (5.6mM finale) was added to dishes to provide energy source to cell as well as to restore the lose of water.

In these conditions it was possible to obtain a 90% cerebellar granule cells culture that was suitable for use starting on the second up to the fifteenth day in culture.

PATCH CLAMP.

Patch clamp recording tecnique originated about 15 years ago from an effort to record currents through individual ion channels in biological membranes. By the early 1970_s it had become clear that discrete molecular entities, integral membrane proteins,underlie the electrical signalling of nerve and muscles. At that time the electronics already permitted to detect current signals as low as the pico amperes (this magnitude had been inferred from analysis of current fluctuations at the neuromuscalar junction) but the noise to signal ratio was too high. Thus, to reduce noise, it was tempting to try to electrically isolate a small area of membrane ("patch") for a localized electrical measurement.The principle on which the tecnique lays is very simple. A glass capillary is pulled with a microforge and fire polished until the tip has a diameter of about 1μm. This pipette,filled by saline and connected to a very fast feedback amplifier,can form a high resistance seal onto cell membranes. The application of a slight suction (10-20 cm H_2O) readly increases the seal leading to the giga seal formation; this is a sudden and physically still not completely understood transition of seal resistence from megaohms to several gigaohms. The method first allowed to see the conformational changes of a single protein (i.e. the stochastic transition between the open and closed conformation of a single membrane ion channel). This is the most sensitive assay known for the study of protein conformational changes.

The way to measure these very small currents (10^{-12} A) is to monitor the voltage drop across a large resistor. So patch clamp set-ups have operational amplifiers with very high feedback resistance (50 GΩ) that implies higher gain. This, on the other hand, increases the noise of the system.In general, at equilibrium, any passive electrical network produces a noise whose spectral density can be expressed as (Sigworth 1983):

$$S = 4kTR_e\{Y(f)\}$$

where Y(f) is the frequency dependent admittance of the network,k is the Boltzmann's constant and T is the absolute temperature. In the patch clamp circuit eq 1.3 becomes

$$S_I = 4kT4\pi^2 \frac{RC^2}{1+4\pi^2 f^2 R^2 C^2}$$

of parallel RC circuit. So to reduce the noise it is possible either to increase R or to decrease C. In practice it is possible to increase R by obtaining a very high resistence seal (giga seal) and to decrease C by coating the pipette tips with sylgard. This is a low dielectric polymer that, furthermore, is highly hydrophobic and prevents also the increase in tip capacity due to creeping of solutions up the external wall of the pipette.

A second noise source is the membrane shot noise due to ions crossing the membrane by leakage or ion pumps. The spectral density of this noise is directly proportional to the unidirectional membrane current and therfore to the surface of membrane patch.

Another source of noise is the so called 1/f noise; this is a low frequency noise whose physical nature is not completely understood yet.

The patch clamp tecnique has four main configurations (fig.2.1)

The cell attached mode is largerly used and the most physiological since the cell is intact.In this configuration however, the actual membrane potential is unknown as the membrane resting

potential (unknown) must be summed to the pipette potential.

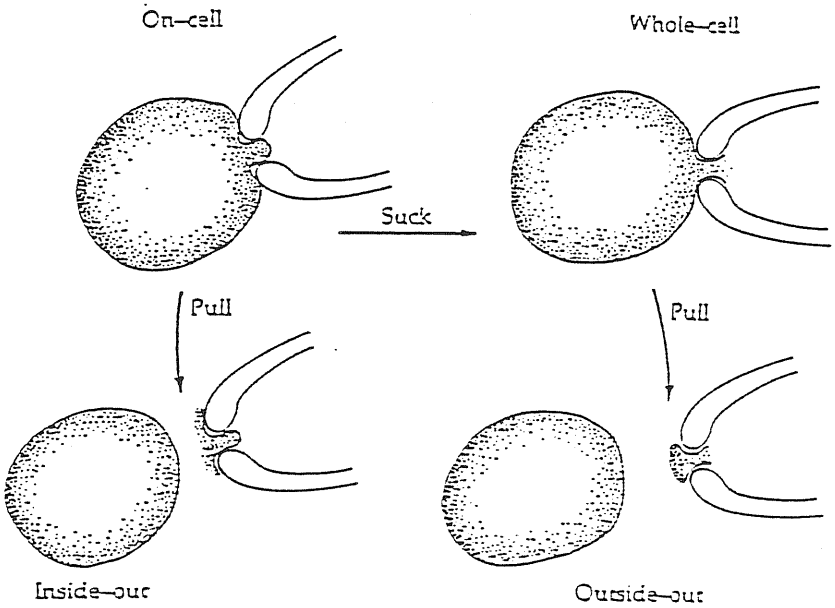

Figure 2.1

Patch-clamp main configurations. All methods starts from cell-attached (on cell) mode. Additional manipulation permits to obtain the other configurations.

(Adapted from Hamill et al. 1981).

The whole cell recording configuration allows to record macroscopic whole cell currents (from several hundreds of pA up to nA). It is obtained from the cell attached configuration: a further suction causes the membrane under the pipette to break; the internal of the cell is so dialized by pipette solution within few seconds (at least for readly diffusible ions).In this configuration, therefore, the dialysis of the intracellular components may lead to loss of modulators acting as second messengers in voltage and/or ligand gated channels.

In inside-out configuration the patch membrane is ripped off the cell and remains on the tip of the pipette. The inner side of the membrane is bathed in the dish so that it is possible to exchan-

ge "intracellular" components changing the bathing solution.

The outside-out configuration is obtained by tearing the pipette off a whole cell patch.The configuration is similar to inside-out but the outer side of the membrane is exposed to the bath; this configuration is mainly used for single channel recording of ligand gated channels.

Both the excised patch methods allows to exactly control the membrane voltage.

EXPERIMENTAL PROCEDURE

Patch pipettes were were pulled from 1.5 mm external diameter borosilicate capillaries (Hilgemberg, Germany) and fire polished to obtain a tip resistance (in working solutions) of 7-10 MΩ for single channel (outside-out) and of 2-5 MΩ for whole cell recordings. Pipettes for excised patches were then coated with Sylgard (Corning) to improve the electrical properties of the glass.

Acutely dissociated as well as cultured cells were observed under an inverted microscope (Zeiss) with Nomarski optics.

A microcomputer (Atari 1040 ST) and D/A converter were used to control the pipette potential. Currents were measured with a standard patch-clamp amplifier (List EPC-7). The outputs of the amplifier was filtered by a built-in 10 KHz 3 pole Bessel filter.One output fed the pulse code modulator (Sony) and then a video tape recorder (Sony ßmax).A second output was filtered by a Butterworth filter (Krohn-Hite 3202) at a cut off frequency of 1-3 KHz and then transferred to an oscilloscope and to the microcomputer by a 16 bit A/D converter (ITC-16 Instrutech) at a sampling time of 200-500 μs for single channel (both outside-out and whole cell noise analysis) and 10 ms for macroscopic currents whole cell recordings.

To prevent current run down the internal solutions contained Mg^{2+} and ATP (Stelzer et al. 1988). GABA, isoguvacine and all the other drugs were applied with a multibarrel perfusion system composed by gravity driven inputs whose openings were controlled by an electrovalves connected to an electronic selector (home mede) and a timer (Medical System Corp. USA). The control solution flow was continuous and stopped only while opening another way of the multibarrel device. Solutions were continuously removed by an aspiration system. The average flow rate in the experimental chamber was 3 ml/minute. With this method the 10 - 90% exchan-

ge time at the patch surface was between 150 and 300 ms and the solution in the dish was totally exchanged in about 90 seconds.

Bathing solution contained: (in mM) NaCl 137, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes-NaOH 10, Glucose 15 (pH 7.4) for acutely dissociated cells experments as well as for cultured cell experiments. Pipette solution for both whole cell and outside-out recordings contained: (in mM) CsCl 120, tetraethylammonium chlride (TEACl) 5, $MgCl_2$ 2, ATP-Na_2 2, $CaCl_2$ 1, EGTA 11, Hepes-TEA-OH 10, (pH 7.3) and CsCl 138, $MgCl_2$ 4, EGTA 5, ATP-Na_2 2, Hepes-TEA-OH 10 (pH 7.3) for hippocampal and cerebellar cells respectively.

In all the experiments liquid junction potentials were checked before and after the experiments; they were either eliminated by using an agar bridge or corrected "a posteriori". All the experiments were carried out at room temperature (22-24^0 C.).

DATA ANALYSIS

Whole cell currents were analyzed either with commercial programs TAC and REWIEW (Instutech) run on an Atari 1040ST microcomputer or with quick basic (Micosoft corporation) written programs developed in our laboratory and running on PC compatible computers.

The decay kinetics of currents were fitted by one or two exponentials according to the following equation where A_2=0 if only one exponential was fitted.

$$I = A_1 \exp -t/\tau_1 + A_2 \exp -t/\tau_2$$

dose/response data were plotted on semilogarithmic scale and fitted using either the simple

logistic Hill equation:

$$I = \frac{I_{max}}{1+(k/c)^n} \qquad \text{Eq.2.4}$$

or, when two components were present, the sum of two Hill functions:

$$I = \frac{I_{max}}{1+(k/c)^n} + \frac{1-I_{max}}{1+(k'/c)^{n'}} \qquad \text{Eq.2.5}$$

where I_{max} represents the normalized maximum current, k is the agonist concentration activating one half of the receptors (Ec50), c is the actual agonist concentration and n is the Hill coefficient that indicates the existence of cooperativity when >1.

The time course of the recovery from desensitization could be fitted with either one or the sum of two exponential functions:

$$R = 1-(Ae^{-t/\tau_1} + Be^{-t/\tau_2}) \qquad where\ A+B=1 \qquad \text{Eq.2.6}$$

where t is the interval between two paired pulses τ_1 and τ_2 are the time constants of the exponential functions, and R is the fraction of the control current that is activated by the agonist after the time t.

Single channels were analyzed using the TAC program (Instrutech) written by F.J. Sigworth. This program uses the 50% criteria for the detection of channel opening.

Open and closed times histograms were fitted with one or more exponential functions according to the method of Sigworth and Sine (1987).

If not otherwise stated, data in this thesis are presented as mean ± SEM.

MEASUREMENT OF SINGLE CHANNEL CONDUCTANCE BY STATIONARY NOISE ANALYSIS

The amplitude of single channel events was calculated also by stationary noise analysis obtained from the analysis of current fluctuations. (Neher and Stevens 1977, Conti and Wanke,1975, DeFelice 1978, Dempster 1994). This method, derived from fluctuation analysis used in statistical physics, allows to estimate amplitude and mean open time of single channel events from macroscopic current recordings.

Two restrictive assumptions are usually necessary for this calculations: i) ionic channels open and close independently. ii) the channels can exist only in two states, either open or closed.

If we consider a cell membrane containing n ion channels each one having a probability p of being open at any given time and capable of passing a current i, then the mean macroscopic current flowing through the membrane is:

$$I = npi \qquad \text{Eq. 2.7}$$

The variance of the current around this mean is:

$$\sigma^2 = i^2 np(1-p) \qquad \text{Eq.2.8}$$

This is a parabolic equation in p. The function showes a maximum for p = 0.5 and two minima for p = 0 and for p = 1, meaning that if all the channel are always open or always closed there

are no current fluctuations at all.

Combining equations 2.7 and 2.8 gives

$$\sigma_I^2 = iI - \frac{I^2}{n} \qquad \text{Eq.2.9}$$

if p is small ($p < 0.1$) or n is large (i.e. there are many channels in the patch) the second term is neglegible and we obtain

$$\sigma_I^2 = iI \qquad \text{Eq.2.10}$$

a proportionality between the variance and the mean current.

It follows that, if p is small or n is large, the unitary current passing through a single channel is given by:

$$i = \frac{\sigma_I^2}{I} \qquad \text{Eq.2.11}$$

and, assuming ohmic behaviour of the channel, the single channel conductance is given by:

$$\gamma = \frac{\sigma_I^2}{I}(V_m - V_r) \qquad \text{Eq.2.12}$$

It is useful to bear in mind that even if these data come from whole cell recordings the sampling

time needed to acquire the data for the analysis of the variance must be quite fast; the current signal fluctuations in fact are spread over a wide range of frequencies that must be represented in the recorded data. The digital sampling interval, t, is determined by the desired upper frequency limit f_h according to the Nyquist theorem as follows:

$$t = \frac{1}{2f_h} \qquad \text{Eq.2.13}$$

Obviously the recorded noise (and the current as well) consists of two components: the background noise due to the instrumentation and to the opening of leak conductances and the signal noise due to the opening of the channels under investigation (in our case the channels opened by the application of the neurotransmitter). Before making the above described calculations it is thus necessary to obtain the "pure" data to process; this can be done by subtracting the background noise, recorded in the very same conditions but before the application of the neurotransmitter, from that recorded at the steady state of the transmitter evoked current.

