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Relative contribution by GABA or glycine to Cl^- mediated synaptic transmission on neonatal rat hypoglossal motoneurons in vitro

Thesis submitted for the degree of *Doctor Philosophiae*

Candidate:
Roberta Donato

Supervisor:
Andrea Nistri

Trieste, October 2000
International School for Advanced Studies

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CONTENTS

NOTE

All work reported arises solely from my own experiments and data analysis. During the accomplishment of this thesis, the following papers and posters have been published and presented:

1) R. Donato, A. Nistri. Relative contribution by GABA or glycine to Cl- mediated synaptic transmission on rat hypoglossal motoneurons in vitro. *J. Neurophysiology*.In Press.

2) Canepari, M., Lagostena, L., Donato, R., Lape, R., and Mammano, F. Rapid Ca2+ transients visualized in different cellular preparations at high spatiotemporal resolution.*British Neuroscience Association Abstracts* 15: 133, 1999 Leeds, UK (Abstract).

3) Lape, R., Donato, R., Canepari, M., and Nistri, A. Properties of the spike afterhyperpolarization (AHP) of rat hypoglossal motoneurons. *Soc. Neurosci. Abstr.* 25: 454, 1999 (Neuroscience Meeting , Oct 23-28/1999, Miami, FL (Abstract).

4) R. Donato, A. Nistri. A study of glycinergic and GABAergic mediated postsynaptic currents on rat hypoglossal motoneurons. *Eur. J. Neurosci.* 12: 148, 2000 (FENS Meeting, June 24-28/2000, Brighton, UK (Abstract)

ACKNOWLEDGMENTS

First, all my sincere gratefulness is for my supervisor, Professor Andrea Nistri (alias Nicetry), who succeeded, thanks to his wide scientific (and human) experience, in the very difficult task to direct my work. Then, I am immensely grateful to Remis, who lost a lot of time in teaching me how to undertake an experimental work, and suggested me many important ideas for my project. Moreover, I admire his patience and courage, because, after four years of working and living together, he is still married with me. A cumulative but equally warm THANKS! is for all friends met during these years, still – or again!- here, or spread all over the world. They contributed to make special this experience, sharing enthusiasms and frustrations, problems and solutions, joys and sorrows.

Finally, I want to dedicate this work to the most beautiful couple that I have never known: my mother and my father.

ABSTRACT

The relative contribution by GABA and glycine to synaptic transmission of motoneurons was investigated using an hypoglossus nucleus slice preparation from neonatal rats. Exogenously applied glycine or GABA (100 μM) induced a comparable and marked reduction in cell input resistance (49±7% and 53±7%, respectively), and inhibited cell excitability. Spontaneous, miniature or electrically evoked GABAergic or glycinergic postsynaptic currents (sPSCs, mPSCs, ePSCs, respectively) were recorded under whole cell voltage clamp. The overall majority of the Cl^- mediated sPSCs was glycinergic, while only one third was GABAergic. 70±10% of mPSCs were glycinergic while 22±8% were GABAergic. Tetrodotoxin (TTX) revealed a major difference between GABAergic and glycinergic transmission since it did not change frequency or amplitude of glycinergic sPSCs whereas it dramatically reduced the frequency (and slightly the amplitude) of GABAergic events. These results indicate that glycine mediated neurotransmission was essentially independent of network activity, while spontaneous GABAergic events required a strong one. A striking difference in the kinetics of GABAergic and glycinergic responses emerged as GABAergic events had significantly slower rise and decay times than glycinergic ones. Such a difference was consistently present whenever sPSCs, mPSCs or ePSCs were measured. GABAergic and glycinergic mPSCs were differentially modulated by activation of glutamate metabotropic receptors (mGluRs) which are abundant in the hypoglossus nucleus. In fact, the broad spectrum mGluR agonist *t*-ACPD (50 μM), which in control solution increased the frequency of both GABAergic and glycinergic sPSCs, enhanced the mPSC frequency of glycinergic events only. Moreover, preliminary data suggested a major difference between glycinergic and GABAergic synaptic plasticity under conditions of high frequency stimulation of afferent fibers. These data do not provide evidence for any substantial co-release of these transmitters at this early postnatal stage and are interpreted in terms of kinetically distinct synaptic roles for glycine and GABA.

INTRODUCTION

GABA and glycine are neutral amino acids that, in addition to their metabolic role, in the mammalian central nervous system (CNS) operate as neurotransmitters via activation of distinct postsynaptic receptors gating Cl^- permeable channels. Cl^- is by far the most abundant physiological anion. In many animal cells it is distributed almost at equilibrium, so that the cytoplasmic Cl^- concentration is often lower (with a few exceptions discussed below) than the plasma concentration, and the equilibrium potential (E_{Cl}) is near the resting potential, or at least within ±15 mV of it. Thus, Cl^- increased permeability is expected to oppose any excitability rise and to have a stabilizing influence on the cell membrane potential.

Binding of GABA or glycine to specific postsynaptic receptors opens Cl^- permeable channels, and, by allowing Cl^- flow following its electrochemical gradient, it helps to repolarize a depolarized cell and to inhibite neuronal firing.

1. GABA and glycine: receptors, pharmacology, properties.

1.1 Glycine

Glycine was suggested as a potential neurotransmitter in the mammalian spinal cord as early as 1965 (Aprison and Werman, 1965). Release of this amino acid upon stimulation (Hopkin and Neal, 1970) and its inhibitory action on motoneurons (Werman et al 1968; Curtis et al 1968) have subsequently established glycine as a major inhibitory transmitter in the CNS. Ligand binding studies have identified a receptor protein specific for glycine (GlyR), which differs from the inhibitory $GABA_A$ receptor.

The only agonists known for the GlyR are a number of α- and β- amino acids, which differ from glycine in the length of the carbon chain or for the presence of hydroxylic groups or for cyclic structures as shown below. Their efficiency in eliciting a response from spinal cord neurons decreases with the order: glycine>β-alanine>taurine>>L-alanine, L-serine>proline (Curtis et al. 1968, Werman et al 1968; Davidoff et al 1969).

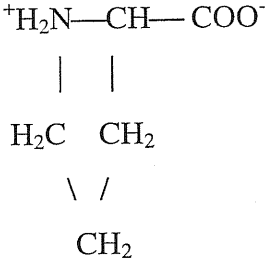
A $^{+}H_3N\text{-}CH_2\text{-}COO^{-}$ glycine

B $^{+}H_3N—CH_2—CH_2—COO^{-}$ β-alanine

C $^{+}H_3N—CH_2—CH_2—SO^{-}_3$ taurine

D $^{+}H_3N—CH(CH_3)—COO^{-}$ alanine

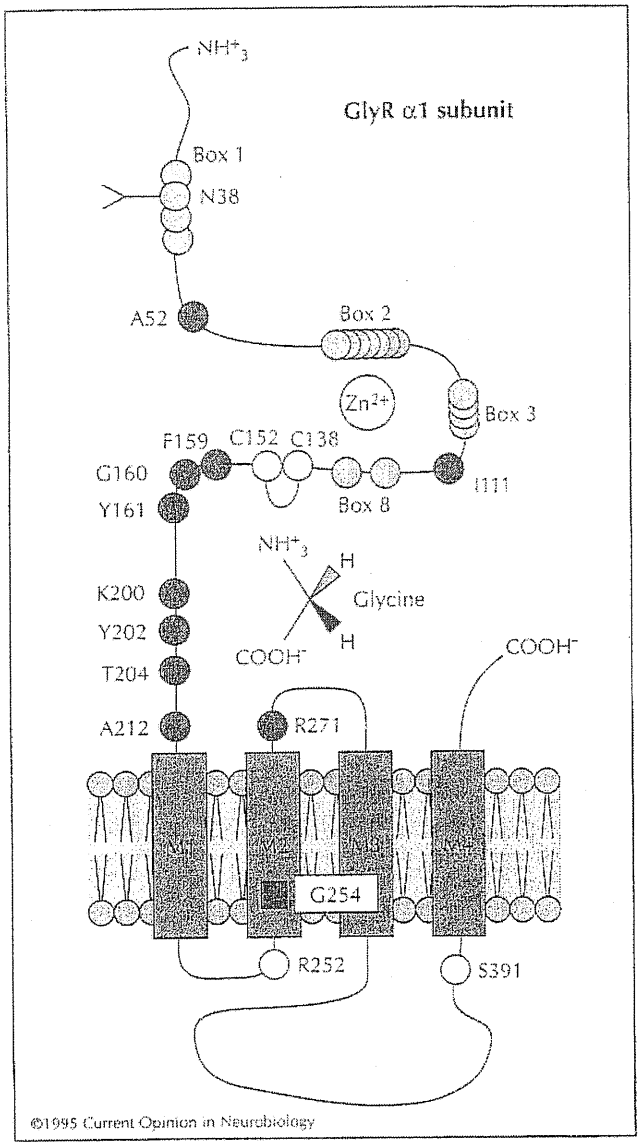
E $^{+}H_3N—CH(CH_2OH)—COO^{-}$ serine

F  proline

G $^{+}H_3N—CH_2—CH_2—CH_2—COO^{-}$ GABA

The plant alkaloid strychnine and its derivatives, and the steroid RU 5135 as well as 1,5-diphenyl-3,7-diazaadamantan-9-ol, are the only antagonists which act in nanomolar concentrations (IC_{50}~ 30 nM, Betz 1990) on glycine receptors. Block of Cl^{-} flux through the GlyR upon strychnine poisoning leads to overexcitation of the motor system, i.e. muscular convulsions. Strychnine blocks the action of glycine but not of GABA on motoneurons (Curtis et al 1971) and interneurons (Davidoff et al 1969), and starts becoming non selective only at very high concentrations. In fact, its IC_{50} for GABA mediated currents is ~ 40 times higher than for glycine mediated ones in spinal motoneurons (Jonas et al 1998, Lewis and Faber 1993). Aprison and co-workers (Aprison et al 1987) described how strychnine, a very complex molecule,

could antagonize glycine, the simplest amino acid known. They employed computational methods to assess the similarities between these seemingly different molecules. With an exhaustive comparison of topological and electronic features of both molecules, they located a glycine-like fragment in the strychnine molecule that, when compared to glycine, exhibits both topological and electronic charge congruence.



Membrane topology and localization of functional sites of the GlyR α1 subunit.
M1-M4 are the four predicted transmembrane regions. Dark gray circles represent amino acids crucial for ligands affinities. For example, residues G160, K200 and Y202 are important for strychnine binding, F159, Y161 and T204 are determinants of agonist specificity and affinity, mutations of R271 change glycine affinity and average conductance states. The conserved cysteine motif is thought to form a disulfide bridge and is located at position C138 and C152. Dark gray square indicate a residue (G254) important for main-state conductances. Light gray circles named assembly boxes represent residues important for subunit processing and assembly. Residue N38 in Box1 is an N-glycosylation site.
S391 in the large cytoplasmic loop is proposed to be phosphorilated by PKC.
Taken from Kuhse J *et al.*, *Current Opinion in Neurobiology* 1995, **5**:318-323

1.1.1 Structure

The postsynaptic GlyR is a pentameric transmembrane protein composed of glycosylated integral transmembrane subunits of 48 kDa (α) and 58 kDa (β) (Betz et

al 1994). The primary structure of the GlyR α and β subunits shows significant similarity to that of the nicotinic acetylcholine receptor (nAChR), $GABA_A$ receptor ($GABA_AR$) and serotonin type-3 receptor ($5\text{-}HT_3R$) (Grenningloh et al 1987, 1990a); consequently, GlyR is classified as a member of the superfamily of ligand-gated ion channels (Betz 1990). Sequence conservation is particularly high around a conserved cysteine motif of the extracellular amino-terminal region and in four hydrophobic domains predicted to form transmembrane regions (M1-M4). Molecular heterogeneity of the GlyRs has been found by biochemical and molecular cloning approaches. cDNAs (from rat and human) of three different genes encode the α subunits (α_1-α_3) (Betz et al 1994), the murine gene (α_4) and the α_2 variant (α_2*). Isoforms for both α_1 and α_2 subunits result from alternative splicing. The GlyR subunit genes are composed of nine coding exons, whose borders are largely conserved between the α_1, α_2, α_4 and β genes (Matzenbach et al 1994). A close evolutionary relationship also exists between the GlyR β subunit and $GABA_AR$ proteins, as indicated by similarities of the respective gene structures (Mühlhardt et al 1994). Co-expression of α_1 and α_2 subunits in Xenopus oocytes reveals that these subunits can generate hetero-oligomeric receptors of variable stoichiometries. In contrast, incorporation of the β subunit invariably generates a stoichiometry of 3α:2β. These differences in assembly behaviour are encoded by diverging amino-acid motifs in the respective amino–terminal domains of the two subunits (Kuhse et al 1993).