METHOD OF LEAST SQUARES LINEAR REGRESSION FITTING

This method is used to find the straight lines that best fitted the experimental data. Lets consider a set of experimental points each defined by (x_i,y_i) and a straight line that passes through these points. Assuming that the variable x_i is choosen by the observer and has no error, any error which does occur will be in the observed experimental value y_i. Lets define d_i the difference between each experimental point ($y_i = mx_i + c$) and the corresponding point on the fitted line ($y = mx + c$). If the line exactly passes through the points then $d_i = 0$. However if two points lie off the line by equal amounts but on opposite sides d_i will still be zero giving the false impres-

sion of a perfect fit. For this reason the measure of the goodness of the fit is given by the sum of the squres of all the d_i. This function of d_i is given by:

$$D = \sum_{i=1}^{n} d_i^2 = d_1^2 + d_2^2 + \ldots + d_i^2 + \ldots d_n^2 \qquad \text{Eq.2.14}$$

and can be used as a measure of the goodness of the fit.

Conversely the two parameters m (slope) and c (intercept) can be varied in order to minimize D. As

$$d_i = y_i - (mx_i + c) \qquad \text{Eq2.15}$$

then

$$D = \sum_{i=1}^{n} (y_i - mx_i - c)^2 \qquad \text{Eq.2.16}$$

which is a function of the two parameters m and c. To have a good fit requires to minimize the value of D that means to find the minimum of the function. This can be done equating the two partial derivatives $\partial D/\partial m$ and $\partial D/\partial c$ to zero.

As

$$\frac{\partial D}{\partial m} = \sum_{i=1}^{n} 2(y_i - mx_i - c)(-x_i) \qquad \text{Eq.2.18}$$

and

$$\frac{\partial D}{\partial c}=\sum_{i=1}^{n} 2(y_i-mx_i-c)(-1) \qquad \text{Eq.2.19}$$

equating expressions 2.18 and 2.19 to zero and considering that the mean values of the x_i and y_i are given respectively by:

$$\bar{x}=\frac{1}{n}\sum_{i=1}^{n} x_i \quad , \quad \bar{y}=\frac{1}{n}\sum_{i-1}^{n} y_i \qquad \text{Eq.2.20, Eq.2.21}$$

hence

$$\sum_{i=1}^{n} x_i Y_i - m\sum_{i=1}^{n} x_i^2 - (\bar{y}-m\bar{x})\sum_{i=1}^{n} x_i = 0 \qquad \text{Eq.2.22}$$

and thus

$$m = \frac{\sum_{i=1}^{n} x_i y_i - \bar{y}\sum_{i=1}^{n} x_i}{\sum_{i=1}^{n} x_i^2 - \bar{x}\sum_{i=1}^{n} x_i} \qquad \text{Eq.2.23}$$

once m has been determined c is given by:

$$c = \bar{y} - m\bar{x} \qquad \text{Eq.2.24}$$

Unfortunately, analytical least squares solutions can only be obtained for mathematical functions that are linear functions of the independent variable. These include the quadratic function and higher order polynomials; to interpolate any polynomial:

$$Y = a+bx+cx^2+dx^3+\ldots \qquad \text{Eq.2.25}$$

we must calculate the minimum of the function:

$$\sum_{i=1}^{n} (y_i-Y)^2 = \sum_{i=1}^{n} (y_i-a-bx_i-cx_i^2-dx_i^3-\ldots)^2 \qquad \text{Eq. 2.26}$$

Some functions that are not themselves linear can be transformed into related linear functions. This is the case of the monoexponential function:

$$Y(t) = Ae^{(-\frac{t}{\tau})} \qquad \text{Eq. 2.27}$$

Taking the natural logarithm of the equation we obtain:

$$\ln(y) = \ln(Ae^{-\frac{t}{\tau}}) \qquad \text{Eq.2.28}$$

that can be expressed as:

$$\ln(y) = c+mt \qquad \text{Eq. 2.29}$$

To this purpose, a very used transform is the logistic:

$$y' = \log \frac{y}{100 - y} \qquad \text{Eq. 2.30}$$

that is often used in processing pharmacological data.

However it is often impossible to obtain convenient analytical solutions for best fitting of non linear functions. In this case a numerical solution is required. Data contained in this thesis were processed using the Simplex method (Nelder and Mead, 1965).

RESULTS

1- hippocampus

The whole cell configuration of the patch clamp technique was used to record currents activated by GABA and isoguvacine (a compound structurally related to GABA selective agonist for $GABA_A$ receptors) in pyramidal neurones acutely dissociated either from neonatal (P0-P7) or from older (>P12) rats. As shown in the example of fig. 3.1A, application of 100 µM GABA at a holding potential of -50 mV evoked, in 150 mM symmetrical chloride concentration, an inward current that peaked in about 400 ms and then slowly declined to a steady state level.

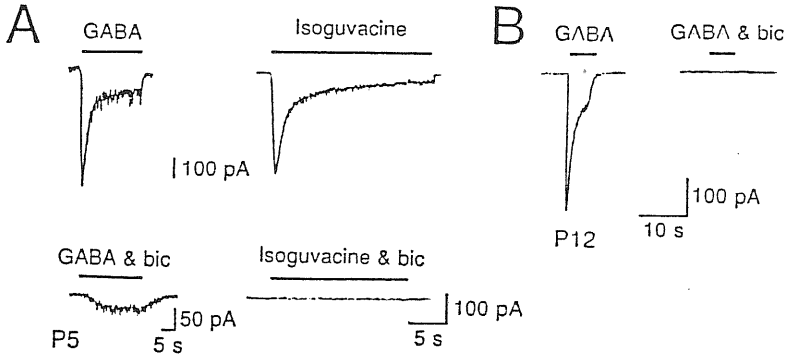

Fig. 3.1 GABA-mediated bicuculline-insensitive Cl--dependent currents
A. Whole cell currents induced by GABA (100 µM) and isoguvacine (50 µM) in the absence or in the presence of bicuculline (100 µM). Holding potential -50 mV. B. At P12 the current elicited by GABA (100 µM) was abolished by bicuculline (100 µM).

The mean amplitudes of the peak currents induced in five neurons by 100 and 500 µM GABA at P3-P5 were 512 ± 137 pA and 913 ± 161 pA respectively. Isoguvacine mimicked the effects of GABA. Bicuculline (100 µM) completely abolished the response to isoguvacine but only reduced, even if consistently, the response to GABA (see fig 3.1A). The mean amplitude of the peak currents induced by 100 and 500 µM GABA in the presence of 100 µM bicuculline were 60 ± 13 pA (n=10) and 364 ± 102 pA (n=8) respectively. There was a trend for bicuculline-resistant GABA responses to disappear with age. After P12 GABA activated currents were always completely and reversibly blocked by 100 µM bicuculline (fig 3.1B). The amplitude of the bicuculline insensitive current was dependent on GABA concentration. The Hill plot for the steady state currents gave an EC_{50} of 120 µM and Hill number of 1.86, thus showing a certain degree of cooperativity (see fig 3.2).

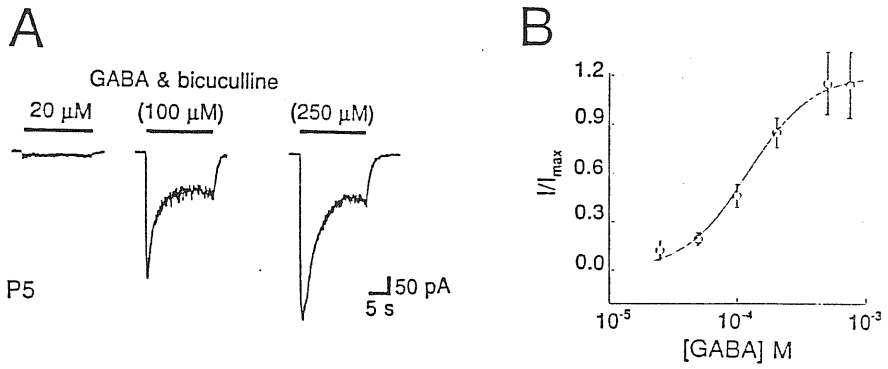

Fig. 3.2 A. GABA currents elicited in the presence of bicuculline (100 µM) by different GABA concentrations. Vh = -50 mV.
B. Dose response curve of the bicuculline insensitive GABA currents. Each point
represents the mean of 7 experiments. Error bars represent the standard deviation.

As it had been suggested that the conformationally restricted analogue cis-4-aminocrotonic acid (CACA) interacts with binding sites that are insensitive to bicuculline and baclofen (Drew and Johnston 1992), we have examined the effects of this compound on our preparation. CACA (300-1000 μM) activated both bicuculline-sensitive and bicuculline-insensitive responses and therefore was not selective for this novel response. The bicuculline resistant response to GABA was blocked by picrotoxin (fig.3.3). The amplitude of GABA (500 μM, n=3) current was reduced to 40 ± 8% and 24 ± 7% in the presence of 10 and 30 μM of picrotoxin respectively. The current was completely abolished by 100 μM of picrotoxin.

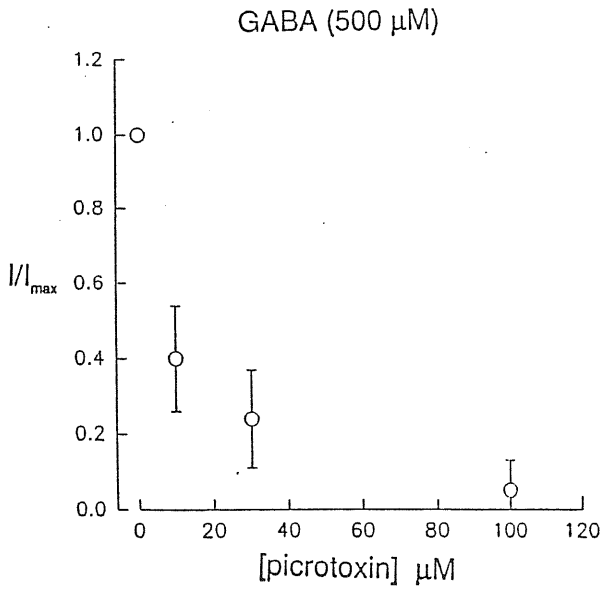

Fig. 3.3 The bicuculline insensitve GABA currents are blocked by picrotoxin. Whole cell currents evoked by GABA (500 μM) in the presence of bicuculline (100μM) and incresing concentrations of picrotoxin (normalized to the response in the absence of picrotoxin). Bars represent the S.D. (n=3).

The bicuculline insensitive GABA current was carried by chloride ions. In symmetrical chloride solutions the reversal potential was -0.5 ± 3 mV (n=12, fig. 3.4). This value was very close to 0 mV, the chloride equilibrium potential predicted from the Nernst equation for these conditions. The current voltage relationship was linear in the potential range between -50 and 30 mV (see fig. 3.4B). When extracellular chloride concentration was reduced from 150 to 50 mM (gluconate or sulfate substitution) the reversal potential shifted from -0.5 ±3 to 27 ± 5 mV in good agreement with the behaviour predicted by the Nernst equation.

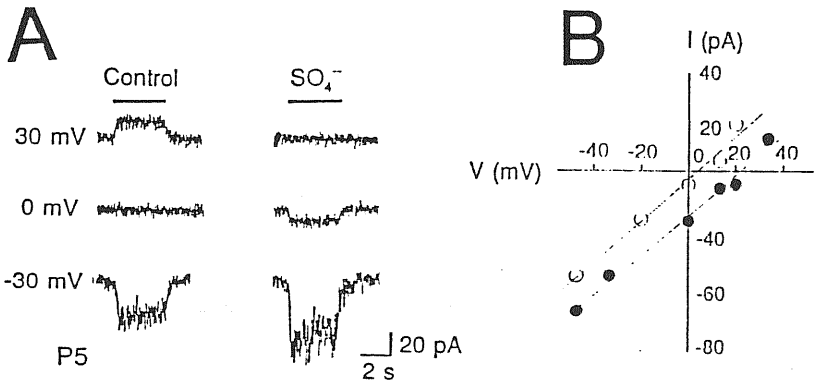

Fig. 3.4 A. Responses to GABA (100 µM) obtained at different membrane potentials in the presence of bicuculline (100 µM) in 150 mM (left) or 50 mM (right) external Cl-. Extracellular NaCl was partially substituted with Na_2SO_4.
B. Relationship between the membrane potential and the amplitude of bicuculline-resistant GABA currents shown in A, recorded in symmetrical (○) or asymmetrical (●) chloride solutions.

This novel GABA response was further characterized by analyzing the desensitization and the time constants of the recovery from desensitization (τ_r). The ratio between the peak (Ip) and the steady state (I_s) GABA current elicited in the absence or in the presence of bicuculline was significantly ($P<0.05$, Student's t-test) different (fig. 3.5A). For instance the peak to plateu ratio of the currents induced by 100 and 1000 µM GABA shifted from 4.6 ± 0.4 and 17.7 ± 2.6 in the absence of bicuculline to 1.5 ± 0.1 and 3.1 ± 0.5 in the presence of bicuculline.