Expression of the GlyR α_1, α_2 or α_3 subunits in *Xenopus* oocytes or mammalian cell lines generates homo-oligomeric chloride channels, gated by glycine, taurine and β-alanine and competitively blocked by strychnine (Schmieden et al 1989, Grenningloh et al 1990b). The glycine responses of this recombinant GlyR display Hill coefficients ranging from ≈2.5 to ≈4, indicating that at least three glycine molecules must bind to open the channel (Kushe et al 1995, Bormann et al 1993). Site directed mutagenesis identified two regions between amino-acid position 170 and 220 of the α_1 subunit that are important for agonist and antagonist binding.

Another region close to the conserved cysteine motif is crucial for agonist selectivity and antagonist affinity (Vandenberg et al 1992). Interestingly, the replacement of a single aromatic hydroxyl group at this site makes the GlyR responsive to GABA (Schmieden et al 1993). Moreover, residues localized amino-terminally of the conserved cysteine motif and the M1 segment are homologous to amino-acid

positions determining ligand affinities of the nAChR and $GABA_AR$, indicating a similar, perhaps common, architecture of the ligand-binding pocket of all these receptors. Mutation of arginine at position 271, located at the extracellular end of the M2 segment, causes a drastic decrease in agonist binding and average main-state conductance. This single amino-acid mutation (Arg271→Gln/Leu) is the cause of hyperekplexia in humans, a neurologic disorder characterized by marked muscle rigidity and exaggerated startle response.

While the agonist and antagonist binding pocket of the GlyR seems to be composed of several discontinuous domains of the α subunits, it is presently unknown if the β subunit also contributes to ligand binding. The M2 segment of the GlyR β subunit determines open channel block by picrotoxin (Pribilla et al 1994), and the same segment of both the α and the β subunits is responsible for different main-state conductances of homo- and hetero-oligomeric GlyRs. These data show the importance of the M2 segment for Cl^- conductance and a central role of the β subunit in channel function. Moreover, the β subunit is associated with gephyrin, a 93-kDa peripheral membrane polypeptide (Meyer et al 1995).

Gephyrin is of primary significance because of its pivotal role in the formation of glycine receptor clusters, which most likely occur through receptor anchoring to the cytoskeleton (Kirsch and Betz 1993, 1995; Kirsh et al 1991; Todd et al 1995, Wheal et al 1998).

As gephyrin binds with high affinity (K_D=2.5nM) polimerized tubulin (Kirsch et al 1991), this protein is postulated to link the GlyR to subsynaptic microtubules. During synaptogenesis in cultured spinal neurons, postynaptic accumulation of gephyrin precedes that of GlyR, and inhibition of gephyrin expression by antisense oligonucleotide prevents the formation of GlyR clusters (Kirsch and Betz 1993). Since high level expression of the GlyR can alter the agonist and antagonist response properties of the receptor itself (ligand affinities are increased after high-level expression of GlyR in *Xenopus* oocytes, Taleb and Betz, 1994), a regulation of GlyR packing density by gephyrin and cytoskeletal elements could modulate the functional properties of the receptor, thus altering synaptic efficacy. However, it has recently been shown (Essrich et al 1998; Levi et al 1999) that gephyrin is also required for clustering and postsynaptic localization of $GABA_AR$ as well (at least the ones containing γ_2 and/or α_2 subunits; Kneussel et al 1999).

1.1.2 Physiological properties of GlyRs

Single channel I-V relations in outside out patches with symmetrical Cl^- concentrations (Bormann et al 1987) revealed a predominant conductance of ~46 pS. In addition to the main conductance state, other less frequently occurring states were observed (approximately 30 pS, 20 pS, 12 pS). These conductance sublevels appear to be very similar for $GABA_A$Rs. However, the fraction of time spent in each level is different for the two receptors (for GlyRs the distribution is 78, 10 and 12 percent, respectively for the three sublevels, while for $GABA_A$Rs is 1, 80, 17). Similar multiple conductance levels are consistently found for glycine receptor channels of spinal neurons in a slice preparation (Takahashi and Momiyama 1991), indicating that, at far as single channel conductance of spinal neurons is concerned, extrasynaptic and synaptic GlyRs are indistinguishable. Nevertheless, there is a high variability in other electrophysiological characteristics, such as decay time and desensitization properties (Lewis and Faber 1996a,b)

1.1.3 Receptor modulation

Glycine activated currents in cultured spinal neurons but also in *Xenopus* oocytes expressing recombinant GlyRs (Bloomenthal et al 1994; Laube et al 1995) are strongly modulated by the divalent cation zinc. This metal ion is stored in synaptic vescicles and co-released with neurotransmitters upon stimulation (Assaf and Chung 1984). Low concentrations of zinc (1-10 μM) potentiate the response about threefold, whereas higher concentrations (≥100 μM) cause inhibition. Analysis of chimeric GlyR proteins assigns a potentiating zinc ion binding site to conserved motifs within the amino-terminal region of the α subunits (Laube et al 1995).

Phosphorylation of ligand-gated ion channels constitutes another important mechanism to modulate their response properties. In culured spinal trigeminal neurons, stimulation of protein kinase A enhances glycine responses (Song and Huang 1990). Similarly, glycinergic currents elicited in oocytes (injected with mRNA from rat spinal cord) are enhanced by activation of protein kinase A (PKA) and inhibited by activation of protein kinase C (PKC, Vaello et al 1994) . Also, in the same study, PKC dependent phosphorylation of serine at position 391 of the GlyR α1 subunit was demonstrated biochemically. Thus, multiple phosphorylation events may regulate

GlyR function, allowing "crosstalk" of intracellular signalling pathways with glycinergic neurotransmission.

1.1.4 Distribution of GlyRs in CNS

The distribution of GlyRs in the CNS has been analyzed with [^{3}H]strychnine autoradiography of CNS sections (Zarbin et al 1981; Frostholm and Rotter 1985). These studies indicate a marked rostro-caudal gradient within the spinal cord. Immunocytochemistry with monoclonal antibodies raised against [^{3}H]strychnine sensitive GlyRs, shows the highest level in the brain stem or at purified rat GlyRs (Pfeiffer et al 1984). The distribution of α subunit antigen and gephyrin generally matches that of [^{3}H]strychnine-binding sites, although there is no perfect overlap (Triller et al 1985; Altschuler et al 1986; Van del Pol and Gorcs 1988). Several higher brain regions, including the olfactory bulb, midbrain and cerebellum, display low, yet significant immunoreactivity.

1.2 GABA

GABA produces its actions on the CNS by binding to two subclasses of ionotropic receptors, $GABA_A$ and $GABA_C$, and one class of metabotropic receptor, $GABA_B$. $GABA_C$Rs are ligand-gated Cl channels that are insensitive to drugs that modulate $GABA_A$ and $GABA_B$ receptor function (Bormann and Feigenspan 1995; Johnston 1997. Compared with $GABA_A$ Cl^- channels, $GABA_C$ receptors are more sensitive to GABA, less prone to desensitization, and have longer open times. Their molecular biology has not been fully characterized. It appears that ρ_1-ρ_2 subunits, which are typically referred to as $GABA_A$ receptor subunits, may form $GABA_C$Rs (Costa 1998; Enz and Cutting 1998; Johnston 1997). The role of $GABA_C$Rs in regulation of neuron excitability is not known. $GABA_C$ type responses have been characterized predominantly in the retina. The distribution of ρ subunits is almost restricted to the retina (Cutting et al 1991,1992) although ρ_2 has additionally been described in other brain areas (hippocampus and cortex; Enz et al 1995). Pharmacological effects indicate the presence of receptors composed of ρ subunits in the spinal cord (Johnston et al 1975), optic tectum (Nistri and Sivilotti 1985; Sivilotti and Nistri 1989), cerebellum (Drew et al 1984; Drew and Johnston 1992), hippocampus (Strata and Cherubini 1994). Little is known about the ρ_3 subunit, which has been obtained from

the cat retina. Furthermore, although ρ_1 mRNA is exclusive to the retina, RT-PCR analysis of ρ_2 indicates its presence throughout the brain, including the spinal cord. Its expression in, or presynaptic to, motoneurons remains to be established (Enz and Cutting 1998).

$GABA_BRs$ are metabotropic receptors coupled through G proteins to second messenger systems, activated by GABA and baclofen (that have approximately the same EC_{50}, around 100 nM) and resistant to bicuculline and picrotoxin, antagonists of $GABA_ARs$, or isoguvacine and imidazoleacetic acid, $GABA_ARs$ agonists. $GABA_BRs$ receptor activation has mainly a presynaptic modulatory effect on the postsynaptic cell. In dorsal root ganglion neurons, the presynaptic inhibitory action of $GABA_BRs$ is proposed to result primarily from a G protein-mediated reduction of voltage-sensitive Ca^{2+} currents (Curtis et al 1997; Kamatchi and Ticku 1990; Lev-Tov et al 1988). A postsynaptic increase in K^+ currents (Gage 1992; Misgeld et al 1995; Mott and Lewis 1994), and a third mechanism independent of effects on leak or voltage-gated currents (Misgeld et al 1995) may also mediate $GABA_BR$ activity. Despite the obvious inhibition induced by baclofen, it is difficult to assess an endogenous role for $GABA_BRs$ in modulating neuron excitability. Part of this difficulty may reflect the use of relatively weak $GABA_B$ antagonists, such as 2-OH saclofen, or that $GABA_B$ antagonists applied at the cell soma do not reach distal synapses (Stuart and Redman 1992), but discrepancies may reflect varying degrees of endogenous activation of the relevant pathways in different experimental models. Another possibility is that $GABA_B$ receptors on afferent terminals are located extrasynaptically (Stuart and Redman 1992). Under such conditions, activation of extrasynaptic receptors is only likely to occur during periods of massive GABA release (or reduced uptake) due to highly effective GABA uptake mechanisms. Paracrine-like activation of extrasynaptic $GABA_B$ receptors is present in hippocampal CA1 cells (Isaacson et al 1993). Thus, efficiency of GABA uptake systems may play an important role in presynaptic inhibition by $GABA_BRs$.

$GABA_ARs$

$GABA_ARs$ are stimulated by muscimol and isoguvacine and inhibited by bicuculline in a competitive manner. This distinguishes them from $GABA_BRs$, which are activated by baclofen and antagonized by phaclofen (Bowery 1989,1993; Kerr and

Ong, 1995; Misgeld et al 1995), and from $GABA_C$Rs, which are bicuculline insensitive.

Bicuculline is a phthalide-isoquinoline alkaloid found in several *Fumariaceae*. The IC_{50} value for bicuculline against GABA binding is 0.58 μM (Jonas et al 1998). As discussed above for strychnine, also bicuculline is rather selective as $GABA_AR$ blocker, since the bicuculline IC_{50} for glycine mediated currents is two order of magnitude higher than for GABA mediated currents (Jonas et al 1998, Lewis and Faber 1993).

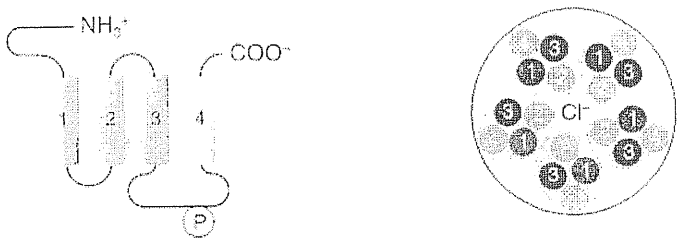
Picrotoxin and TBPS (*t*-butylbicyclophosphorothionate) inhibit $GABA_A$Rs in a non competitive manner (Yoon et al 1993). Penicillin (in high concentrations) is known as a $GABA_AR$ blocker (Twyman et al 1992). Other substances are competitive antagonists at the GABA-binding site, such as the amidine steroid Ru 5135 and the arylaminopyridazines SR 95103 and SR 95531 (Hevers and Lüddens 1998).

Concentration response curves for GABA are sigmoidal with Hill coefficients between one and two (Sakmann et al 1983; Bormann and Clapham 1985), suggesting that binding of at least two GABA molecules is required to open the channel. Binding studies with [^{3}H]bicuculline and [^{3}H]GABA (Olsen and Snowman 1983; Olsen et al 1981) revealed high and low affinity sites with K_D values in the low and high nM range, respectively, which could be seen in support of two different binding sites.

1.2.1 Receptor structure

In line with the view that $GABA_A$Rs are part of a structurally conserved superfamily that includes 5-HT3 receptors, nAChRs and GlyRs, a pentameric subunit structure had been predicted for this receptor. In analogy with the other members of the superfamily, $GABA_A$Rs combine the ligand-binding site as well as the ion-permeating pore within the same homo- or hetero-oligomeric complex. Modern molecular biology has revealed that the pleiotropic pharmacology of $GABA_AR$ mediated responses is due to a vast diversity of possible receptor subtypes. Presumably, this diversity is caused by the combination of five of at least eighteen different subunits known at present (α_1-α_6, β_1-β_3, γ_1-γ_3, δ, ε, ρ_1-ρ_3, π; Davies et al 1997; Lüddens and Wisden 1991; Macdonald and Olsen 1994; Seeburg et al 1990; Sieghart 1995). Each subunit has a molecular weight between 40-60 kDa as predicted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), resulting in pentameric

receptors of approximately 240-290 kDa. For five subunits splice variants have been described.



Each subunit comprises four transmembrane domains (TM1-TM4; in figure named 1-4); the large intracellular loop between TM3 and TM4 contains consensus sites for phosphorilation by protein kinase (P). The amphiphilic TM2 provides the lining of the Cl^- pore intrinsic to the pentameric structure.
Taken from Bormann, J, TiPS 2000, **21**: 16-19

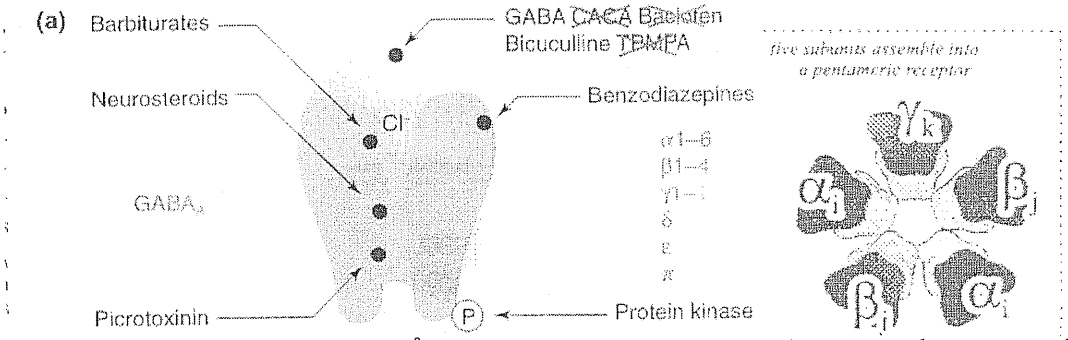
Each subunit comprises a large extracellular N-terminal domain that putatively includes the ligand-binding site, four hydrophobic membrane-spanning domains labeled TM1-TM4, and a small extracellular C-terminus. The N-terminal domain also contains a cysteine loop, conserved (like in the case of GlyRs) throughout all members of this superfamily.

In some subunits a larger intracellular loop between TM3 and TM4 may represent a potential phosphorylation site. In the pentameric arrangement of subunits, it is likely that the α-helical TM2 region faces the channel pore (Xu et al 1995; Unwin 1995). The N-terminal domains of both α and β subunits appear to contribute to the agonist site.

The effects of a number of psychoactive drugs on $GABA_AR$ activity differ dramatically with the subunit combination. All this makes the identification of native receptor isoforms, their characteristics and perhaps the significance of their diversity in the CNS a major task for future studies.

Most $GABA_ARs$ are made up of three types of subunits: an α subunit, a β subunit and the γ_2 subunit. The stoichiometry of $GABA_AR$ subunits is not firmly established. In an electrophysiological study on the recombinant receptor subtype $\alpha_3\beta_2\gamma_2$, the most likely subunit stoichiometry was found to be two α_i, one β_j and two γ_k subunits, (Backus et al 1993), but, more recently, another combination has been proposed, namely two α_i, two β_j and one γ_k (with i=1-6, j=1-3, k=1-3; Sieghart 1995; Tretter et

al 1997). If all combinations were possible, the number of possible receptor isoforms could exceed 100,000. Immunoprecipitation studies, localization studies of mRNA, immunolabelings of receptor subunits, and also developmental and functional trascriptional control drastically reduce the possible number of different isoforms.



The $GABA_AR$ is a Cl^- pore with ~5Å diameter and modulatory sites for many substances, such as benzodiazepines, barbiturates and neurosteroids. $GABA_AR$ responses are blocked competitively by bicuculline and noncompetitively by picrotoxin; they are modulate intracellularly by protein kynases A and C. The agonist CACA (cis-4-aminocrotonic acid) and antagonist TPMPA (1,2,5,6-tetrahydropyridine-4-yl(methylphosphinic acid)) of $GABA_CR$ have no effect on $GABA_AR$. Likewise, the $GABA_BR$ agonist baclofen is inactive. The vertebrate $GABA_AR$ complex is built from five subunits belonging to different families (α1-6, β1-4, γ1-4, δ, ε and π), the most abundant isoform being 2α:2β:γ.Taken from Bormann, J, TiPS 2000, **21**: 16-19 and Hevers, W. and Lüddens, H., Molecular Neurobiology 1998, **18**: 35-86.

1.2.2 Distribution of subunits

The α1 subunit appears to be the most abundant subunit in the CNS, with only a few regions devoid of it (Laurie et al 1992a,b; Benke et al 1991a; Fritschy and Mohler 1995). Often it is colocalized with the β_2 subunit. Also the γ_2 subunit is found in nearly all brain regions, albeit with different intensities, and it is often described to be colocalized with $\alpha_1\beta_2$, so that $\alpha_1\beta_2\gamma_2$ is the most widely found isoform. α variants other than α_1 show more limited distribution. The α_2 and α_3 subunits show stronger mRNA and immunolabeling where weaker traces of α_1 are found. Other subunits, although found in minor amounts in the CNS, confer to the receptor important pharmacological characteristics. For example, the α_5 subunit, found in adult hippocampus and olfactory bulb (Wisden et al 1992; Fritschy and Mohler 1995), constitutes a receptor with low affinity for certain benzodiazepines (BZs) ligands like the imidazopyridine zolpidem (Lloyd et al 1990; Pritchett and Seeburg 1990; Arbilla et al 1986). Similarly, the α_4 and α_6 subunits have a distinct BZ pharmacology

(diazepam insensitive). Major amounts of the α_4 subunit are found in the hippocampus and thalamus and often colocalize with the δ subunit (Wisden et al 1991). The α_6 subunit, also found to colocalize with the δ subunit, appears to be almost exclusively restricted to cerebellar granule cells (Lüddens et al 1990) with some traces found in the dorsal cochlear nucleus (Varecka et al 1994).

The role and distribution of β subunits have been studied in less detail. However, β_1 mRNA signals are strongest in the hippocampus, less pronounced in part of basal nuclei and septum, and weak in the amygdala and hypothalamus (Wisden et al 1992). The β_2 subunit shows a more widespread distribution, which is of low intensity where strong concentration of β_1 and β_3 are found. The β_3 subunit signals are strong in CA1 and CA2 of hippocampus, olfactory bulb, cortex, part of the basal nuclei (caudate putamen and nuclei accumbens) and hypothalamus.

The ubiquitous presence of the γ_2 subunit is contrasted by the restricted distribution of the γ_1 and γ_3 variants. Whereas there is some overlapping distribution of the γ_2 and γ_3 subunits, mRNA encoding for γ_1 subunit is limited to regions of the amygdala, septum and hypothalamus and does not appear to coexist with γ_2 or γ_3 in a single receptor (Quirk et al 1994). The γ_3 subunit is scarce in cerebellum, but present in higher amounts in the olfactory bulb, cortex, basal nuclei and medial geniculate of the thalamus (Wisden et al 1992; Herb et al 1992).

The δ subunit, which colocalizes with the α_4 and α_6 subunits, is concentrated in granule cells of the cerebellum, but present in a minor amount also in the cerebral cortex, thalamus and olfactory bulb (Laurie et al 1992b; Shivers et al 1989; Benke et al 1991b).

Recently, it has been shown (Nusser et al 1998) that in cerebellar granule cells the δ subunit is not localized to synaptic junctions but is abundantly present on the extrasynaptic dendritic and somatic membrane. Cerebellar granule cells thus contain an unusual receptor composition, since the six subunits α_1, α_6, β_2, β_3, γ_2 and δ colocalize in these cells. Which subunits combine within a single pentameric receptor remains however unclear.

The ε subunit shares approximately 45% amino acid identity with the γ subunit and is indicated to coassemble with α and β subunits. It is found mainly in subthalamic nuclei and, to a lesser extent, in the amygdala and thalamus (Davies et al 1997).

1.2.3 Physiological properties

Many studies employed mouse spinal cord neurons for single channel analysis of native $GABA_AR$s and their pharmacology (Bormann et al 1987). In response to GABA, these channels open to 12, 17-20 and 27-30 pS conductance levels, the largest accounting for approximately 95% of the current. Conductance sublevels do not greatly vary among different $GABA_AR$ isoforms. The competitive antagonist bicuculline reduces the open frequency and mean duration of GABA-induced single channel event. Similarly, the noncompetitive antagonist picrotoxin reduces the channel open frequency whereas the channel conductance and other time constants remained unaltered according to noise analysis (Newland and Cull-Candy 1992). Indeed, differences in subunit composition determine GABA sensitivity of the various isoforms. Although data obtained by different groups for one type of subunit composition are often almost incomparable because of different experimental conditions, α6 containing receptors are consistently reported as the most sensitive binary and ternary isoforms, with EC_{50} values in the sub-to low micromolar range (0.1-2.2 μM). The α_1 containing isoform shows an intermediate sensitivity, whereas α_4 and α_5 are less sensitive and α_3 containing receptor has an EC_{50} approximately ten fold higher than the $\alpha_1\beta_n\gamma_n$ isoform. But α subunits are not the only determinants of GABA sensitivity, as differences due to β or γ subunits have been reported (Ducic et al 1993; Ebert et al 1994; Ymer et al. 1989).

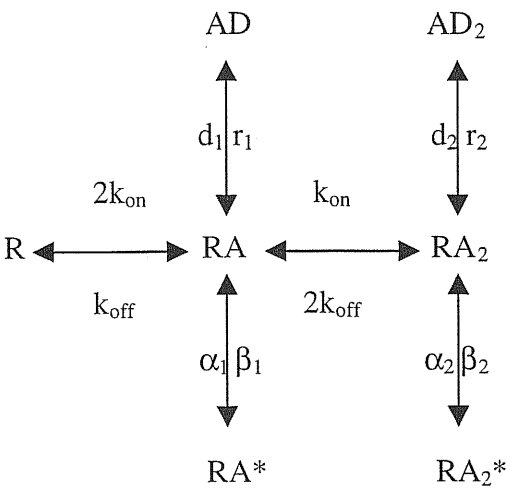
Different kinetics have also been reported for distinct $GABA_AR$ isoforms. For example, activation and deactivation rates of recombinant $GABA_AR$ channels have been shown to be dependent on the α subunit (Lavoie et al 1997; McClellan and Twyman 1999). In particular, responses by the $\alpha_2\beta_1\gamma_2$ isoform peak twice faster than those by $\alpha_1\beta_1\gamma_2$ (0.5 ms versus 1.0 ms), whereas deactivation of $\alpha_2\beta_1\gamma_2$ is two to six times slower.

Concerning the γ subunit, it has recently been shown (Essrich et al 1998; Kneussel et al 1999) that, although the γ_2 subunit is not necessary for receptor assembly and translocation to the cell surface, it is required for clustering of major postsynaptic $GABA_AR$ subtypes (namely the αβγ types). Essrich et al (1998) have demonstrated loss of $GABA_AR$ clusters in neurons of mice deficient in the γ_2 subunit, and parallel loss of gephyrin and synaptic GABAergic function. Conversely, inhibiting gephyrin expression causes loss of $GABA_AR$ clusters (Kneussel et al 1999). It has thus been

suggested that γ_2 subunit and gephyrin are interdependent components of the same synaptic complex. A similar role can probably be accomplished by γ_3 subunit, as transgenic overexpression of γ_3 subunit in γ_2-deficient mice restores benzodiazepine binding sites, benzodiazepine-modulated whole cell currents, postsynaptic GABAergic miniature currents, all data suggesting the formation of functional postsynaptic receptors (Essrich et al 1998).

1.2.4 Pharmacology

A number of psychoactive drugs exert their effect mainly via $GABA_A$Rs. Aside from the natural agonist GABA, among the most clinically relevant there are benzodiazepines (BZ). At single channel level, benzodiazepines are believed to increase the number of channel openings (Twyman et al 1989; Vicini et al 1987). Although photoaffinity labeling studies on native receptors originally located BZ modulatory site in the α subunit (Sieghart 1985), recombinant receptors are responsive to classical BZ site ligands only when α and β subunits are coexpressed with the γ_2 subunit (Pritchett et al 1989; Pritchett and Seeburg 1990). Thus, the γ subunit is a prerequisite for BZ sensitivity. Other drugs are sedative and anesthetic barbiturates, but in this case the allosteric modulatory site is less definite. However, barbiturates are known to exert at least two separate actions on the $GABA_A$Rs: an enhancement of the effects of $GABA_A$ agonists and a direct channel opening action (when moderate to high concentration of barbiturates are applied in absence of GABA agonists). The barbiturate potentiation of $GABA_A$R responses seems to be due to change (increase) in the mean open time of the channel, i.e. to an enhanced probability of channel opening to the longest open state, rather than to a change in channel conductance. As pointed out by Mcdonald et al (1989), the complexity of kinetic models for $GABA_A$R channel gating makes it difficult to establish which rate constant(s) may be altered by barbiturates. The effect by benzodiazepines or barbiturates observed at the level of macroscopic currents, is an increase in peak amplitude (if the control response is not maximal) and a substantial prolongation of the decay time.