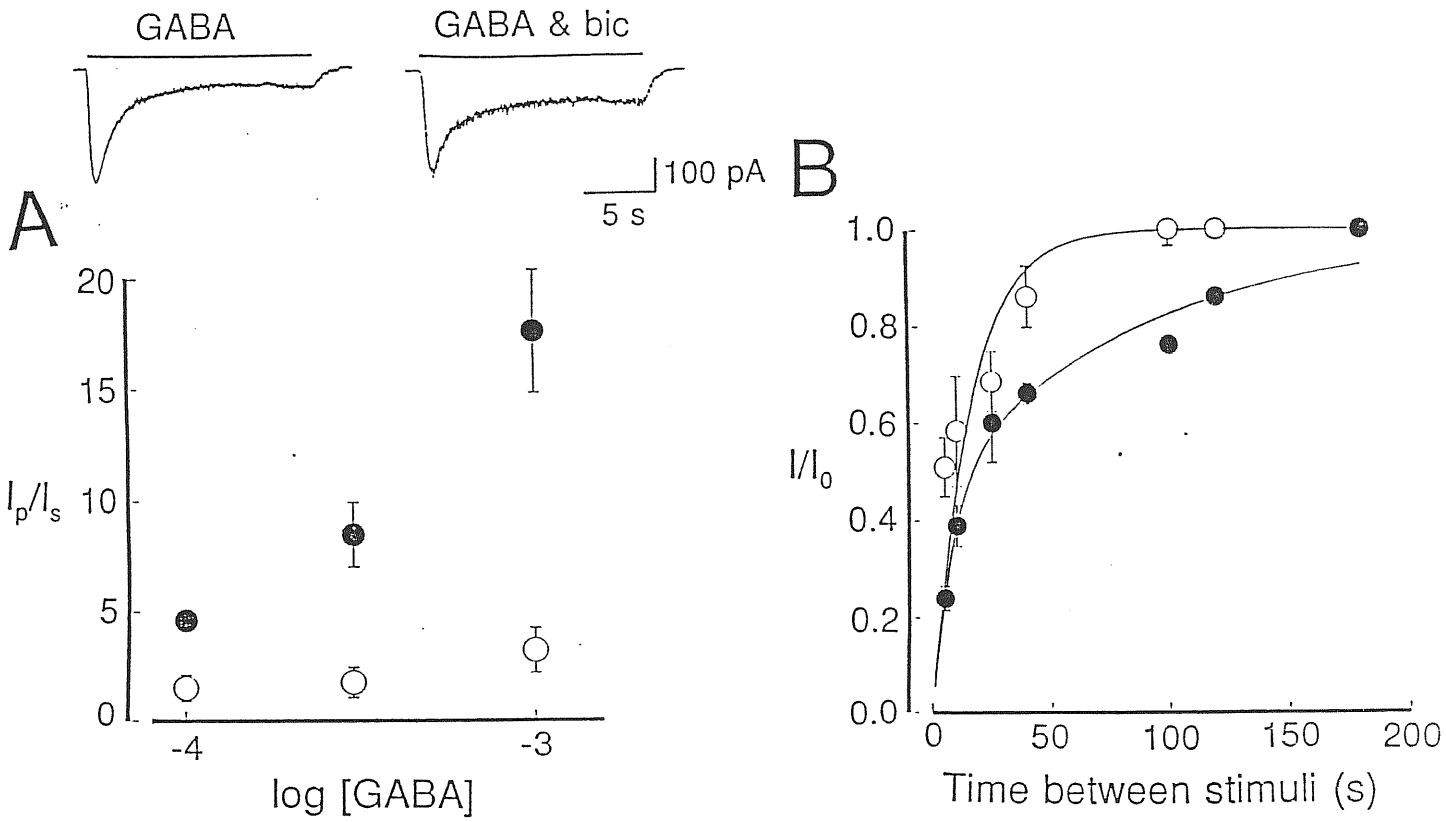

Fig. 3.5 Slow desensitization kinetics of bicuculline-insensitive GABA currents.
A.Plot of the peak (I_P) to plateau (I_S) ratios of whole cell currents evoked by different GABA concentrations in the absence (•) or in the presence (○) of bicuculline (100-200 µM).
Each point represents the mean of 5-7 experiments. Standard deviation error bars are within the symbols.
B. Recovery from desensitization, express as the ratio I/I_0 versus time between stimuli in the presence (○) or in the absence (•) of bicuculline (100 µM). I_0 represents the peak amplitude of the response to the first GABA (100 µM) application and I the amplitude of the peak current elicited by GABA application at time t after the first stimulus. The best fitting of the data points was obtained with the sum of two exponentials (with time constants of 8.5 and 98.6 seconds for bicuculline-sensitive and 0.1 and 17.6 seconds for the bicuculline insensitive GABA current). Each point represents the mean of 4-8 experiments; error bars represents the standard deviation.

As desensitization of GABA current could be dependent on the ampitude of the peak response, the different I_P to I_S ratio could be dependent on the absolute magnitude of the responses. To overcome this problem, we compared the ratio between I_P and I_S of responses of the same amplitude obtained in different experimental conditions. A significantly ($P<0.01$) different ratio (5.4 ± 0.6 and 3.2 ± 0.4, see fig. 3.5A) corresponded to responses of equal peak amplitude (172 ± 35 pA and 196 ± 61 pA, n=10, in the absence or presence of bicuculline respectively). The value of the time constants of recovery from desensitization of whole cell GABA currents evoked in the presence of bicuculline at a holding potential of -50 mV significantly differed from those found for $GABA_A$ currents (Fig. 3.5B).

Single channel currents

The outside out configuration of the patch clamp technique was used to study the bicuculline resistant GABA mediated currents at single channel level. In the presence of 100 to 200 μM bicuculline GABA (20 μM) activated multiconductance state channels (fig. 3.6A); in most (n=6) cells two main conductance states of 14 ± 0.5 and 30 ± 1 pS were observed. In two other cells, however, a main conductance state of 23 pS was found. The mean open time was 1.4 ± 0.4 ms at -70 mV. Conductances and mean open time were similar to those previously found in the same preparation for $GABA_A$ receptor channels (Hosokawa et al. 1994). an estimation of the single channel conductance was also obtained by analyzing, in eight different experiments, the membrane noise at the steady state of the macroscopic GABA current obtained in the presence of bicuculline (Fig 3.6C). Fig. 3.6D shows the plot of the conductance variance versus the mean membrane conductance. The linear regression fit gives an estimate of a single channel conductance value of 17 pS. This value is compatible with the values of the conductance states found in single channel experiments.

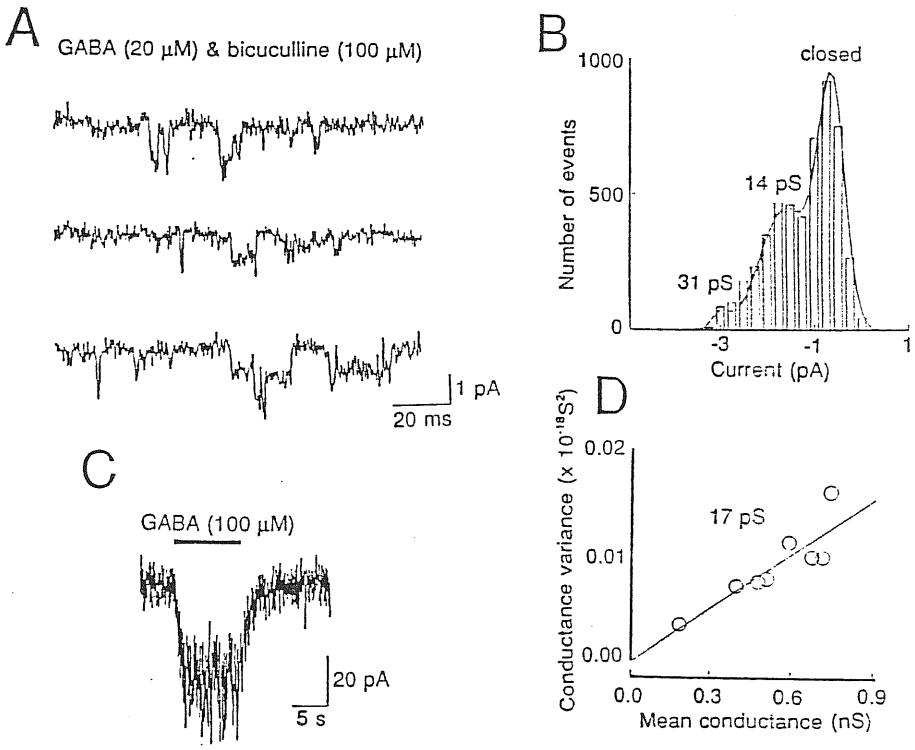

Fig. 3.6 Single channel conductances of bicuculline-insensitive GABA currents.
A. Continuous recordings of single channel currents (outside out configuration) activated at P5 by GABA (20μM) in the presence of bicuculline (100μM). Holding potential -70 mV. Band width 1 kHz. B. Amplitude histograms of single channel currents shown in A. C. Whole cell GABA current elicited at P5 in the presence of bicuculline (100 μM) from a holding potential of -60 mV. Note the increase of the current noise level. D. Relationship between mean membrane conductance, μ_g, and the conductance variance, σ_g^2, produced by applications of GABA (100μM)in the presence of bicuculline (n=6). The slope of the plot gives a single channel conductance of 22 pS.

BICUCULLINE SENSITIVE OR INSENSITIVE GABA ACTIVATED CHLORIDE CURRENTS OF DEVELOPING HIPPOCAMPUS ARE DIFFERENTLY MODULATED BY ZINC

We tested the effects of zinc on both the bicuculline sensitive and the bicuculline-resistant response to GABA. Zinc inhibited bicuculline-sensitive GABA currents in a concentration dependent way. The effect of zinc had a rapid onset and a slower wash out. As shown in the example of Figure 3.7A, the whole cell current evoked by GABA (50 μM) was completely blocked by zinc (1 μM). On average, zinc (1 μM) almost completely blocked the responses elicited by 50 μM GABA whereas it only reduced the responses evoked by 100 μM GABA.

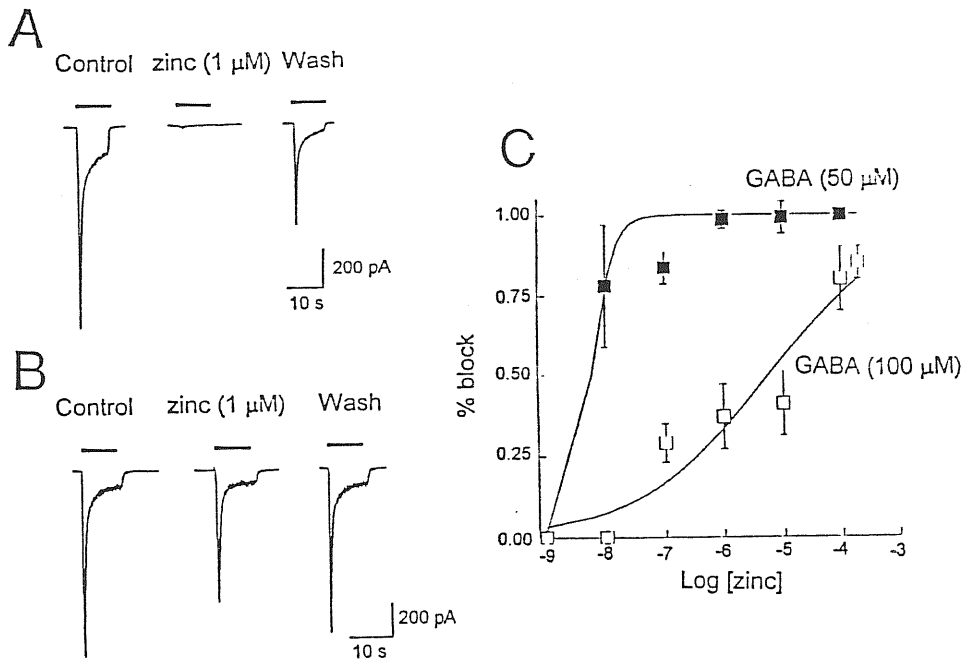

Fig. 3.7 Zinc inhibition of $GABA_A$ currents in hippocampal pyramidal neurons.
A. Responses evoked by 50 μM GABA are completely abolished by zinc (1μM).
B. Responses evoked by 100 μM GABA are much less sensitive to zinc inhibition.
C. Plot of the zinc blocking effect versus zinc concentration for currents activated either by 50 or by 100 μM GABA. The extrapolated EC_{50} were 6.6 nM and 5.8 μM for currents elicited by 50 or 100 μM GABA respectively.

In Figure 3.7 C whole cell currents elicited by GABA (50 and 100 µM) in the presence of increasing concentrations of zinc, normalized to the control responses are plotted versus different zinc concentrations. The value of the EC_{50}, extrapolated by fitting the data to the logistic Hill equation (see methods) was 6.6 nM (n=5) for currents activated by 50 µM GABA.