The simplest and more general kinetic scheme for the $GABA_AR$. Here, R represents the closed unbound state; RA and RA_2 the closed states in which one or two GABA molecules are bound, respectively; AD and AD_2 the closed desensitized states with one or two GABA molecules bound, respectively; RA^* and RA^*_2 the open states with one or two molecules bound.d_1,d_2, α_1,α_2 are the forward rate constants to pass from the closed bound state with one or two molecules to the desensitized states AD or AD_2 and to the open ones RA^* or RA^*_2, respectively. Analogously, r1,r2,β1,β2 are the rate constants for the corresponding backwards transitions. k_{on} and k_{off} are the rate constant for binding and unbinding of one molecule.

Other important compounds that modulate $GABA_AR$s response are steroids. In this case, the α subunit influences the degree of potentiation; probably the site of action is different from the barbiturate site, and may be of particular physiological significance, since the brain is capable of synthesizing neurosteroids that affects $GABA_AR$ function.

The functional diversity of $GABA_AR$s is further increased by modification at putative intracellular phosphorylation sites. Those have been found both in native and recombinant $GABA_AR$s (Angelotti et al. 1993; Macdonald and Olsen 1994). Consensus sites for phosphorylation by PKC are found in subunits α_4, α_6, $\beta_{1\text{-}3}$, γ_2 and ρ_2 (Enz et al 1995; Kellenberger et al 1992; Moss et al 1992a); subunits amenable to phosphorylation by PKA include all β subunits and the α_4 and α_6 subunits.

Since the pharmacological profile of the many different isoforms is influenced by the particular subunit composition, pharmacology can be used as tool to differentiate between native receptor isoforms.

1.2.5 Diversity of subunit composition: implications

The physiological significance of the potential diversity of GABA and glycine receptors conferred by multiple subtypes, subunits, and posttranscriptional modification remains one of the major unanswered questions of amino acid mediated transmission.

Although GlyRs show some degree of variability in terms of isoforms, this is much less than that of $GABA_A$Rs. Concerning $GABA_A$ receptors, the major functional implication of $GABA_A$ receptor diversity may be associated with changes in the channel-gating potency of GABA (Costa 1998). The EC_{50} of 19 different subtypes of recombinant $GABA_A$Rs varies from 0.3 to 15 μM (Ducic et al 1995). In hippocampal and cortical pyramidal neurons, $GABA_A$R diversity is postulated to be critical for synchronizing the firing rate and coordinating neuronal interactions in columnary cortical activity (Costa 1998; Banks et al 2000).

For a number of transmitters, it has been suggested that distinct receptor subtypes may be targeted to specific regions of the neuronal membrane, possibly enabling cells to respond in different ways to the same transmitter. Cerebellar granule cells are an excellent model to study this issue. In fact, they express a particularly wide range of $GABA_A$ subunits (up to ten), despite having a relatively simple morphology and receiving most of their inhibitory input from a single cell type, namely the Golgi cell. It has been suggested (Nusser et al 1998) that a difference in subunit composition can be responsible for a different form of inhibition that has been observed to occur on these cells. In fact, cerebellar granule cells receive both tonic and phasic $GABA_A$R mediated inhibition (Brickley et al 1996; Wall and Usowicz 1997). The former type of inhibition is apparently mediated mainly by extrasynaptic $GABA_A$Rs, probably of the $\alpha_6\beta_{2/3}\delta$ isoform, whereas the latter should occur mainly via activation of synaptic $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2$ and $\alpha_1\alpha_6\beta_{2/3}\gamma_2$ isoforms. Moreover, as previously noted, the α_6 subunit endows receptors with an affinity for GABA that is an order of magnitude higher than the more generally occurring α_1 subunit (Ducic et al 1995; Saxena and MacDonald 1996), and thus provides a potent basis for spillover-mediated inhibitory cross-talk within the glomerus-synapse between the Golgi and the granule cell (Rossi and Hamann 1998). In general, neurochemical and morphological evidence has shown that some neurotransmitters may be released from both synaptic and nonsynaptic sites and may diffuse, or spill-over, from the release site to target cells more distant than those studied during standard synaptic transmission (Barbour and Haüsser 1997; Isaacson et al 1993; Rusakov et al 1999; Walmsley et al 1998).

There are functional interactions between neurons even in the absence of synaptic contacts. For example, besides cabled information signaling (through synapses), there is a "wireless" nonsynaptic interaction between axon terminals (Herkenham 1987).

This would be a form of communication transitional between discrete classical neurotransmission and the relatively nonspecific neuroendocrine secretion (Vizi 2000). Nonsynaptic interaction between neurons mediated via receptors and high affinity transporters has the potential to be an important contributor to the properties and function of neuronal networks.

1.3 Inhibitory synaptic transmission: membrane properties

Inhibitory postsynaptic potentials were first recorded in cat motoneurons (Coombs et al 1955) and subsequently attributed to activation of glycine and $GABA_A$Rs (Curtis et al 1968; Krnjevic et al 1977; Werman et al 1968). Activation of glycine (Chase et al 1989; Curtis et al 1968; Kohlmeier et al 1997; Krnjevic et al 1977; Takahashi 1984, 1992; Takata 1993) or $GABA_A$Rs (Curtis et al 1968; Davidoff and Hackman 1983; Krnjevic et al 1977; Malcangio and Bowery 1996; Takata 1993) in adult spinal and cranial motoneurons by exogenous or synaptically released agonists elicits similar responses comprising opening of Cl^- selective channels, inward movement of Cl^-, decrease in membrane resistance, and membrane hyperpolarization. Although predominantly hyperpolarizing, GABA and glycine responses can also be depolarizing if intracellular Cl^- concentration is elevated, so that E_{Cl^-} is less negative than cell resting membrane potential (E_{rest}).

Recently it has been demonstrated that a K^+/Cl^- co-transporter, KCC2, is the main Cl^- extruder (antisense oligonucleotide inhibition of the expression of this transporter produces a marked positive shift in the reversal potential of $GABA_A$ responses; Rivera et al 1999). The expression of KCC2 in hippocampus shows a pronounced developmental upregulation, which is almost parallel to the developmental shift in the reversal potential for GABA.

Thus, in neonatal (or fetal) motoneurons, in which intracellular Cl^- is higher than in the adult, responses to $GABA_A$ and glycine agonists, either exogenously applied (Gao and Ziskind-Conhaim 1995; Wu et al 1992) or synaptically released (Gao and Ziskind-Conhaim 1995; Jahr and Yoshioka 1986; Jonas et al 1998; Singer et al 1998; Takahashi 1984), are typically depolarizing (HCO_3 flux does not contribute to the depolarizing response in motoneurons, Gao and Ziskind-Conhaim 1995, Perkins and Wong 1997). The ontogeny of many aspects of GABAergic and glycinergic transmission has been examined in spinal motoneurons (Gao et al 1998; Gao and Ziskind-Conhaim 1995; Wu et al 1992); however, a clear description of the

developmental stage when depolarizing GABA/glycine responses become hyperpolarizing is lacking. Whole cell recordings, while providing valuable information on kinetics of GABAergic and glycinergic IPSP/Cs of neonate and adult motoneurons (Takahashi 1992; Takahashi et al 1992; Takahashi and Momiyama 1991), are not well suited for these measurements because of disruptions in internal Cl^- concentration. Perforated-patch recordings using the Cl^- impermeant ionophore gramicidin indicate a change in reversal potential of glycinergic IPSC in hypoglossal motoneurons from -37 to -73 mV between postnatal days 0 and 18 (Singer et al 1998). However, although Cl^-dependent potentials can be depolarizing, large decreases in input resistance are believed to underlie the fact that they remain inhibitory (Gao et al 1998; Gao and Ziskind-Conhaim 1995; Singer et al 1998; Wu et al 1992).

Although membrane depolarization depends on the internal concentration (and hence the reversal potential) of Cl^-, it is not obvious that in an intact neuron the Cl^- concentration is homogeneous in space (in processes versus soma, for example) or in time. Periodic, post-episode depression of GABA mediated currents during spontaneous rhythmic activity in chick embryo spinal cord has been suggested to be due to a transient decrease in intracellular Cl^- concentration during prolonged (40-50 sec) opening of Cl^- channels during a burst lasting 40-80 sec with a period of 10-15 min (Chub and O'Donovan 1999). Another example of periodic changes in the concentration of intracellular Cl^- comes from mature cells in the suprachiasmatic nucleus (SCN) of the hypotalamus (Wagner et al 1997), responsible for the generation of circadian rhythms in mammals. In parallel with the spontaneous periodic change in their electrical properties these cells show a daily rhythm in the GABA equilibrium potential (E_{GABA}). During day-time E_{GABA} is several millivolts positive to the E_{rest} of SCN cells, while during night-time this condition is reversed. Wagner and co-workers suggest that the temporal switch in E_{GABA} is due to a switch in intracellular Cl^- concentration.

In summary, activation of $GABA_A$ and glycine receptors decreases excitability, apparently regardless of whether the responses are depolarizing or hyperpolarizing. In adults, membrane hyperpolarization combines with a significant reduction in input resistance (that shunts excitatory inputs to reduce excitability). In neonates, the reduction in input resistance and associated shunt has the dominant effect on

excitability, decreasing action potential generation despite membrane depolarization (Gao and Ziskind-Conhaim 1995; Krnjevic et al 1977; Wu et al 1992). Note, however, that a depolarizing versus hyperpolarizing shunt will have different effects on voltage-gated channels. It is possible, for example, that depolarization eventually takes the membrane potential to such a level that Na^+ channels are inactivated; in this way depolarization of the membrane can result in inhibition of firing (Zhang and Jackson, 1995).

1.4 Co-localization and co-release of GABA and glycine

A recurrent theme in studies of inhibitory transmission is the extensive degree of overlap between GABAergic and glycinergic systems. As mentioned above, GABA is the principal inhibitory neurotransmitter of supraspinal interneurons while glycine is predominant for spinal interneurons. Nevertheless, there is strong anatomical evidence for colocalization of $GABA_A$ and glycine receptors at single postsynaptic densities (Bohlhalter et al 1994; Todd et al 1996; Triller et al 1987b) and for GABA and glycine colocalization in presynaptic terminals in the spinal cord (Örnung et al 1994, 1996, 1998; Shupliakov et al 1993; Taal and Hoolstege 1994; Todd et al 1996). Electrophysiological measurements are consistent with GABAergic and glycinergic contributions to IPSP/C in cranial motoneurons (Kolta 1997), as well as recurrent (Schneider and Fyffe 1992), afferent, and descending inhibitory inputs to spinal motoneurons (Gao et al 1998; Gao and Ziskind-Conhaim 1995; Pinco and Lev-Tov 1993, 1994; Stuart and Redman 1992; Wu et al 1992). In fact, GABA and glycine can apparently be coreleased from the same presynaptic terminal (Jonas et al 1998; O'Brien and Berger 1999).

The postsynaptic complement of receptors, however, is not constant between synapses. For example, analysis of evoked and mIPSCs suggests three types of inhibitory synapses on spinal motoneurons: GABA only, glycine only, and mixed synapses corresponding to 15, 41, and 44% of the total (Jonas et al 1998). In contrast, when analyzed in the Mauthner cell, the situation is different. Double labeling with anti-glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, and monoclonal antibodies (MAbs) against glycine receptors (Triller et al 1987a) showed that only a minority of GAD-positive endings are close to postsynaptic membranes displaying GlyR immunoreactivity. Interestingly, in this preparation presynaptic contacts are instead observed between serotonin-containing profiles and terminals

near glycine receptors; this observation is consistent with physiological data showing that on Mauthner cells serotonin presynaptically controls glycine release (Mintz et al 1989).

An important difference between $GABA_AR$ and GlyR distribution has been underlined by Triller et al (1990). Glycine receptors are concentrated opposite to presynaptic terminals, while $GABA_ARs$ are in general more diffusely distributed (Somogyi et al 1989). In the cerebellum of the cat, rat and monkey positive immunolabeling of dendritic and somatic membrane occurs at sites that do not correspond to synaptic loci.

Banks and Pearce (2000) have recently suggested the presence of two receptor populations on CA1 hippocampal pyramidal cells: one synaptic, which generates fast IPSCs, and another one, that dominates the responses of excised patches to exogenous GABA application and exhibits slower kinetics. The presence of extrasynaptic $GABA_ARs$ apparently responsible for the generation of slow IPSCs has been suggested to occur on lamina I neurons in adult rat spinal cord slices (Chery and de Koninck 1999).

Thus, $GABA_ARs$ are often sparsely distributed on the postsynaptic membrane, whereas GlyRs are more concentrated on the postsynaptic membrane facing the presynaptic active zone.

These data raise a number of important questions regarding how a nerve terminal type is determined, how transmitters are packaged, how postsynaptic densities are constructed to match terminal type (Nicoll and Malenka 1998), how much (and if) any interplay between two neurotransmitters is developmentally regulated. The functional significance of co-localization and of co-release to synaptic integration and neuron excitability is not clear. Moreover, it is still unclear how many inhibitory synapses can operate in this fashion, if co-release takes place also in the case of network-mediated, spontaneous events (sPSCs) and of responses evoked by electrical stimulation of afferent inputs (ePSCs). Furthermore, it is not yet known if the GABA and glycine co-release process might be subjected to modulation via receptors located on presynaptic fibres.

One way to study possible interaction between the two systems of neurotransmitter (GABA and glycine) is to analyze the effect of modulators on GABAergic and glycinergic transmission.

1.5 Modulation: metabotropic glutamate receptor

Glutamate, in addition to its role as the principal fast excitatory neurotransmitter, also modulates neuronal excitability by activating a large family of metabotropic glutamate receptors (mGluRs), which are known to up or down regulate synaptic transmission in several areas of the CNS.

Metabotropic glutamate receptors (mGluR) are coupled through GTP-binding proteins to intracellular second messenger cascades (Schoepp et al 1992; Sladeczek et al 1985; Sugiyama et al 1987; Winder and Conn 1992). There are at least eight subtypes of mGluR (mGluR1 to -8) (Abe et al 1992; Houamed et al 1991; Masu et al 1991; Nakajima et al 1993; Okamoto et al 1994; Tanabeet al 1992, 1993) that can be divided into three groups (groups I, II, and III) on the basis of sequence homology and pharmacological profiles (Conn and Pin 1997; Pin and Duvoisin 1995). Group I mGluR (mGluR-1, 5) are coupled to phospholipase C (PLC), increase the synthesis of inositol 1,4,5-trisphosphate (IP3), and trigger intracellular Ca^{2+} release (Abe et al 1992; Aramori and Nakanishi 1992; Chan et al 1986). They are highly sensitive to quisqualate, 3,5-dihydro phenylglycine, and (+)-1-amino cyclopentane-*trans*-1,3-dicarboxylic acid (*t*-ACPD). Group II (mGluR2, -3) and group III mGluRs (mGluR-4, 6-8) are negatively coupled to adenylyl cyclase and inhibit the formation of cAMP (Duvoisin et al 1995; Nakajima et al 1993; Okamoto et al 1994; Saugstad et al 1994; Tanabe et al 1992, 1993). Group II mGluRs are also sensitive to t-ACPD and selectively antagonized by metyl-L-carboxycyclopropylglycine (MCPG) whereas group III mGluRs are not sensitive to t-ACPD but respond selectively to L-2-amino-4-phosphonobutyric acid (L-AP4). However, the mechanisms by which these systems are activated are poorly understood.

The release of transmitters is modulated by a number of receptor systems located at the presynaptic terminal. The effects of activation of mGluRs on synaptic transmission are complex. Indeed, mGluR activation may either enhance (McBain et al 1994; Miles and Poncer 1993; Cochilla and Alford 1998) or depress release (Lovinger 1991; Baskys and Malenka 1991). These actions are mediated by multiple mechanisms which include inhibition of presynaptic Ca^{2+} channels (Dunlap and Fischbach 1978; Takahashi et al 1996), activation of presynaptic K^{+} channels (Morishige et al 1996; Saugstad et al 1994), direct actions on release proteins (Scanziani et al 1995) and alteration of Ca^{2+} release from presynaptic Ca^{2+} stores (Peng 1996). Although it would be interesting to study the intracellular mechanisms

of modulation triggered by activation of mGluRs, at first it seems necessary to use mGluR activation as a possible tool to obtain differential modulation of glycine or GABA mediated transmission.

Metabotropic glutamate receptors are widely distributed throughout the CNS (Baude et al 1993; Del Negro and Chandler 1998; Hay et al 1999; Shigemoto et al 1997; Tanabe et al 1993). Group I mGluRs appear to be localized postsynaptically (Baude et al 1993; Lujan et al 1996; Martin et al 1992; Shigemoto et al 1996) where they act to increase neuronal excitability (Netzeband et al 1997; Schoppa and Westbrook 1997; Schrader and Tasker 1997). Group II and III receptors are predominantly localized on presynaptic terminals (Bradley et al 1996; Kinoshita et al 1996; Li et al 1997; Shigemoto et al 1996, 1997) where they inhibit transmitter release (Baskys and Malenka 1991; Forsythe and Clements 1990; Gereau and Conn 1995; Macek et al 1996; Salt and Eaton 1995; Schrader and Tasker 1997; Trombley and Westbrook 1992; Vignes et al 1995). This differential localization is by no means exclusive (Gereau and Conn 1995; Manzoni and Bokaert 1995; Romano et al 1995). Nevertheless, the discrete cellular localization of each mGluR subtype suggests that the precise placement of receptors is a crucial factor contributing to the control of neuronal excitability.

2. A model system to study the relative role of GABA and glycine

GABA and glycine are thus considered two main inhibitory neurotransmitters at central synapses. Glycine is mainly present in the spinal cord and brainstem, while GABA is prevalent in higher regions of the neuraxis. In brain stem cranial nuclei, and, in particular, in the nucleus of hypoglossus, both inhibitory neurotransmitters are present. The co-existence of GABA and glycine in this nucleus raises the question of their relative contribution to synaptic microphysiology, their interplay as transmitters and their differential role at functional level.

Motoneurons generate the motor output as they integrate and relay patterns of interneuronal activity into commands for skeletal muscle contraction.

One of the main goals for studies of motoneuronal excitability is to understand how integration of synaptic inputs affects and modulates motoneuron firing.

Motoneuronal inputs are affected by the presynaptic release of various transmitters (amino acids, amines, peptides) acting on postsynaptic receptors; several of these transmitters can also affect signaling by acting on presynaptic receptors. The complex integration of the effects of these transmitters and the intrinsic motoneuron properties (passive and active) determine the generation of the efferent signals. Effects via ionotropic receptors induce (or reduce) localized current flow that spreads according to membrane properties and cellular morphology. Actions via metabotropic receptors initiate second messenger cascades that can have many different results, including altering channel or receptor function, or their number.

2.1 Nucleus of hypoglossus

The nucleus of hypoglossus is the XII cranial nucleus, and is located in the lower medulla. Hypoglossal motoneurons innervate tongue muscles and are thus important for functions such as swallowing, respiration, suckling, vocalization (Lowe 1981). Disorders of hypoglossal motoneurons appear to be implicated in syndromes like sleep apnea in man (Gauda et al. 1987; Wiegand et al. 1991).

2.1.1 Architecture

This nucleus consists of several longitudinally oriented cell columns stacked in the dorsoventral plane. Muscles responsible for tongue movements derive their innervation from XII and include: extrinsic muscles (the hyoglossus (HG), styoglossus (SG), genioglossus (GG) and geniohyoid (GH); the first two are protuders, the other retractors) and intrinsic muscles, that are responsible for changes in the tongue form.

Investigations of the myotopic organization of this nucleus in a number of animal species have suggested that hypoglossal motoneurons are functionally segregated into discrete areas of the nucleus. Although specific tongue movements result from various combination of retractor, protuder and intrinsic muscle activity, the mechanism of integration of XII motoneuron activity during complex motor behaviors is unknown.

Up to 90% of the cells within the XII nucleus are motoneurons (Viana et al 1990, see also results of the present study), although there is anatomical evidence for a small population of interneurons. However, these neurons are smaller (10-18 μm) than

motoneurons (25-50 μm), have fewer dendritic processes and are confined to the ventrolateral or dorsolateral borders of the nucleus.

At ultrastructural level, interneurons have invaginated nuclei and little cytoplasm, in contrast to motoneurons that contain a large, centrally-placed round nucleus and abundant cytoplasm (Takasu and Hashimoto 1988). It is established that the XII nucleus contains a small population of interneurons that are probably GABAergic (as suggested both by radioactive uptake studies and immunohistochemical staining for GABA and its enzymatic precursor glutamic acid decarboxylase (GAD), Aldes et al 1988; Takasu et al 1987). However, it is still unclear whether hypoglossal interneurons receive recurrent axon collaterals from motoneurons, similar to spinal cord Renshaw cells, or whether their relatively few synaptic terminals (Takasu and Hashimoto 1988) are derived solely from external projections (Takasu et al 1987).

Cytoarchitectonic characteristics of size, shape and dendritic orientation differentiate motoneurons within different regions of the hypoglossal nucleus (Fukunishi et al 1999); whether these cytoarchitectonic characteristics may reflect also differences in the electrophysiology of different motoneuron pools within the nucleus is still unknown.

2.1.2 Afferents

Afferent projections reach hypoglossal nucleus from cells in the midbrain, pons and medulla (Aldes 1980; Borke et al 1983; Cooper 1981; Travers and Norgren 1983). These projections originate from well-defined cell groups in identified brain stem nuclei and from more widely distributed, poorly defined regions of the reticular formation (RF). These projections define the final common pathway for several motor systems that make use of the tongue, including ingestive consummatory response (mastication, licking, swallowing) as well as grooming and respiration.

Projections onto hypoglossal motoneurons are represented at ultrastructural level by a variety of axon terminals and synaptic specializations (Boone and Aldes 1984; Sumner 1975): asymmetric S-type synapses characterized by spherical vesicles, symmetric F-type synapses with flattened vesicles, symmetric P-type synapses with pleiomorphic vesicles, C-type synapses with pleiomorphic vesicles and subsynaptic cysternae, asymmetric T-type ones with spherical vesicles and subsynaptic dense bodies.

There appear to be no direct cortical projections to XII in the rat (Travers and Norgren 1983), emphasizing the general scheme that voluntary motor commands to motoneurons likely pass through various group of brain stem (and/or spinal) premotor neurons. Descending pathways, however, may activate neurons in the brain stem RF that organize oromotor responses of mastication and licking.

Proprioceptive information from the muscles of mastication reaches the trigeminal (V) and hypoglossal nucleus via the trigeminal mesencephalic nucleus. Afferent information from other peripheral sensors enter the brain stem through the vagal (X), glossopharingeal and accessory (XI) nerves and is conveyed to the trigeminal, facial (VII) and hypoglossal nuclei via premotor neurons in the nucleus of the solitary tract. The central subnucleus of the solitary tract contains the pattern generator for swallowing and conveys direct synaptic information to the hypoglossal motoneurons and motoneurons of the compact formation of the ambiguus nucleus. The largest concentration of premotor neurons to orofacial motor nuclei is in the medullary and pontine reticular formation adjacent to the motor nuclei themselves. In particular, hypoglossal premotor neurons are ventrolateral and dorsolateral in the medullary reticular formation (Borke et al 1983; Dobbins and Feldman 1995; Travers and Norgren 1983). In addition to these regions, other afferents to the hypoglossal nucleus come from pontine nuclei (parabrachial and intratrigeminal nuclei and Kölliker-Fuse nucleus; Aldes 1980; Borke et al 1983; Saper and Loewy 1980; Travers and Norgren 1983). Descending projections from limbic structures to the midbrain provide additional pathways through which forebrain structures involved in feeding can ultimately influence the activity of hypoglossal motoneurons. Few cells in the controlateral reticular formation project to XII motoneurons (as demonstrated with HPR; Aldes 1980; Aldes and Boone 1984; Travers and Norgren 1983).

2.1.3 Input specificity

Although there are noradrenergic, serotoninergic and peptidergic inputs to hypoglossal motoneurons, most premotor neurons are GABAergic, glycinergic or glutamatergic. Within the brainstem, a large part of GABAergic or glycinergic inputs to hypoglossal motoneurons originate from the RF (Li et al 1996, 1997), but also from the respiratory centres (ventral tegmental area and, in the ventrolateral medulla, the Pre-Bötzinger complex; Paton and Richter 1995).

2.1.3.1. Localization of glycinergic receptors on motoneurons.

On cranial and spinal motoneurons, glycine receptors appear confined to the postsynaptic membrane, with few differences among the various motoneuron pools.

The differential expression of glycine receptor subunit proteins on motoneurons is poorly described. Specific antibodies are only available for α1-subunits and gephyrin. Few motoneuron studies have used the α1-antibody (Bechade et al 1996; Triller et al 1987a, b, 1985). Most have used a nonspecific antibody or the antibody against gephyrin (Bohlhalter et al 1994; Geyer et al 1987; Shefchyk et al 1998; Todd et al 1995, 1996; Van den Pol and Gorcs 1988). Evidence that gephyrin may also be expressed at nonglycinergic synapses indicates the need for caution in using gephyrin immunoreactivity on its own as a marker for glycinergic synapses (Sassoe-Pognetto et al 2000; Levi et al 1999; Wang et al 1999).