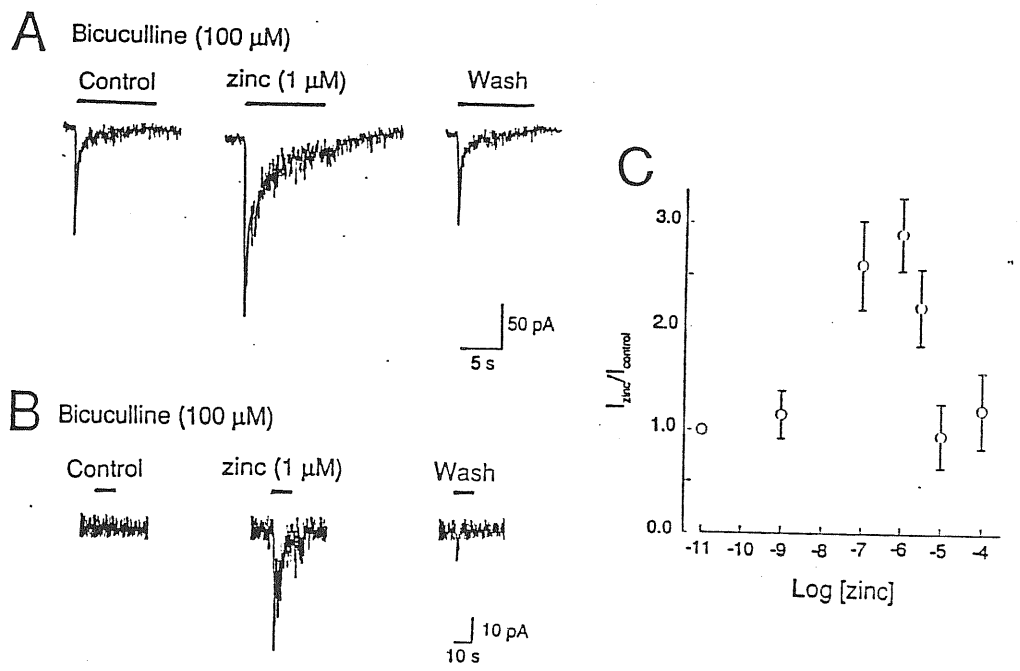

Fig. 3.8 Zinc potentiates the bicuculline insensitive GABA responses.
A. Bicuculline (100µM) resistant currents elicited in a P4 neuron By 100 µM GABA are potentiated by low zinc concentrations.
B. Sometimes the addition of zinc could elicit responses to GABA (100 µM) in cells in wich bicuculline (100 µM) had completely abolished the response. Vh = -50 mV.
C. Plot of the bicuculline insensitive currents (normalized to the response in the absence of zinc) versus the applied zinc concentrations. The curve is bell shaped with a maximum for 1 µM zinc. Each point represent the mean of 5-9 cells. Error bars represent the SEM.

In hippocampal neurons (Legendre and Westbrook, 1991) as well as in many other preparations (Smart and Constanti 1990, Smart 1991, Kilic et al. 1993), the effect of zinc on GABA currents was non competitive.In particular it was found that, on cultured hippocampal neurons, a similar zinc inhibition was obtained on chloride currents activated by GABA concentrations ranging from 2 to 200 μM (Legendre & Westbrook, 1991). Interestingly, in our experiments, the zinc inhibition of the currents evoked by 100 μM GABA was much less effective (Figure 3.7C), the extrapolated EC_{50} value being 5.8 μM, (n=9).

Zinc was also tested on GABA currents which persisted in the presence of bicuculline (100 μM). Surprisingly this divalent cation at very low concentrations (nanomolar range, the same that inhibited the $GABA_A$ currents) markedly potentiated the bicuculline-resistant responses to GABA (up to 3 fold, Figure 3.8C).

In four cells (out of nine) in which whole cell GABA currents were completely abolished by bicuculline (100 μM), in the presence of 100 nM of zinc, GABA (100 μM) was still able to evoke inward currents (Figure 3.8B), that, in comparison with those elicited in the absence of bicuculline, had different kinetics. Zinc (100 nM) *per se* did not produce any change in membrane conductance or inward current. Zinc (100 nM) failed to restore the response to isoguvacine (50 μM) when this was blocked by 100 μM bicuculline (Fig. 3.9).

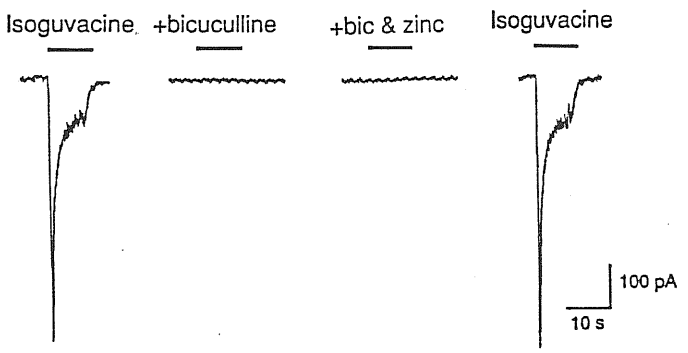

Fig. 3.9 Zinc did not compete with bicuculline for a common binding site.
Zinc could not restore responses to isoguvacine (50 μM) that had been abolished by bicuculline (100 μM). This fact suggests that zinc does not interfere with bicuculline binding. Vh = - 50 mV.

In a few cases (n= 2) in which the bicuculline block was incomplete, addition of zinc (1 μM) caused a further reduction of isoguvacine currents. Moreover, zinc did not change the reversal potential for GABA that in our experimental conditions (symmetrical chloride solutions) was close to 0 mV before and after zinc application. These data suggest that zinc potentiates responses to GABA mediated by a receptor distinct from the conventional $GABA_A$. To test whether other transition metals were capable of modulating bicuculline-insensitive GABA currents we tested the effects of cadmium. As zinc, cadmium (100 nM) also potentiated the bicuculline-resistant GABA responses but in a less effective way (26 ± 9%, n=3). For concentrations from 1 μM to 10 μM zinc was less effective or ineffective. At higher concentrations zinc had a moderate inhibitory effect on bicuculline insensitive currents also (n = 2, not shown). As shown in Figure 3.8C, the plot of the bicuculline-insensitive GABA currents elicited in the presence of increasing concentration of zinc and normalized to the control responses versus increasing concentrations of zinc is bell shaped with a peak at 1 μM.

The potentiating effect of zinc on bicuculline-resistant currents seemed to have little, if any, voltage dependence (n=3, fig. 3.10).

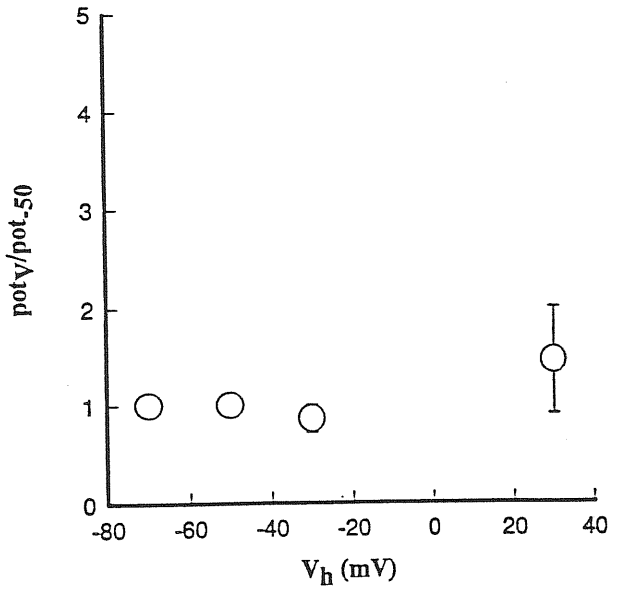

Fig 3.10 The zinc potentiation of bicuculline resistant GABA currents is not voltage dependent. The potentiation values were normalized to that one obtained at Vh = -50 mV and plotted versus the applied holding potentials. Error bars represent the SEM (n=3).

2- cultured cerebellar granule cells.

The whole cell and outside out configuration of the patch clamp technique were used to study GABA evoked currents in cerebellar granule cells cultured in low (5mM) potassium medium.

Whole cell experiments

In a first set of experiments we studied the macroscopic currents evoked by GABA.

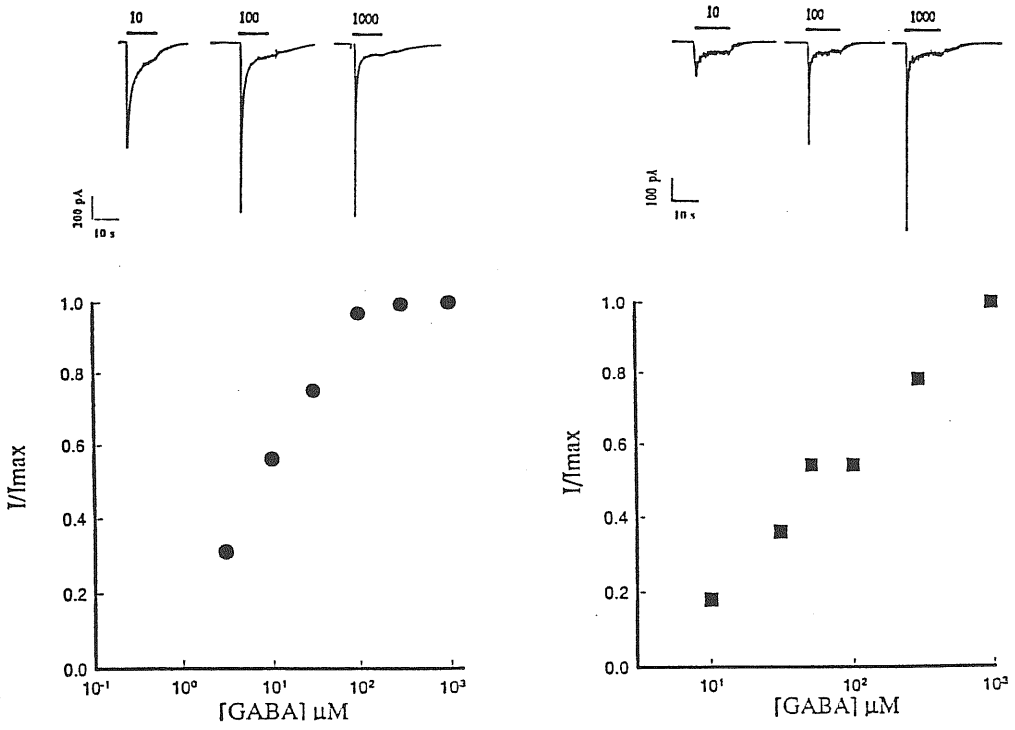

Fig. 3.11 Dose/response plot for GABA obtained in two different cells.
A. This cell only expressed high affinity GABA receptors. The EC_{50} was 7.6 µM and the response was saturated at 100 µM GABA.
B. This cell showed biphasic behaviour and the dose/response curve could be fitted with the sum of two Hill functions; EC_{50} were 13 and 255 µM for the high and low affinity component respectively. Note the plateu in the curve for GABA concentrations equivalent to those that were saturating for the cell in A. No saturation was evident for GABA concentrations up to 1 mM.
In the insets above the plots: currents evoked in two neurons by the GABA concentrations indicated above the bars. Note the different sensitivity to GABA of the two cells.

At a holding potential of -50 mV, in symmetrical chloride solutions, GABA induced inward currents whose amplitude and kinetics were dependent on the concentration of the agonist applied.

Plotting the peak amplitude of GABA responses versus agonist concentrations revealed two distinct populations of cells. As shown in the example of Figure 3.11, in the first population the dose dependence of the normalized currents was described by a sigmoidal curve. The threshold GABA concentration for a detectable current was in the range of 0.5 - 1μM and saturating responses were obtained for GABA concentrations around 100 μM. For instance, for the cell in figure 3.11A the concentration eliciting half-maximal response (EC_{50}) was 7.6 μM. In the second population, detectable currents were still obtained with low threshold concentrations of GABA (3 μM). The dose response curve of GABA currents, however, after reaching a first plateau, started to increase again with higher agonist concentrations (see example of Figure 3.11B). Normalized peak current amplitudes were distributed along two sigmoidal curves with different apparent affinities for GABA. In the example of figure 3.11B the EC_{50}s were 19 and 280 μM for the first and second component respectively. In a few cases (n= 3), cells expressing only the low affinity GABA responses were found (not shown).