Distribution of glycine receptor subunit transcripts assessed with *in situ* hybridization indicates marked developmental changes in expression patterns but similar patterns between the different motoneuron pools of the adult. In general, transcripts for α_1- and β-subunits are highly expressed in adult cranial and spinal motoneurons (Fujita et al 1991; Malosio et al 1991; Sato et al 1991, 1992). α_2-transcripts are reduced relative to α1 (Malosio et al 1991; Watanabe and Akagi 1995) but show similar patterns of distribution (Racca et al 1997, 1998). Gephyrin transcripts are high in spinal motoneurons (Kirsch et al 1993), whereas α_3-mRNA is barely detectable in spinal ventral horn and hypoglossal motoneurons (Malosio et al 1991; Singer et al 1998). Details of the α_4-distribution are also unclear, but low levels are expressed in the mouse spinal cord. The only marked spatial difference in expression is that, relative to spinal and cranial motoneuron pools innervating striated muscle, α_1-transcripts appear reduced in motoneurons innervating the eye muscles (Sato et al 1991). Consistent with immunohistochemical data, α_1-, α_2-, and β-transcripts are also lower in visceromotor nuclei (EW and DMV) relative to motoneurons innervating striated muscle (Fujita et al 1991; Sato et al 1991, 1992).

Subcellular distribution of glycine receptor transcripts may, however, differ between spinal and cranial motoneuron pools. In the majority of spinal cord motoneurons, α_1- and α_2-transcripts are localized to the soma and dendrites, whereas β-subunit and gephyrin mRNAs are restricted to the soma (Racca et al 1997, 1998). In contrast, in

facial, hypoglossal, and ambigual motoneurons, α-transcripts are restricted to the soma (Racca et al 1998).

2.1.3.2 GlyRs: developmental changes

The potential diversity of glycine receptors is less than that of $GABA_A$ receptors. However, variations in subunit composition affect gating properties and may account for heterogeneity in the voltage dependence and desensitization of glycine responses (Rajendra et al 1997). The most obvious change in GlyR structure and function occurs during the first 2-3 postnatal weeks, when the fetal/neonatal receptor (most probably an homomeric α_2-receptor) matures to the adult heteromeric form that lacks the α_2-subunit (Becker et al 1988; Malosio et al 1991; Kushe et al 1991). The open time of recombinant α_2-receptors is much greater than for homomeric α_1- and native adult receptors (Singer et al 1998; Takahashi et al 1992). These changes are consistent with developmental decreases in the decay time course of inhibitory postsynaptic currents (IPSC) in spinal neurons. In hypoglossal motoneurons, a postnatal switch from α_2- to α_1-glycine receptor subunit expression correlates with shorter channel open times and faster PSC/P decays, matching kinetic properties of glycinergic synaptic potentials to membrane properties of the motoneurons (Singer et al 1998). Thus, changes in glycine receptor structure appear to significantly alter glycinergic transmission during development.

2.1.3.3 Localization of $GABA_AR$ subunits in cranial motoneurons

GABAARs are ubiquitous on cranial motoneurons (Aldes et al 1988; Mugnaini and Oertel 1985), but expression patterns of the various receptor subunits appear to differ between cranial and spinal motoneurons (Table 1). Facial and trigeminal motoneurons of adult rat express transcripts for α_1-, α_2-, $\beta_{1/2}$-, and γ_2-subunits but very low levels, or absence, of α_3-, α_5-, α_6-, β_1-, γ_1-, and δ-transcripts (Persohn et al 1992). α_1-mRNA is also strongly expressed in the dorsal motor nucleus of vagus (DMV), XII, facial, III, IV, VI, with only weak labeling in V nucleus (Hironaka et al 1990). Immunohistochemical analyses with antibodies specific against $\alpha_{1/3}$-, α_5-, $\beta_{2/3}$-, γ2-, and δ-subunits indicate intense to moderate labeling for α_1-, α_2-, and γ2-subunits and weak labeling for α_3 and α_5 in III, V, VII, ambigual, DMV, and XII adult motoneurons. The only exceptions to this general pattern are that V and DMV show

minimal labeling for α_1 and that $\beta_{2/3}$ has only been detected in the VII nucleus (Fritschy and Mohler 1995). Thus, the major feature distinguishing cranial from spinal motor pools is the presence of α_1- and α_3-subunits in cranial motoneurons. It is also of considerable interest the apparent lack of $\beta_{2/3}$-subunits in all adult motoneuron pools (spinal and cranial), because β-subunits are widely expressed in many other neuron types. Differences between functional groups of cranial motoneurons are not yet apparent.

	α1		α2		α3		α5		α6		β1		β2		β3		γ1		γ2	
δ Motoneuron nuclei	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH	I	ISH
CRANIAL																						
Oculomotor (III)	2-3	3	2-3		1		1						nd		nd				2	2-3	nd	
Trochlear (IV)		3																				
Trigeminal (V)	1	1	2-3	1	1	A/L	1	A/L		A/L		nd	1	1	1	1		A/L	2	2-3	nd	A/L
Abducens (VI)		3																				
Facial (VII)	2-3	2-3	2-3	2	1	A/L	1	A/L		A/L		A/L	1	1	1	1		A/L	2	2-3	nd	A/L
Vagal (X)	1	3	2-3		1		1						nd		nd				2	2-3	nd	
Ambigual	2-3		2-3		1		1						nd		nd				2	2-3	nd	
Hypoglossal (XII)	2-3	3	2-3		1		1						nd		nd				2	2-3	nd	
SPINAL	A/L	A/L	2.5	3	A/L	A/L	1-2			A/L			A/L	nd	A/L	2		nd	2	2		nd

Table 1 I= immunohistochemical, IHS= *in situ* hybridization detection of subunit; nd= not detected, A/L= absent/low, 1= low, 2= moderate, 3= strong; blank entry indicate data not available (from Rekling et al 2000)

2.1.3.4 $GABA_A$Rs: developmental changes

The GABAergic system undergoes substantial developmental regulation (Lauder et al 1986; Ma et al 1992, 1993; McKernan et al 1991; Poulter et al 1993a,b; Xia and Haddad 1992). Transcripts for α_2, α_3, α_5, $\beta_{2/3}$, and $\gamma_{2/3}$ are all present in presumptive motoneurons of the mantle zone by embryonic day 13 in the rat. Peak expression of subunits occurs between embryonic day 17 and 20 when mRNAs for $\alpha_{2\text{-}5}$, $\beta_{1\text{-}3}$, and $\gamma_{1\text{-}3}$ subunits are all present. $\alpha_{3\text{-}5}$, $\beta_{1/2}$, γ_1, and γ_3 then decrease and are almost absent by the end of the second postnatal week (Ma et al 1993). Developmental changes in transcript expression appear to result in changes in the amount of receptor protein subunit (Fritschy et al 1994; Laurie et al 1992a).

The precise role of $GABA_AR$s in the development of motoneuron circuits and modulation of motoneuron excitability is not known. GABA itself can alter $GABA_A$ (and $GABA_B$) receptor expression (Liu et al 1998; Malcangio et al 1993, 1995; Schousboe and Redburn 1995). In addition, the abundant expression of $GABA_AR$s in embryonic and neonatal spinal cord may serve a trophic role in regulation of neuronal differentiation and synaptogenesis (Meier et al 1991). In early development depolarization due to $GABA_AR$ activation could elevate intracellular Ca^{2+} levels, a potentially important signal in modulating early differentiation (Spitzer 1994). In addition, GABA (and glycine) mediated depolarization, in conjunction with the observation that in some systems glutamatergic synaptic transmission is strongly mediated by NMDA receptors early in development (Ziskind-Conhaim 1990), has led to the suggestion that in hippocampus GABA receptor activation may facilitate activation of NMDA receptors (Ben Ari et al 1997). In such a scheme, GABA (and glycine) mediated depolarization would play the role in activity-dependent organization of neuronal circuits which, later in development, is brought about by AMPA receptors.

2.1.4 Spatial distribution of amino acid receptors over the somatodendritic tree

Ultrastructural characteristics of excitatory and inhibitory synapses on motoneurons are well established. Analysis of the distribution of presumptive excitatory (glutamatergic; S- or M-type terminals) and inhibitory (GABA or glycinergic; F-type terminals) boutons (Bernstein and Bernstein 1976; Bodian 1975; Bras et al 1987; Lagerback and Ulfhake 1987; Rose and Neuber-Hess 1991), combined with postembedding immunohistochemical studies of amino acid transmitters in boutons synapsing with motoneurons, have provided important insight into the spatial distribution of synapses on motoneurons. Although many of the original studies focused on the soma and proximal dendrites (Destombes et al 1992, 1996; Holstege and Calkoen 1990; Örnung et al 1996; Ramirez-leon and Ulfhake 1993), extension of these studies to include distal dendrites has been essential, since dendrites comprise >90% of the motoneuron receptive domain. A number of general organizational features are emerging. 1) Between 85 and 95% of boutons on spinal motoneurons (lumbar, phrenic, sacral) are immunoreactive for glutamate, GABA, and/or glycine (Murphy et al 1996; Örnung et al 1998). 2) Terminals that are exclusively glycinergic exceed those that are exclusively GABAergic; there is a population of terminals

positive to both neurotransmitters, at least in the spinal cord (Jonas et al 1998; Örnung et al 1994, 1996, 1998; Taal and Holstege 1994). 3) Almost 60% of boutons apposing dendrites of spinal and brain stem motor nuclei of cat and rat contain glycine and/or GABA (Destombes et al 1992; Örnung et al 1998, 1996, 1994; Ramirez-Leon and Ulfhake 1993; Saha et al 1991; Shupliakov et al 1993; Taal and Holstege 1994). 4) Glutamate-enriched boutons appear to comprise just over one-third of all boutons in lumbar and sacral motoneurons (Örnung et al 1998) and between ~48 and 58% of boutons in the phrenic nucleus (Murphy et al 1996, Tai and Goshgarian 1996). 5) The proximal compartment (soma and stem dendrites) appears to be under powerful glycine/GABA inhibitory influence. The proximal compartment of lumbar motoneurons has a glycine/GABA-to-glutamate synaptic ratio of 3.5-4:1, and the distal compartment has a ratio of ~1.5:1 (Örnung et al 1998). 6) Inhibitory synapses are not limited to the soma and proximal dendrites. Distal compartments of lumbar motoneurons show a uniform balance between inhibitory and excitatory synapses, with glutamate accounting for 40% of synapses (Örnung et al 1998). The importance of inhibitory synapses in dendrites has long been recognized. Not only can inhibition gate action potential production at the soma, but it can control the weight of excitatory inputs from different dendritic regions (Skydsgaard and Hounsgaard 1996).

AIMS

The present study of neonatal rat hypoglossal motoneurons aimed at addressing:

- relative contribution by glycine or GABA to Cl^- mediated synaptic events;
- effect of GABA and glycine on the excitability of hypoglossal motoneurons
- basic properties of glycinergic and GABAergic currents: characteristics of spontaneous (sPSCs) and miniature (mPSCs) events;
- sensitivity of glycinergic or GABAergic transmission to tetrodotoxin (TTX);
- modulation of glycine or GABA sPSCs and mPSCs by the broad spectrum metabotropic receptor agonist (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*t*-ACPD);
- basic properties of glycinergic or GABAergic electrically evoked post synaptic currents (ePSCs);
- short term plasticity properties of glycinergic or GABAergic synapses;
- presence and extent of co-release process for glycine and GABA.

METHODS

1. Slice preparation

Experiments were carried out on brainstem slices obtained from 0 to 5 day old Wistar rats terminally anesthetized with 0.2 ml urethane (10 %; i.p. injection). The entire procedure (including animal handling and care) is in accordance with the Animal Welfare Act and was approved by the Local Authority Veterinary Service. Thin brainstem slices were prepared following the procedure described by Viana et al. (1994). In brief, the brainstem was isolated and placed in modified ice-cold Krebs solution (see below) to dissect out the lower medulla which was then pinned to an agar block placed inside a Vibratome chamber (filled with ice-cold Krebs solution gassed with 95 % O_2/5 %CO_2). 200 μm thick slices were cut and transferred to an incubation chamber for 1 h at 32°C containing continuously oxygenated Krebs medium. The incubation temperature was later lowered to ambient level and the slices maintained under this condition for at least 1 h before use. The slice was then transferred to a small recording chamber, held in place by fine nylon strands glued to a horseshoe-shaped platinum wire form, and superfused with a continuous stream of recording Krebs solution (see below) gassed with 95 % O_2/5 %CO_2.

2. Electrophysiological recordings

For electrophysiological experiments brainstem slices placed in a small recording chamber were viewed with an infrared video-camera to identify single hypoglossal motoneurons within the XII nucleus. For voltage clamp experiments cell recordings were obtained with whole-cell patch-clamp electrodes (3-5 MΩ DC resistance), pulled from thin walled borosilicate-glass capillaries with a L/M-3P-A puller (List Medical), using a L/M – PCA amplifier (List Medical) while cells were clamped at -70 mV holding potential (V_h). For current clamp experiments recordings were obtained with whole-cell patch-clamp electrodes (12-15 MΩ DC resistance) pulled from thick walled borosilicate-glass capillaries and using an Axoclamp-2A amplifier (Axon Instruments). A positive pressure was applied to the patch pipettes to prevent blocking while advancing through the tissue. Offset potentials were nulled using the DC offset control. Seal resistance was usually higher than 2 GΩ. After seal rupture,

series resistance (R_s; 5-25 MΩ) was routinely monitored and compensated (usually by 30%, range 20-60%). Voltage clamp recordings were performed only after stabilization of R_s; if changes in R_s exceeded 10% during recordings, data were not considered for analysis. The bridge was balanced routinely in current clamp experiments. Voltage and current pulse generation and data acquisition were performed with a PC running pClamp 6.1 software. Voltage pulse (or current pulse, for the experiments in current clamp) generation and data acquisition were performed with a PC using pClamp 6.1 software. Currents elicited by voltage steps were filtered at 3-10 kHz and sampled at 5-10 kHz. All electrophysiological recordings were performed at room temperature.