A two sigmoidal dose response curve was obtained when results from the whole set of cells were pooled together (Figure 3.12). Data points were fitted with the sum of two logistic Hill equations. EC_{50} values were 13 and 255 μM for the high and low affinity component respectively. Interestingly, peak amplitude responses obtained with increasing concentrations of isoguvacine, a structural analogue of GABA, selective agonist for $GABA_A$ receptors, were distributed along a single sigmoidal curve (Figure 3.13). Data points obtained pooling together data from six different neurons were fitted with the logistic Hill equation. The EC_{50} was 16 μM, a value very

close to that obtained for the high affinity GABA component, suggesting that at least one population of GABA responses was mediated by $GABA_A$ receptors.

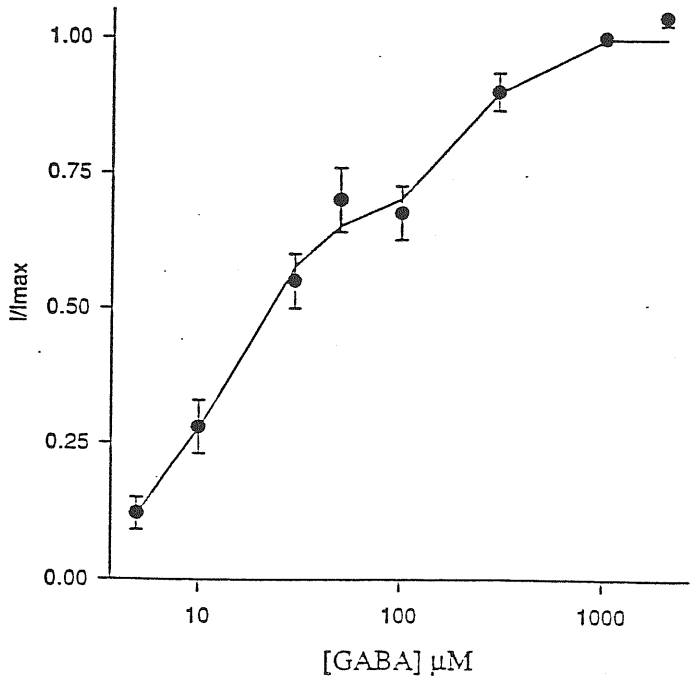

Fig. 3.12 Dose/response plot for GABA obtained in the entire cell population. The curve showed a bimodal behaviour and could be fitted with the sum of two Hill functions. Ec_{50} were 13 and 256 µM for the high and low affinity component respectively. A clear plteau was present for GABA concentrations around 50 µM. Each point represents the mean of 9 - 22 cells. Bars represent the SEM.

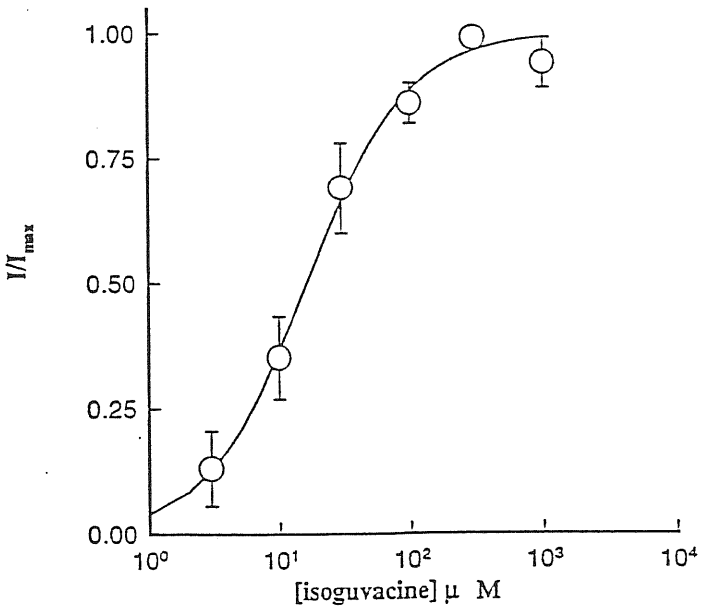

Fig 3.13 Dose/response plot for isoguvacine. The curve showed monophasic behaviour. EC_{50} was 16.3 µM, very similar to that of the first component of GABA responses. Each point represents the mean of 6 different experiments. Bars represent the SEM.

If we assume that, in some neurons, GABA activates more than one receptor whereas isoguvacine only activates one receptor, a saturating concentration of isoguvacine should give a response which should not be occluded by co-application of GABA. A typical experiments is shown in Figure 3.14. When a submaximal concentration of GABA (300 µM for the cell in figure) was applied after complete desensitization of the response to a saturating concentration of isoguvacine (100 µM in the example), GABA was still able to induce a small response of about 40 pA. Occlusion did occur however, when the same concentration of isoguvacine was applied during the desensitizing phase of GABA-evoked current.

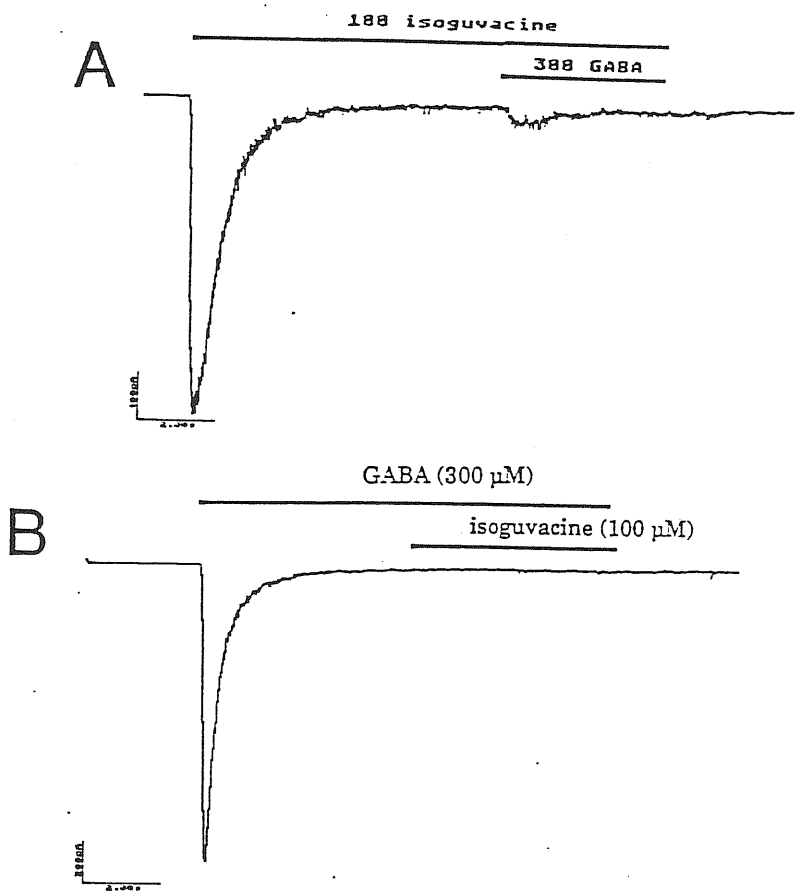

Fig. 3.14 Cross desensitization experiments.
A. GABA concentrations (300 µM) supposed to activate both the high and the low affinity receptor could still evoke a response when applied after complete desensitization of the current evoked by a long lasting application of a saturating concentration (100µM) of isoguvacine.
B. In the same cell, on the contrary, isoguvacine (100 µM) was not able to evoke any response after complete desensitzation of the response to GABA (300 µM). Vh = -50 mV.

Similar results were obtained in 2 cells, in which a GABA current was still elicitable during complete isoguvacine desensitization. In three additional neurons GABA responses were completely occluded by isoguvacine ones.

Because bicuculline is a selective $GABA_A$ receptor antagonist, known to compete with GABA binding, in the following experiments the sensitivity of high or low affinity GABA responses for this antagonist was tested. As shown in the examples of Figure 3.15 A, in 3/25 cells bicuculline (10 μM), completely abolished the response to GABA (100 μM). This effect was reversible upon wash out of the antagonist. In others cells (3 out of 25) the same concentrations of bicuculline only slightly reduced the peak amplitude of GABA evoked response and no further reduction in peak current amplitude was obtained when a ten fold higher concentration of the antagonist (100 μM) was applied. Among these extremes, a third population of responses was found in which the antagonizing effect of bicuculline was evident (30 ± 3 % reduction for 10 μM bicuculline, n=19 out of 25) but never complete. In Figure 3.15B, whole cell current evoked by GABA (100 μM, n= 25) in the presence of increasing concentrations of bicuculline and normalized to the control responses are plotted against different antagonist concentrations. It is clear from the figure that bicuculline exhibited at least two different antagonist efficacies depending on the presence of high or low affinity responses. The first ones showed maximum blocking effect for concentrations of bicuculline ranging between 1 and 3 μM whereas the others for concentrations two order of magnitude higher (range around 100 μM). In both cases however, the inhibitory effect of bicuculline was, on average, never complete. The blocking effect of bicuculline on responses elicited by 10 μM GABA, supposed to selectively activate only the high affinity population of GABA receptors, was also tested. Antagonist sensitivity of responses to GABA 10 μM was then compared to that of responses to 100 μM GABA, supposed to

activate both high and low affinity receptors (Figure 3.16). As shown in the figure, a non paral-

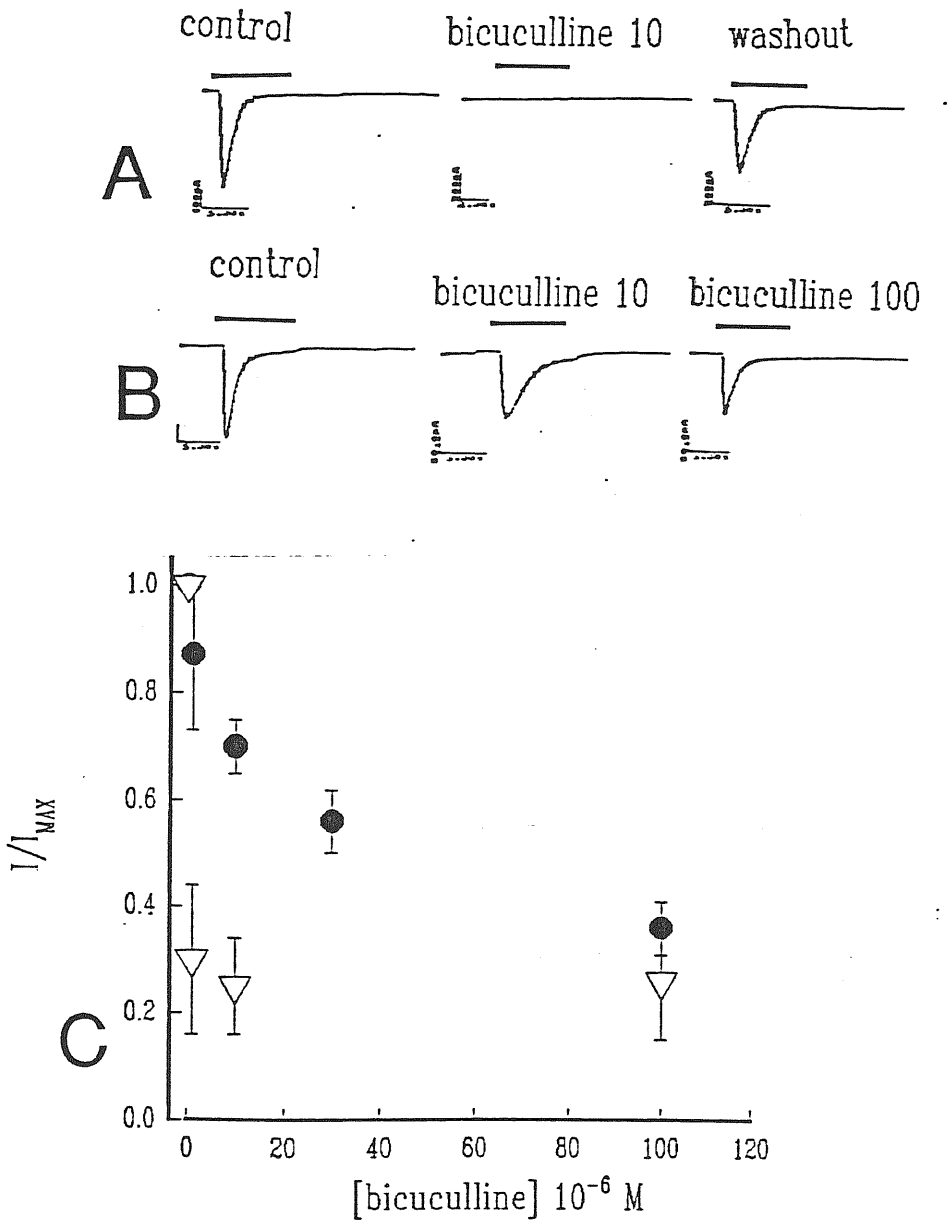

Fig. 3.15 Bicuculline sensitivity of the GABA responses was highly heterogeneous.
A. In 3 out of 25 cells bicuculline (10 μM) completely abolished the response to GABA (100 μM).
B. In 3 other cells responses to 100 μM GABA were insensitive to bicuculline concentrations up to 100 μM.
C. Amplitude of GABA (100μM) currents obtained in the presence of different concentrations of bicuculline and normalized to the response obtained in the absence of bicuculline plotted versus bicuculine concentrations. Circles represent cells that were less sensitive to bicuculline (n=16-21).
Triangles represent cells that were more sensitive to bicuculline (EC_{50} < 3μM, ν=6). Note that both curves saturate before reaching the zero current level.
Error bars represent the SEM.

lel shift in the regression lines fitted to the data points obtained in the two experimental conditions was obtained, giving support to the hypothesis that two (or more) different GABA receptors having different sensitivity to the antagonist were activated by different GABA concentrations. The dose/response curve for GABA was repeated in the presence of bicuculline (100 μM). Peak amplitude responses were distributed along a single sigmoidal curve and an EC_{50} value of 209 μM was obtained (Figure 3.17).