2.1. Extracellular stimulation

For extracellular stimulation at low frequency (0.2 Hz; 0.2 ms; variable intensity) of GABAergic or glycinergic cells a single bipolar tungsten electrode was placed in the lateral reticular formation. After stabilization of the synaptic response stimulus intensity was adjusted to obtain 25-50% failures for 100 stimuli. Evoked synaptic currents were then stored in a PC as individual files and averaged with pCLAMP software (version 6.1) after discarding failed events.

The high frequency stimulation protocol was as follows: a train of 100 stimuli was delivered at chosen frequency (2, 5, 10 or 20 Hz), and ≥ 5 trials (depending on the stability of recording) were repeated at ≥ 5 min intervals (to allow for synaptic recovery). Responses to each trial were stored as individual files and averaged over different trials. Average responses to a tetanus were then normalized to the first response. An exponential function of the form: $a + (1 - a) \exp(-t/b)$ was then fitted to the time course of the normalized responses (using SigmaPlot software, see below).

2.2. Solutions and drugs

For slice preparation and subsequent incubation the solution (mM) was NaCl 130, KCl 3, $NaHCO_3$ 26, Na_2HPO_4 1.5, $CaCl_2$ 1, $MgCl_2$ 5, glucose 10 (290-310 mOsm). For electrophysiological recordings the extracellular control solution (mM) was NaCl 140, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10 (pH 7.4, 290-310 mOsm). Concerning voltage clamp experiments, unless otherwise stated, 2 mM kynurenic acid was routinely added to block glutamatergic ionotropic currents. The patch pipette

solution (mM) was CsCl 120, NaCl 9, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, EGTA 10, Mg-ATP 2 at pH 7.2 (270-290 mOsm) for voltage clamp experiments and (mM): K-methyl-SO_4 110, KCl 20, NaCl 10, $MgCl_2$ 2, HEPES 10, EGTA 0.5, ATP-Mg 2 (pH 7.2, 260-270 mOsm) for current clamp experiments. The liquid junction potential (V_j) between external and internal solutions (Barry and Lynch 1991; Neher 1992) was measured with a 3 M KCl-agar bridge (Neher 1992). V_j between the extracellular solution and the pipette solution containing CsCl was $\approx$ 0 mV, and hence no correction was added, whereas V_j between external and pipette solution containing $KMeSO_4$ was measured to be 10 mV. All potential values of the experiments done under current clamp conditions were thus corrected off-line subtracting this value.

Drugs were applied via the extracellular solution (superfused at 2-5 ml/min) for a minimum of 5-10 min to reach equilibrium conditions.

The following drugs were used: kynurenic acid (Sigma), bicuculline methiodide (Sigma), strychnine hydrochloride (Sigma), GABA (Sigma), glycine (Sigma), TTX (Affiniti, UK), (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (*t*-ACPD; Tocris, UK), 3-[[(3,4-dichlorophenyl)methyl]amino]propyl]diethoxymethyl)phosphinic acid (CGP 52432, gift from Novartis Pharma) and sodium pentobarbital (B.D.H.).

2.3. Data Analysis

Cell input resistance (R_{in}) was calculated by measuring the current response to 10 or 20 mV hyperpolarizing pulses (from -70 mV V_h), or from the slope of the linear part of the I/V relation obtained by applying a slowly rising voltage signal (ramp test).

Detection of single postsynaptic currents was done with AxoGraph 3.5 (Axon Instruments, Foster City, CA) software which uses the method of minimizing the sum of squared errors between data and a template function approximating the width and time-course of a typical synaptic event as described by Clements and Bekkers (1997).

For the characterization of pharmacologically isolated GABAergic or glycinergic PSCs, trace recordings of the duration of 3-5 min were selected after reaching apparent equilibrium conditions for drug application.

Rise time of captured single events was measured between 10-90% of peak amplitude, while for average events it was measured between 20-80% of peak amplitude to minimize errors due to any single event misalignment. Exponential fitting of captured event decay was carried out with the Chebychev algorhythm

provided by AxoGraph software. The decay of the vast majority of pharmacologically identified glycinergic or GABAergic mPSCs was best fitted by a monoexponential function. For a small minority of mPSCs (≤ 20 % of all events) the timecourse of decay was multiphasic and, as a first approximation, was fitted by a single time constant, representing the weighted average of the individual components. Sigma Plot (Jandel Scientific, San Rafael, CA) and Clampfit (Axon Instruments) softwares were used for linear regression analysis of experimental data. Paired or unpaired *t*-test was used to assess differences in mean values; $p<0.05$ was considered as the acceptable level of statistical significance.

3. Histochemical staining

3.1. Neurobiotin

Neurons were filled with neurobiotin (0.2%) via the patch pipette during electrophysiological recordings. Afterwards, slices were transferred to fixative solution (4% paraformaldeyde in phosphate buffered saline; PBS), at pH 7.2 at 4 ^{0}C for more than one day. Thereafter, after quenching for 15 min in H_2O_2 (1% in PBS) and leaving for 1 hour slices in Triton (2% in PBS), they were incubated overnight at 4^{0}C in avidin-biotin-horseradish peroxidase complex (ABC kit, Vectastain Elite kits, Vector Laboratories) in PBS containing 2% Triton. Each step was followed by wash in PBS. After a 1/2 hour rinse, slices were reacted in a solution of diaminobenzidine in PBS (5mg/10ml) containing nickel ammonium sulfate (150 µl of 1% per 10 ml); H_2O_2 (0.1%; 30 µl per 10 ml) was added to speed up the reaction. The tissue was reacted for 10-15 min, during which time the reaction was monitored under a dissecting microscope and stopped when reaction product began to appear in the background. Slices were then mounted on glass slides, dehydrated in ethanol, cleared in xylene and covered with Kristalon mounting medium and coverslip glass.

3.2. Immunohistochemistry

Immunohistochemical detection of neuronal choline acetyltransferase was carried out as described by Ballerini et al. (1999) using anti-choline acetyltransferase polyclonal antibodies (Chemicon Int., Temecula, CA). Immunocytochemical detection of α_1, α_2, β_2, β_3 subunits of the $GABA_A$ receptor was performed by using polyclonal antisera

(Santa Cruz Biotechnology, Santa Cruz, CA) against a peptide mapping the amino terminus of the α_1 or α_2 subunits, and a monoclonal antibody against the $\beta_{2/3}$ subunit of the same receptor (Mize and Butler 1997). For this purpose the whole brainstem was removed and fixed in paraformaldheide (4% in PBS) for 24 h at 4^0C, then in sucrose (30 % in PBS) for cryoprotection for the same period of time. Transverse sections (20 µm thin) were cut with a sliding microtome at the level of the lower medulla from frozen blocks of tissue and placed in wells containing the fixative until further use. After washing with Triton X-100 (0.2% in PBS), incubating for 30 min in H_2O_2 (3 % in PBS) and further washing, free floating sections were incubated overnight at 4^0C in the solution containing primary antibodies (diluted 1:100 in PBS containing 0.2% Triton X-100 and 10 % fetal calf serum; FCS). After washing with PBS, slices were incubated for 1 h in the solution containing the secondary antibody (diluted 1:100 or 1:50 in 10% FCS in PBS). After further washing sections were then incubated at room temperature for 1 h with the ABC kit for alkaline phosphatase, washed and developed for 20-30 min in buffer containing (for 10 ml): 1 ml Tris (1 M; pH 9.5), 0.2 ml NaCl (5 M), 0.5 ml $MgCl_2$ (1 M), 10 µl levamisol (1 M) and, just before starting the reaction, 45 µl of nitrobluetetrazolium (75 mg in 1 ml of 70% dimethylformamide) and 35 µl of bromocloroindolilphosphate (BCIP; 50 mg in 1ml of 100% dimethylformamide). The reaction was stopped with PBS and slices mounted on gelatinized slides, dried for 30 min at 55°C, dipped in solutions of decreasing methanol concentration (100-50-0%) and increasing xylene strength (0-50-100%) and finally covered with coverslips.

RESULTS

The database of the present study comprises 137 motoneurons with 64±3 pF somatic capacitance and 290±20 MΩ input resistance (R_{in}).

1.Histochemical staining

1.1 Immunohistochemistry: choline acetyltransferase and α1, α2, β2-3 $GABA_AR$ subunits detection

The vast majority of cells in this area were positively stained for choline acetyltransferase (see Fig. 1 A), and were thus identified as motoneurons (cf. Viana et al. 1990). While the presence of glycine receptors on neonatal rat hypoglossal motoneurons has previously been reported (Singer and Berger 1999; Singer et al. 1998), the localization of $GABA_A$ receptors on motoneurons of the same age is uncertain. For this reason we investigated the immunocytochemical presence of α_1, α_2, β_{2-3} subunits of $GABA_A$ receptors. The α_1 subunit (Fig. 1 B) or the β_{2-3} subunits (not shown) were not detected. Conversely, Fig. 1 C, D show, at low and high power, motoneuron somata extensively labeled by the α_2 subunit antibody.

1.2 Neurobiotin staining

To visualize the extension of the dendritic tree, some neurons were injected with neurobiotin during electrophysiological recordings. Fig. 2 A shows an example of motoneuron occupying a ventrolateral position within the nucleus of hypoglossus. It had an extensive dendritic arborization; in particular, three major dendrites (one of which bifurcates in the dorsolateral direction) were present in the slice plane, while a fourth went deeper into the slice. Another long process, probably the axon, extended along the direction of the axons forming the hypoglossal nerve. Fig 2 B shows another example of two hypoglossal motoneurons sequentially recorded and injected in the same slice, within the ventomedial part of the hypoglossal nucleus (note that are visible the boundaries between hypoglossal and vagal nuclei). Fig. 2 C is the same than is Fig 2 B at higher magnification, so that it is possible to see soma shapes. As described by Fukunishi et al (1999), hypoglossal motoneuron shapes are variable. Two are the most frequently observed shapes in the dorsal and in the ventral part of

the ventromedial nucleus, respectively: one ovoidal with a smaller diameter (40.6±1.8 μm, Fukunishi et al 1999), like the one in Fig. 2 B (C) (smaller neuron on the right), and the other one multipolar with a larger diameter (45.2±3.2 μm, Fukunishi et al 1999), similar to the Fig 2B (C), left.

2. Glycine or GABA actions on hypoglossal motoneurons: general properties

2.1 GABA or glycine bath application: voltage clamp experiments

Motoneurons of the hypoglossal nucleus responded both to glycine and GABA applied via the extracellular solution. Fig. 3 A shows an example of the inward currents evoked (on the same cell) by adding 100 μM glycine (left) or GABA (right) to the control solution containing TTX and kynurenic acid. Addition of 100 μM glycine induced a 49±7% decrease in input resistance (n=16 cells), reversible after glycine washout, while the same concentration of GABA also induced a comparable (53±7%) and reversible decrease (n=15 cells). Strychnine (0.4 μM) completely abolished the current evoked by glycine (Fig. 3 B, left), whereas bicuculline (10 μM) fully blocked the GABA evoked current (Fig. 3 B, right). Interestingly, on the cell in Fig. 3Bb left, strychnine fully suppressed all synaptic events even if exogenous application of GABA (100 μM) still elicited inward currents as large as in control solution; a similar effect was observed in other 4 cells.

Since in the present study glycinergic or GABAergic currents were pharmacologically dissected by using their respective antagonist strychnine (0.4 μM) or bicuculline (10 μM), experiments were performed to assess the receptor selectivity of these antagonists. On cells bathed with a solution containing 1 μM tetrodotoxin (TTX) and 2 mM kynurenic acid, bicuculline (10 μM) did not significantly ($p<0.01$; n=10 cells) reduce the amplitude of peak currents evoked by application of 100 μM glycine (sample traces are shown in Fig. 3 C). On some cells (n=4), bicuculline reversibly enhanced glycine mediated currents (22±7 %, $p<0.01$). Likewise, strychnine (0.4 μM) reduced the amplitude of the peak current evoked by 100 μM GABA by 5±3% only (n=7 cells), as shown in Fig 3 D. These data are summarized in Fig 3 E, are consistent with results found on spinal motoneurons (Jonas et al 1998) or cultured medullary neurons (Lewis and Faber 1993) and demonstrate that at the concentrations used in the present study bicuculline or strychnine retained their antagonism selectivity

against $GABA_A$ or glycine receptors, respectively. For this reason, 10 μM bicuculline or 0.4 μM strychnine added to the control solution could be used as pharmacological tools to separate glycinergic or GABAergic currents, respectively.