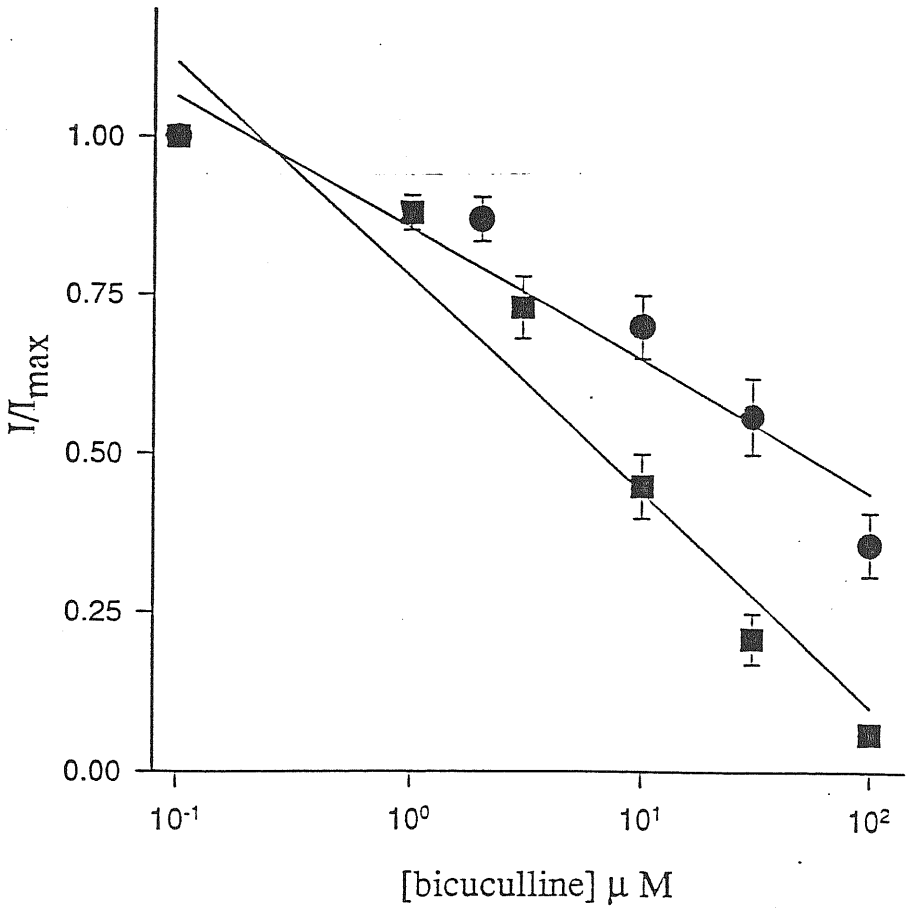

Fig. 3.16 Dose/response plots to bicuculline for currents activated either by 10 (supposed to activate only the high affinity GABA receptor, squares, n = 9) or by 100 μM GABA (supposed to activate both high and low affinity receptors, circles, n = 16). Note the non-parallel shift in the regression lines fitted to the experimental data. Bars represent the SEM.

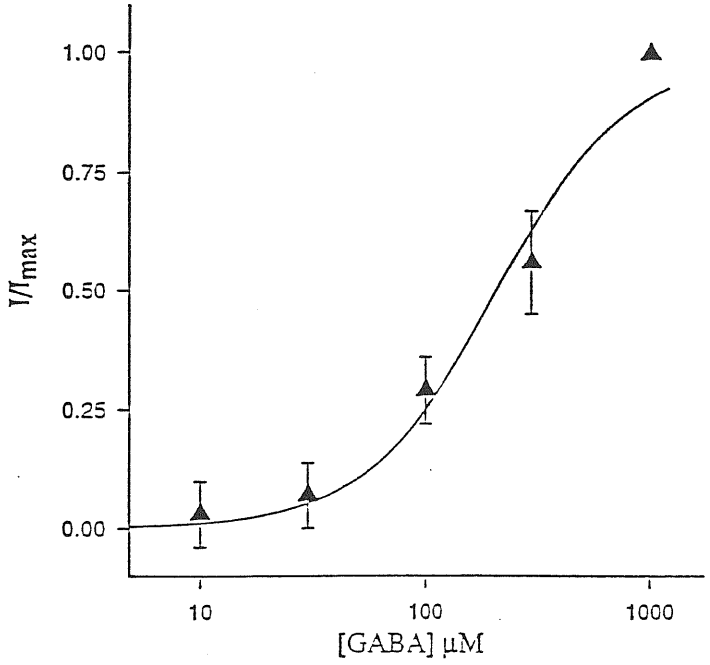

Fig. 3.17 Dose/response curve for GABA currents activated in the presence of 100 µM bicuculline. Note that data could well be fitted with a single Hill function. The EC_{50} for GABA was 209 µM, in good agreement with the value obtained for the second component of the curve obtained in the absence of bicuculline (256 µM). This fact suggests that the receptors showing high affinity for GABA are inhibited by 100 µM bicuculline. Bars represent the SEM.

This value was in good agreement with that one calculated for the second component of the dose response curve obtained in the absence of bicuculline. Picrotoxin (100 µM), a well known non competitve antagonist of GABA evoked chloride currents (Yoon, 1993), induced a further reduction of the bicuculline-resistant GABA response in a reversible way.

Thus these data further support the notion that in cerebellar granule cells cultured in low potassium medium one or more GABA receptor subtypes can be distinguished on the basis of their sensitivity to bicuculline. Therefore in the following experiments we have focussed our attention only on the bicuculline-resistant GABA responses.

Also the bicuculline resistant currents were chloride mediated as shown by their reversal potential, very close to that predicted by the Nernst equation for chloride permeant channels and from

their sensitivity to picrotoxin (not shown).

One feature of the bicuculline-resistant chloride-mediated GABA responses recently described in retinal horizontal cells is the lack of desensitization (Qian and Dowling, 1993). We have therefore investigated whether a similar property was also present in low potassium cultured cerebellar granule cells.

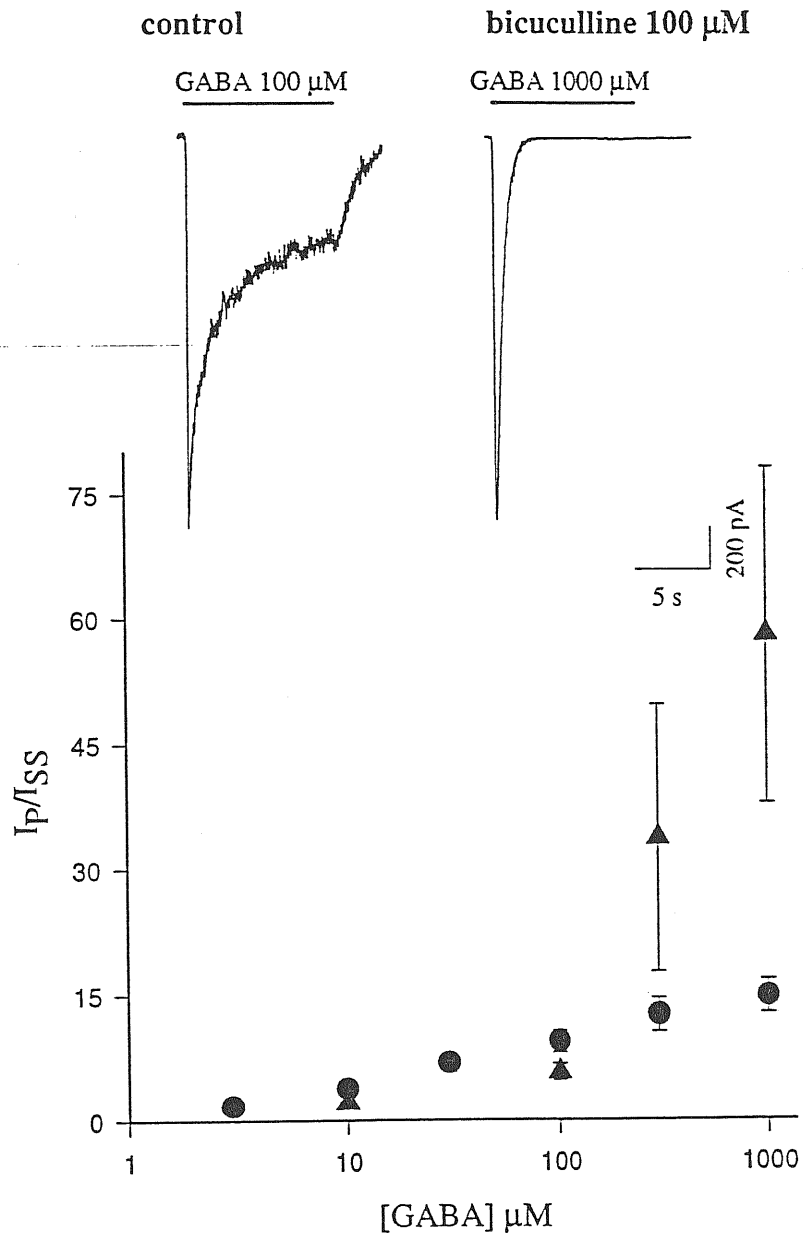

Fig 3.18 Desensitization is different in bicuculline sensitive and insensitive receptors.
Plot of the peak to plateu ratio (I_P/I_{SS}) versus GABA concentration of currents elicited either in the absence (circles) or in the presence (triangles) of 100 µM bicuculline. Note that the behaviour became different only for GABA concentrations that activate the low affinity GABA receptor. This fact suggests that the low affinity GABA receptor currents are unmasked by the bicuculline block of the high affinity receptors. Error bars represent the SEM.

Desensitization was measured as the ratio between the peak (I_P) and the steady state current (I_{SS}) elicited by increasing concentrations of GABA in the presence or in the absence of bicuculline. In cases of complete desensitization, in which the decay of GABA-evoked currents was very fast and reached the zero current level (see inset of Figure 3.18) the ratio between I_P and I_{SS} was arbitrarily considered 100 (instead of infinite). The ratio between I_P and I_{SS} of bicuculline sensitive and insensitive responses was significantly ($P<0.05$, Student's *t*-test) different when currents were elicited by concentrations of GABA higher than 100 μM (Figure 3.18). For instance, the peak to plateau ratio induced by 0.3 or 1 mM GABA was 12.6 ± 4.4 and 14.8 ± 3.6 in the absence or 33.7 ± 10 and 58 ± 18 in the presence of bicuculline, respectively (n = 6). These data further indicate that a low affinity GABA receptor type, that has different bicuculline sensitivity and kinetics and is activated by high (EC_{50} - 200 µM) GABA concentrations, was unmasked in the presence of bicuculline. As it could be that the steady-state current is proportional to the peak current, we compared also the peak to steady-state ratios obtained from two pools of cells showing equiamplitude responses to GABA (490 ± 49 and 528 ± 29 pA for currents evoked in the presence or in the absence of bicuculline respectively, n = 6, P = 0.49, Student's *t*-test). Also in this case the ratio I_P/I_{SS} was significantly different being 27.6 ± 8.5 and 3.8 ± 0.7 for currents recorded in the presence or in the absence of bicuculline respectively ($P<0.02$, Student's *t*-test).

Single channel experiments

In additional experiments from excised outside out patches (n= 5), the responses to GABA, obtained in the presence of bicuculline (100 μM) were studied at the single channel level. Figure 3.19A shows bicuculline-insensitive GABA-activated single channel currents recorded at three different holding potentials.

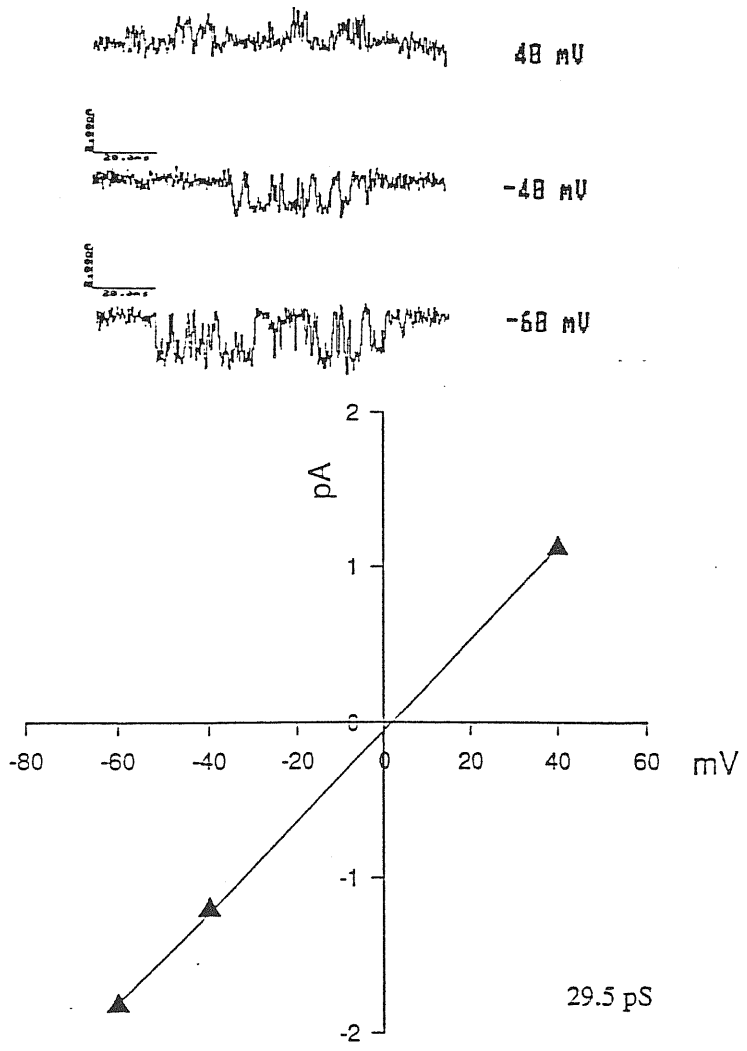

Fig. 3.19 Single channel conductance of bicuculline resistant GABA currents.
A. outside out recordings obtained in symmetrical chloride solutions at three different holding potentials exposing the membrane to 20 µM GABA in the presence of 100 µM bicuculline.
B. I/V plot from the same traces. The reversal potential was close to 0 mV (as expected for a chloride mediated current). The main conductance state was 29.5 pS.

The I/V relationship was linear in the potential range between -70 and 30 mV and, as expected for symmetrical chloride solutions, the reversal portential was close to 0 mV (fig.3.19B). A main single channel conductance of 29.5 ± 1.2 pS (n= 5) was found. Subconductance states of 19 ± 1 pS were sometimes present. These values are in good agreement with those obtained in cerebellar granule cells cultured in high potassium medium when GABA was applied in the absence of bicuculline (Kilic et al., 1993, Martina et al., 1994). Moreover there was no difference in the conductance level of channels activated (in the presence of 100 μM bicuculline) either by 20 or by 100 μM GABA (see Fig. 3.19 and 3.21).

GABA-activated spontaneous synaptic currents

Cerebellar granule cells grown in a low potassium medium exhibit spontaneous GABA-mediated synaptic currents (Virginio et al. 1995). In the presence of kynurenic acid (1 mM) to block the excitatory drive through glutamatergic ionotropic receptors, in 10 out of 13 cells these currents were completely and reversibly abolished by bicuculline (10 μM). In the remaining 3 cells bicuculline (10 μM) only partially blocked the spontaneous events. No further reduction was obtained when an increased concentration of bicuculline (30 μM) was applied. Addition of tetrodotoxin (TTX, 1 μM) in bicuculline revealed spontaneous miniature currents that occurred at a much lower frequency. These currents were chloride dependent since, as the bicuculline-sensitive ones, they reversed around 0 mV, as expected for symmetrical chloride solutions, and were abolished by 100 μM picrotoxin (figure 3.20). These currents, moreover, were insensitive to strychnine (1 μM).

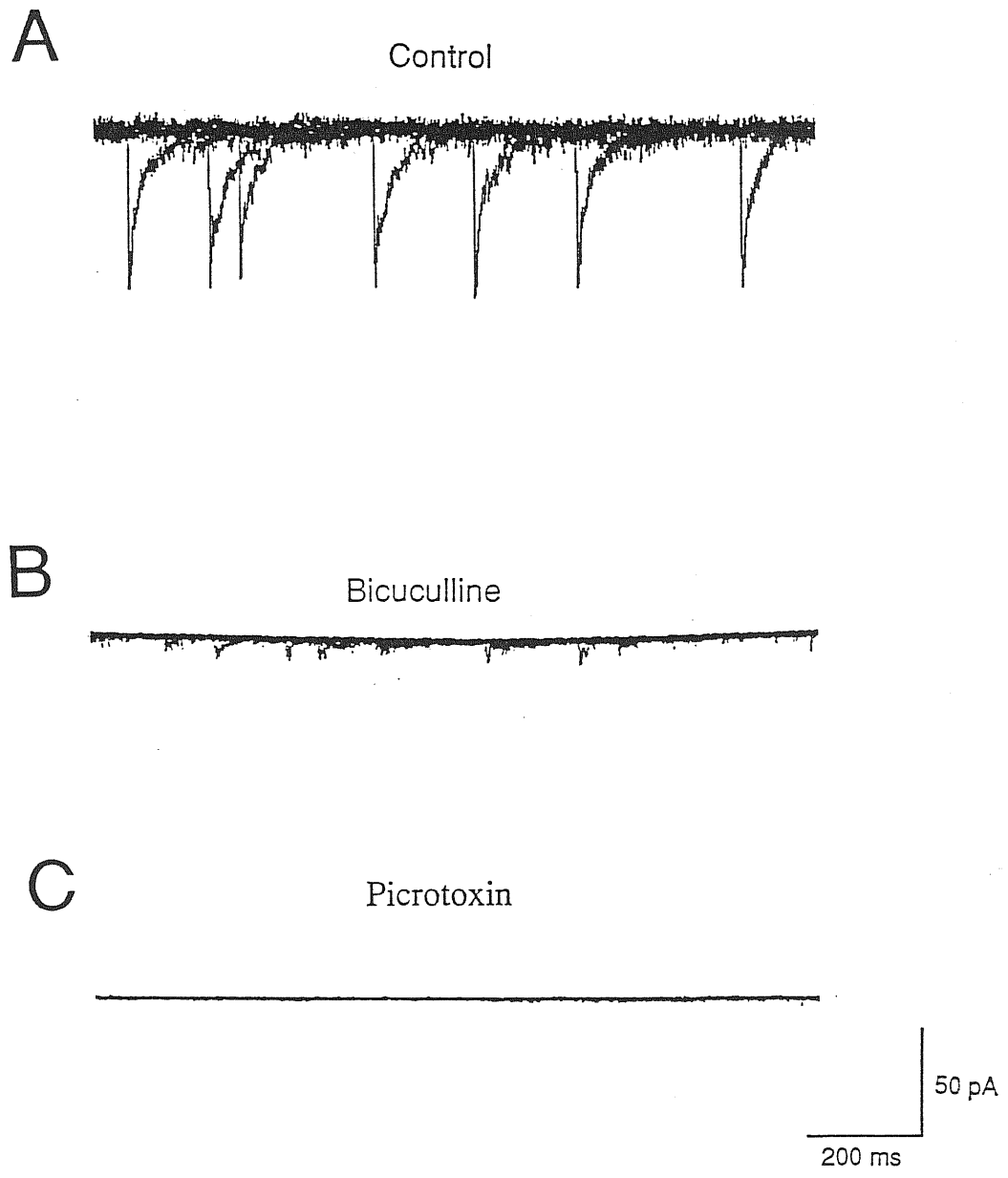

Fig. 3.20 Spontaneous bicuculline sensitive and insensitive GABA mediated spontaneous synaptic currents.
A. Synaptic currents recorded in the absence of bicuculline (V_h = - 50 mV, symmetrical chloride solutions).
B. Bicuculline (30 µM) reduced the amplitude of spontaneous events but did not abolish them.
C. Picrotoxin (100 µM) completely abolished the sponatneous synaptic activity.

In low noise recordings (n=2), channel closures could be resolved directly during the decay phase of spontaneous miniature postsynaptic currents. The estimated single channel conductance was 29 pS, a value that is in good agreement with that found for the bicuculline resistant single channel events activated by exogenously applied GABA (figure 3.21).

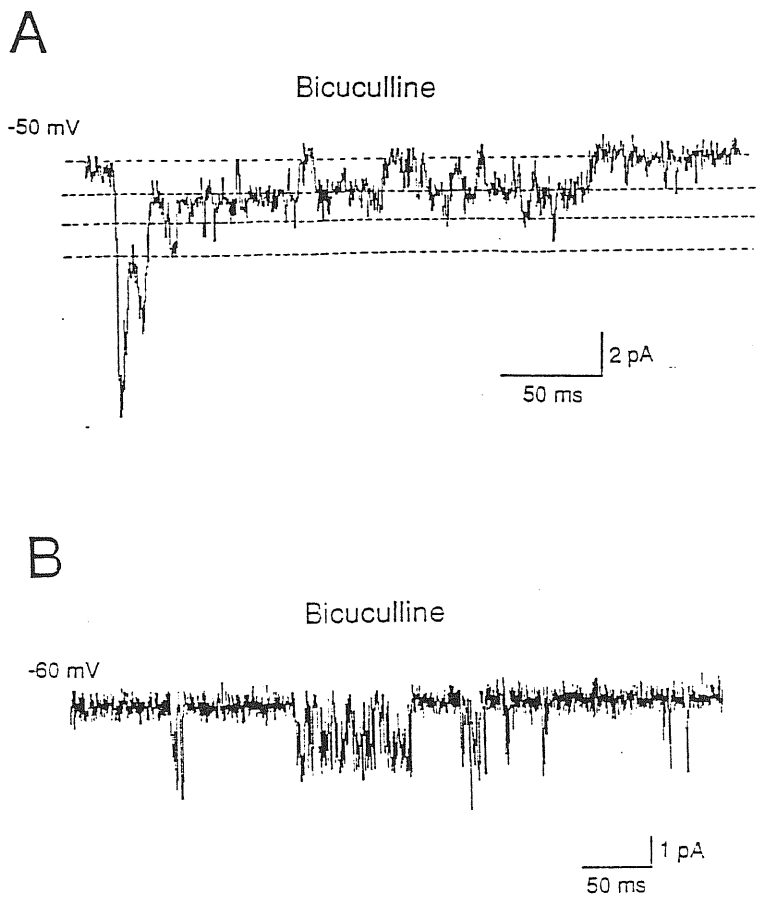

Fig. 3.21 A. Low noise recording of bicuculline resistant synaptic activity. In the decay phase single current steps could be resolved. The single channel conductance (29 pS) is very similar to that one of channels evoked, in the presence of bicuculline (100 µM), in a outside out patch by exogenous application of 100 µM GABA (B).

Discussion

1- Acutely dissociated hippocampal neurons.

The present data show that in acutely dissociated neonatal hippocampal neurons, GABA elicits two distinct chloride dependent currents: the classical $GABA_A$ activted current defined by its conventional inhibition by the competitive antagonist bicuculline and a new one, insensitive to bicuculline but sensitive to picrotoxin. This current which is probably mediated by a novel receptor type is transiently expressed in the hippocampus where it mediates a bicuculline insensitive depolarization during a critical period of postnatal development (Strata and Cherubini 1994). For its characteristics this new current resembles that one recorded from fish and rat horizontal and bipolar cells (Feigenspan et al. 1993; Qian and Dowling 1993), from frog optic tectum (Sivilotti and Nistri 1985), from guinea pig superior colliculus (Arakawa and Okada 1988) or from Xenopus oocytes injected with bovine retinal mRNA (Polenzani et al. 1991; Cutting et al. 1991). In contrast to our findings however, in rat retinal bipolar cells, GABA activated only a low conductance channel clearly different from that obtained for $GABA_A$ receptor in the same preparation (Feigespan et al 1993). It is conceivable that this channel is an hetero-oligomeric protein assembled from the recently cloned $\rho 1$, which is highly expressed in the retina (Cutting et al. 1991) in combination with another subunit, maybe the β one of the glycine receptor (Feigespan et al. 1993). In our experiments, although we

can not rule out the presence of the ρ type low conductance channel, the most frequently occurring channels had conductance states similar to those obtained for $GABA_A$ receptors. This suggests that the subunits composition of the new receptor type does not affect the pore of the channel itself. The combination of α5, β2 and γ2 was identified as the minimal requirement reproducing consensus properties of the vertebrate $GABA_A$ receptor channel (Sigel et al. 1990). The α subunit carries an important part of the agonist antagonist binding site (Sigel et al. 1992). In this respect, the novel response to GABA described here, is reminiscent of that obtained in Xenopus oocytes injected with an alfa 1 subunit in which position 64 was occupied by Leu instead of Phe (Sigel et al. 1992). This mutation changed the binding properties of the receptors for the agonist and antagonist. Different isoforms of GABA receptors with distinct pharmacological properties may subserve different functions. The functional significance of this new receptor type is still unclear. It is however possible that, due to the slow desensitization and fast recovery from desensitization, GABA will prolong the membrane depolarization favouring calcium entry through voltage activated channels. This calcium signal may be essential for synaptogenesis during a critical period of postnatal development. In support to this hypothesis is the recent finding of a developmentally regulated increase in intracellular calcium by GABA which is not mediated through conventional bicuculline-sensitive, chloride-linked GABA receptors (Segal 1993).

Modulation of bicuculline sensitive and insensitve chloride dependent GABA currents by zinc.

The experiments in this thesis show that zinc depressed bicuculline-sensitive GABA currents while potentiating the bicuculline resistant ones.

The depressing effect of zinc on $GABA_A$ mediated responses in the hippocampus as well as in other brain regions has been already well documented (for a review see Smart et al. 1994).

Zinc inhibited the responses to GABA (50 μM) in a non-competitive way. The sensitivity of GABA responses to zinc seem to be inversely related to the expression of the γ_2 subunit (Draguhn et al. 1990; Smart et al, 1991). The IC_{50} value for zinc, found in the present experiments, was more than three order of magnitude lower than that obtained in reconstituted $GABA_A$ receptors lacking the γ_2 subunit (Drauguhn et al. 1990; Smart et al. 1991), suggesting that in early postnatal life different subunit combinations apparently lacking the γ_2 subunit are present in native $GABA_A$ receptors. Alternatively the presence of the α_2 subunit (instead of the α_1) which is particularly abundant during development may confer maximal zinc sensitivity also to GABA receptor complexes containing β and also the γ_2 subunits, (White and Gurley, 1995). However, this hypothesis seems to clash with the observation that in our experiments GABA currents were insensitive to benzodiazepines (Strata & Cherubini, unpublished observations; see also Rovira and Ben Ari, 1991) whose effects depend on the presence of the γ_2 subunit (Pritchett et al. 1989). The much lower potency of zinc blockade in the case of 100 μM of GABA (in comparison with 50 μM) is compatible with the activation by GABA of two distinct, high and low affinity, receptors sensitive or insensitive to bicuculline respectively as discussed above. Thus, because the concomitant potentiating effect of zinc on the low affinity receptor type, the blocking effect of this divalent cation will be

less effective. The most striking and novel finding is the potentiation of bicuculline resistant GABA currents by very low concentrations of zinc. An enhancement of GABA responses by zinc has been already described by Smart and Constanti (1990) in olfactory cortex neurons. In those experiments however, very high concentrations of zinc were used (at least 3 to 4 order of magnitude higher than those used in our experiments), consistent with the view that zinc, like barium, would change the electrical properties of the neuron, following a reduction in resting membrane conductance mainly at the dendritic level (Smart and Constanti, 1990). Moreover zinc did not produce any shift in membrane current as in the case of hippocampal neurons in culture where facilitation of neurotransmission depends on the inhibition of a background cationic conductance (Nakazawa et al., 1995). Interestingly, zinc was also able to restore GABA response once this was completely abolished by bicuculline. This effect can not be due to displacement of bicuculline by zinc, since, in bicuculline, zinc failed to restore the response to the $GABA_A$ receptor agonist isoguvacine. As the blocking effect, also the potentiating action of zinc was voltage independent suggesting that its binding site was not in the membrane's electric field. Although the mechanisms underlying the inhibitory or potentiating effect of zinc are still unclear, it seems plausible that this divalent cation modulates bicuculline sensitive or resistant GABA currents acting on two distinct binding sites possibly located on the extracellular domains of the channel protein.

It is worth to note that, in contrast to our results, the $GABA_C$ respones observed in cone horizontal cells acutely dissociated from the catfish retina (Dong and Werblin, 1995) or in *Xenopus* oocytes expressing ρ_1 type of GABA receptor subunit (Calvo et al. 1994) were downregulated by zinc. This suggests that the bicuculline resistant, baclofen insensitive GABA receptors transiently expressed in the hippocampus during a critical period of postnatal development are different from those present

in the retina.

In conclusions, the down or up regulation of bicuculline sensitive or insensitive receptors by zinc may be functionally important in regulating GABA-mediated synaptic activity during development.

2- cultured cerebellar granule cells.

Cerebellar granule cells express a heterogeneous population of high and low affinity GABA receptors.

From the dose response curves to GABA, at least two populations of receptors could be detected, one having high and the other low affinity for the agonist. In spite of the same morphological features, the two populations could either be present on distinct cells or coexist on the very same cell. A third cell population expressing only low affinity receptors was much less frequent. When data from the entire cell population were pooled together a bimodal behaviour clearly appeared (see fig. 3.11). The similarity of the values of the EC_{50} obtained for the high affinity GABA component and the isoguvacine response suggests that they should act on the same receptor. These values, moreover, fit with data obtained on cells grown in high potassium medium (Kilic' et al. 1993). Further support to the hypothesis of the coexistence of two different receptor populations on the same neuron is given by the occlusion experiments in which responses to submaximal concentrations of GABA could still be obtained when the receptors were completely desensitized by a long lasting application of isoguvacine at saturating concentrations.

Bicuculline blocks GABA responses with different efficacies.

GABA responses were also highly heterogeneous regarding their sensitivity to bicuculline. The results ranged from a complete inhibition of the response to a very low sensitivity. Again the whole population response was probably the result of the sum of the features of two (or more) distinct receptors. Interestingly when the dose-response curve to GABA obtained in the presence of 100 μM bicuculline (that should be saturating for one type of receptor) was plotted an EC_{50} value similar to that of the second component of the response obtained in the absence of bicuculline was found. This suggests that bicuculline binds with high affinity to receptors showing high affinity for GABA. A different bicuculline sensitivty for the high or low affinity GABA receptor is further supported by experiments in which the dose response relationship to bicuculline was tested on currents evoked by two different GABA concentrations (10 or 100 μM) supposed to activate either the high affinity or both the high and low affinity GABA receptor types. If only one type of receptor was present one would expect a parallel shift in the regression lines fitted to the experimental data due to the fact that an increasing bicuculline concentration just causes a reduction of the actual effective GABA concentration. However this was not the case; there is a clear difference in the slope of the lines fitted either to the 10 μM or to the 100 μM GABA points that implies the existence of at least two different receptors with different affinities for the agonist and different sensitivity to the bicuculline effect.

Similarly to the high affinity GABA receptors, the low GABA affinity bicuculline resistant receptors gated chloride permeable channels, as shown by the reversal potential of the currents (that was very close to the equlibrium potential for chloride ions) and by their sensitivity to picrotoxin.

Desensitization properties of high and low affinity GABA receptors differ.

A typical feature of the bicuculline insensitive $GABA_C$ receptors recently described in the retina (Qian and Dowling, 1993; Feigespan et al., 1993) is the lack of desensitization. Moreover, heterogeneous desensitization properties have already been described for GABA responses obtained from homogeneous preparations (Pearce,1993, Schonrock and Bormann 1993). A difference in the desensitization properties of high and low affinity receptors was thus likely. To address this item the peak to steady state ratios (taken as an indicator of the desensitization process) of currents elicited by increasing GABA concentrations either in control condition or in the presence of 100 μM bicuculline were compared (fig. 3.18). It is worth to stress that for GABA concentrations lower than 100 μM (the threshold for the activation of the low affinity receptor) the data obtained in the presence of bicuculline only showed the parallel down-shift expected for the competitive action of bicuculline; for higher GABA concentration on the contrary an abrupt change in the slope appeared proving that at this concentration a second receptor type was recruited. The data of figure 3.18 can be explained assuming that the high affinity GABA receptors are less desensitizing than the low affinity ones; this is shown in the plot for controls by the increase in the size of the standard error of the mean bars for GABA concentrations higher than 100 μM; in the presence of bicuculline and for GABA concentrations high enough to activate the low affinity GABA receptor, that is also less sensitive to the inhibition by bicuculline, there is an unmasking effect that uncovers the strong desensitizing behaviour of the low affinity GABA receptor. This interpretation is supported by the fact that bicuculline seems to almost selectively affect the steady-state component of the currents.

Single channel conductances of the different receptors are the same

It was tempting to envisage that the activation of the different GABA receptors could gate chloride channels having different single channel conductances (Brickley et al. 1995). Excised patch experiments showed however that the main conductance value was for both the bicuculline sensitive and insensitive receptors the same that was already observed in cerebellar granule cells cultured in high potassium medium (Kilic' etal. 1993, Martina et al. 1994).

Spontaneous bicuculline resistant GABAergic currents.

Cerebellar granule cells cultured in low potassium medium develop spontaneous GABAergic synaptic activity (Virginio et al. 1995). Interestingly in 3 out of 13 cells the spontaneous activity was only reduced in the presence of bicuculline concentrations up to 30 μM (fig.3.20) whereas in the other cells 2-10 μM bicuculline could completely abolish the spontaneous activity (not shown). The bicuculline resistant spontanous activity was chloride dependent (as it reversed at the potential predicted for chloride equilibrium potential) and GABA elicited as proven by its sensitivity to picrotoxin (FIG. 3.21). When low noise recordings of this bicuculline resistant GABAergic spontaneous activity were performed it was possible to resolve the single amplitude steps of the decay phase of the synaptic events and their conductance was indistinguishable from that one of channels induced by exogenous GABA application. Thus it seems that, at least in some cells, this bicuculline resistant GABA receptors are present also at synaptic level.

The possible heterogeneity of the $GABA_a$ receptors even inside a single tissue or tissue culture is a well establishd phenomenon (Schonrock and Bormann 1993, Pearce 1993). The heterogeneity is most probably due to the assembly of different receptor subunits to form an heteroligomeric channel whose functional properties are the fingerprints of the molecular composition. Binding studies

showed that in cerebellar membranes a third "non A, non B" GABA receptor exist that is insensitive to both the $GABA_A$ selective antagonist bicuculline and the $GABA_B$ selective agonist baclofen (Drew and Johnston 1992). Moreover binding studies showed that also the $GABA_A$ receptors expressed either in cerebellar membranes or in cultured cerebellar granule cells are not homogeneous (Meier and Schousboe 1982, Belhage et al. 1991). Using binding tecniques it was also shown that cerebellar granule cells cultured in standard conditions (25 mM potassium) only expressed the high affinity receptors but could be induced to express the low affinity ones if cultured in the presence of GABA (Meier et al. 1984). Nevertheless this heterogeneity never appeared in electrophysiological studies. It was on the contrary suggested that the low affinity receptor could be not coupled to a choride channel, even if it shared at least one common β subunit with the high affinity one (Hansen et al. 1991), because its action was insensitive to the action of both benzodiazepines and the non competitive antagonist picrotoxin that is a blocker of GABA induced chloride currents (Yoon et al. 1993).

Very recently it was suggested that synaptic and extrasynaptic $GABA_A$ response in cerebellar slices could result from the activation of distinct receptors (Brickley et al. 1995). Here we first demonstrate that cerbellar granule cells cultured in low (5 mM) potassium medium (condition that favours the development of functional synapses (Virginio et al. 1995)), express at least two biophysically and pharmacologically distinct receptors. We also show that this heterogeneity could be present also at synaptic level. This fact is important also because it shows that changing simply the extracellular potassium concentration can switch on the expression of different kind of GABA receptors. It would be definitely interesting to understand if the peculiar functional features of these receptors are due to the expression of different genes, to single points mutations, that have been shown to dramaticaly

change the affinity of the GABA receptor to both GABA and bicuculline (Siegel et al.1992), or if they could be the result of an intracellular regulation of a unique receptor (Moss et al. 1995). The latter hypothesis seems however unlikely since the receptors can coexist on the same cell and they maintain their features (i.e bicuculline insensitivity) also in excised patches.

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APPENDIX A

SOLUTION	1	2	3	
NaCl	124	124	124	mM
KCl	5.37	5.37	5.37	""
NaH_2PO_4	1.01	1.01	1.01	""
$MgSO_4$	1.2	1.2	1.7	""
D-glucose	14.5	14.5	14.5	""
HEPES	25	25	25	""
Phenol red	0.027	0.027	0.027	""
BSA*	3 mg/ml	3 mg/ml	3 mg/ml	
Trypsin**	_ _	0.25 mg/ml	- -	
DNase**	_ _	- -	25.6 µg/ml	
Trypsin inhibitor**	- -	- -	166.4 µg/ml	

* Bovine serum albumin fraction V

** Sigma, USA.

SOLUTION	4	5
NaCl	124	124
KCl	5.37	5.37
NaH_2PO_4	1.01	1.01
$MgSO_4$	2.75	2.44
$CaCl_2$	- -	0.1
D-glucose	14.50	14.50
Phenol red	0.027	0.027
BSA	3 mg/ml	- -
DNase	80 µg/ml	- -
Trypsin inhibitor	0.52 mg/ml	- -