2.2 Current clamp experiments: shunt inhibition

To test the effect of glycine or GABA on the excitability of motoneurons, a set of esperiments were done in current clamp configuration. Repetitive firing like the one in Fig. 4 A was evoked by injecting cells with a small current pulse (ΔI=0.2 nA, Δt=160 ms). Application of 100 μM GABA to cells at resting membrane potential of -70±3 mV, evoked a depolarization (Fig. 4 B) which was on average 12±2 mV (n=6 cells). In this condition the cell failed to fire action potentials for the same current step. To determine if inhibition of firing could be due to inactivation of voltage – gated channels, the membrane potential was repolarized to its resting value (as in control condition) with steady DC current. Indeed, also in this case firing was inhibited, as shown in Fig 4 C. After washing out GABA, the cell recovered its normal firing pattern (Fig. 4 D). After GABA washout, when the cell membrane potential was manually raised to the same potential induced by GABA, the cell retained its ability to fire, as shown in Fig. 4 E. A similar inhibition of cell firing was observed after adding 100 μM glycine to the extracellular solution, and performing the same protocol described for GABA application. In particular, the example of Fig 5 shows a cell onto which glycine or GABA were applied (Fig. 5 B, Bi, respectively); in both tests comparable depolarization of the cell membrane potential and inhibition of firing ensued, even after injecting steady DC current to repolarize the cell (Fig. 5 C, Ci). These effects were reversible after wash (Fig. 5 D, Di). Fig. 5 E shows cell firing in control solution in response to injection of the short current step when its resting potential was manually (by steady DC current injection) taken to the same level reached after glycine or GABA application.

These data indicate that, despite their ability to evoke membrane depolarization, exogenous application of GABA or glycine (100 μM) effectively inhibited motoneuron firing, presumably because of a large fall in input resistance.

3. Characteristics of GABAergic and glycinergic postsynaptic currents on hypoglossal motoneurons

3.1 Spontaneous postsynaptic currents (sPSCs)

In kynurenic acid solution spontaneous synaptic activity routinely consisted of inward currents occurring on average at 1.5±0.3 Hz (n= 19 cells; see example in Fig. 6 A). Under our recording conditions (symmetrical Cl^- concentration across the cell membrane and -70 mV V_h) glycinergic as well as GABAergic events were inwardly directed. To separate them pharmacologically, we used 10 μM bicuculline or 0.4 μM strychnine, respectively, as shown in the middle and lower traces of Fig. 6 A; note that strychnine was applied after bicuculline had been washed out and synaptic events had returned to control frequency and amplitude. This approach is exemplified in Fig. 6 B, which depicts the scatter plots of peak amplitudes (top) and inter-event intervals (middle) of spontaneous events recorded before and during drug application and their washout. Note that bicuculline washout was rapid and complete in < 1min, whereas the action of strychnine could not be fully reversed even after >30 min washout

3.1.1 Glycinergic sPSCs

On a random sample of 11 cells exposed to bicuculline spontaneous currents occurred at an average frequency of 1.3± 0.4 Hz, a value (90±20 %) not significantly different from control. When strychnine was further added in the presence of bicuculline (n=7), in all cases it completely abolished any residual spontaneous activity, indicating that under the present conditions spontaneous currents were mediated by activation of glycine and $GABA_A$ receptors.

3.1.2 GABAergic sPSCs

After addition of strychnine to the control solution spontaneous GABAergic currents occurred at a frequency of 0.9±0.3 Hz (n=13 cells) which corresponded to a decrease by 69±8 % in control event frequency. Any residual activity was completely abolished by subsequent addition of bicuculline as shown in the sample trace of Fig. 6 B (bottom), in which events spontaneously occurring in a solution containing kynurenic acid (2 mM) and strychnine (0.4 μM), are rapidly suppressed by bath application of bicuculline (10 μM) with recovery after washout. Fig. 6 C (left) exemplifies the mean time-course of glycinergic or GABAergic sPSCs (obtained by averaging all events recorded from two cells in the presence of bicuculline or

strychnine). After scaling, the GABAergic response had slower decay (and rise-time) than the glycinergic one (Fig. 6 C, right). Table 1 summarized the mean characteristics (in terms of kinetics and frequency) of Cl^- mediated sPSCs and confirms that GABAergic sPCSs were significantly slower than glycinergic ones.

Table 1. Mean characteristics of sPSCs.

	Rise (ms)	Amplitude (pA)	Decay (ms)	Frequency (Hz)	
Glycinergic	2.5 ± 0.3	-24 ± 4	12 ± 1	1.3 ± 0.4	n=11
GABAergic	4.2 ± 0.3 **	-15 ± 2	23 ± 2 **	0.9 ± 0.3	n=13

Double asterisks indicate a statistically significant difference between glycinergic and GABAergic sPSCs average values with $p<0.001$ (*t*-test). n= number of motoneurons. Values are means ± S.E.

3.2 Miniature postsynaptic currents (mPSCs)

3.2.1 Glycinergic mPSCs

Fig. 7 A (left) shows sample traces (obtained at -70 mV V_h in control solution; see top two records) which comprised spontaneously occurring inward currents (mPSCs) in the continuous presence of TTX. These events appeared at random (at 2.2±0.5 Hz frequency) with relatively fast onset (2.7±0.1 ms) and monoexponential decay (14.3±0.7 ms; n=22 cells). Fig. 7 A (left, middle) shows that bicuculline reduced (by 17 %) the frequency of all mPSCs and did not abolish those with multiphasic decay (see asterisks) in accordance with a recent report (Singer et al. 1998). Subsequent addition of strychnine (plus bicuculline; left, bottom row of Fig. 7 A) completely suppressed any synaptic activity, indicating that in kynurenic acid solution mPSCs were due to activation of GABA and glycine receptors. The right panel of Fig. 7 A shows the time profile of changes in the event peak amplitude and the inter-event interval during antagonist application (same cell as in the left panel). Note the slight decrease in event peak amplitude after < 1 min of bicuculline application and the increase in the inter event interval with rapid disappearance of all events after further addition of strychnine.

Pharmacologically isolated glycinergic mPSCs occurred at 1.6±0.5 Hz (n=19 cells) and made up 70±10% (n=16 cells) of the total events. Fig. 7 B shows that there was no correlation between glycinergic mPSC amplitude and rise-time, making it unlikely that they were merely shaped by electrotonic filtering (Soltesz et al 1995; Ulrich and Lüscher 1993). Likewise, we found no correlation between mPSC amplitude and decay time (r=0.11, slope= 0.09±0.03 ms/pA). An electrical signal generated at electrotonically remote sites may be attenuated because of passive cable spreading and the shape of that signal may be distorted (the amplitude reduced and the time course slowed down) when recorded at a locus far from the site of generation (Rall et al 1992; Shepherd and Koch, 1990). Whenever an electric signal such as a synaptic event originating far from the recording site (the soma, in our experimental conditions) is attenuated, it is possible to find evidence for such a distortion in a correlation (Spruston et al 1993) between its kinetic parameters (namely amplitude and decay or rise time). Our data showed no such correlation and thus suggest that there is no preferential spatial segregation of glycinergic synapses (somatic versus dendritic), which seem to be randomly distributed.

Histograms of the amplitude of glycinergic mPSCs displayed a skewed distribution as shown in Fig. 7 C (see also Singer and Berger 1999). In accordance with the stochastic nature of the underlying releasing process (Fatt and Katz 1952), glycinergic mPSCs occurred at random as indicated by the histogram of the inter event intervals (Fig 7 D, in which their distribution is well fitted by a single exponential). As shown in Fig. 7 E glycinergic mPSCs reversed at -3±5 mV, a value close to the one predicted for Cl^- mediated currents.

3.2.2 GABAergic mPSCs

GABAergic mPSCs were pharmacologically isolated by adding 0.4 µM strychnine to kynurenic acid and 1 µM TTX containing solution (Fig. 8 A, left). Subsequent application of bicuculline blocked any residual activity. Note that on 4 cells (out of 17 tested with strychnine) 0.4 µM strychnine fully abolished all synaptic events even if exogenous application of GABA (100 µM) still elicited inward currents as large as in control solution (98±5 %). Whenever present, pharmacologically-isolated GABAergic mPSCs occurred at 0.5±0.2 Hz (n=13 cells), and represented 22±8% (n=7 cells) of all mPCSs. The large majority of GABAergic mPSCs decayed monoexponentially as only ≤ 15 % of them had complex kinetics of decay. The right panel of Fig 8 A shows

an example of the time course of event peak amplitude (top) and inter-event interval (bottom) during antagonist application and washout. Note that co-application of strychnine and bicuculline completely suppressed event occurrence. The effect of bicuculline was rapidly and fully reversed after washout.

The amplitude of GABAergic mPSCs was not correlated to their rise-time (Fig. 8 B) or decay (r=-0.34, slope= -0.18±0.03 ms/pA), and was distributed in a skewed fashion (Fig 8 C). As in case of glycinergic mPSCs, this result suggest that most GABAergic synapses are not located far from motoneuron somata, indeed they are probably spread out over somata and processes.

The inter-event distribution could be fitted with a monoexponential function to indicate the stochastic occurrence of such events (Fig 8 D). Fig. 8 E shows that GABAergic mPSCs reversed at -2±1 mV in accordance with the calculated reversal potential for Cl^-. Fig. 9 A shows the average kinetics of glycinergic and GABAergic mPSCs, which differed significantly in rise time, amplitude, and decay (see Table 2 for full description of data). Fig. 9 B compares histograms for the mPSC decay in control solution and after pharmacological application of bicuculline or strychnine to dissect mPSCs into glycinergic or GABAergic ones. The control histogram and the one for the glycinergic mPSCs appear similar because glycinergic responses represent the large majority of events. However, the cumulative plots for control, glycinergic or GABAergic event decay were significantly different (Fig. 9C; $p<0.0001$, Kolmogorov-Smirnov test).

3.2.3 Effect of bicuculline (or strychnine) on the baseline current

On 12 out of 16 cells bathed with TTX (1μM) and kynurenic acid (2mM) addition of 10 μM bicuculline to the extracellular solution reduced the baseline noise and caused a small outward shift in the leak current, revealing the presence of a residual "background" current not blocked by TTX or kynurenic acid and presumably mediated by tonic activation of $GABA_A$ receptors. A similar effect was observed only on 1 out of 7 cell after that 0.4 μM strychnine was added to the control solution (1 μM TTX plus 2 mM kynurenic acid).

Table 2. Mean characteristics of mPSCs.

	Rise (ms)	Amplitude (pA)	Decay (ms)	Frequency (Hz)	
Glycinergic	2.9 ± 0.2	-23 ± 2	12.8 ± 0.8	1.6 ± 0.5	n=19
GABAergic	4.4 ± 0.6 **	-12 ± 1 *	25 ± 3 **	0.5 ± 0.2 *	n=13

Double asterisks indicate a statistically significant difference between glycinergic and GABAergic mPSCs average values with $p<0.001$, simple asterisk $p<0.05$ (*t*-test).
n= number of motoneurons. Values are means ± S.E.

3.2.4 Effect of pentobarbital on GABAergic mPSCs.

In a preliminary set of experiments 50 μM sodium pentobarbital was added to the control solution containing TTX and kynurenic acid. It elicited an inward current (ΔI= 20±5 pA, n=3 cells), accompanied by an increase in baseline noise as it is shown in the example of Fig. 10 A. In this conditions, it was not possible to assess the effect of pentobarbital on individual synaptic events because the noise induced by this concentration of the barbiturate made it impossible to identify single events. However, both effects (increase in baseline noise and inward current) were reversed by bicuculline, as shown in Fig. 10 B and quantified in the histograms of Fig. 10 C, thus suggesting that at this concentration pentobarbital operated as $GABA_A$ receptor agonist.

Pentobarbital was then tested at a lower concentration (15 μM) on pharmacologically dissected GABAergic mPSCs. In this case it was possible to discriminate single events from the baseline noise. The effect of pentobarbital was to increase slightly but significantly the amplitude of GABAergic mPSCs, and to almost double their half width and decay time, as indicated in the sample trace in Fig. 11 A and summarized in the histograms in Fig. 11 B (n=4). These data indicate that pentobarbital at moderate (15 μM) concentrations retained its known effect of potentiating $GABA_AR$ mediated currents.

Although pentobarbital (15 μM) had such a marked effect on the kinetics of GABAergic mPSCs, it was however impratical to use this drug as a routine to distinguish GABA mediated events from glycinergic one, as GABAergic mPSCs had such a low occurrence with respect to glycinergic mPSCs to make sufficient

collection of data very difficult. In fact, Fig 11 C shows an histogram of the decay time of the total mPSC in control, and after addition of 15 μM pentobarbital. Indeed, there is a small surplus of events with long (> 40 ms) decay time after addition of pentobarbital; however, the cumulative distributions of decay time in control and in pentobarbital are not significantly different (Kolmogorov-Smirnov test, p=0.369), as shown in Fig. 11 D.

3.3 Network dependent activity of glycinergic and GABAergic synaptic transmission.

The experiments reported so far relied on protocols in which TTX was applied from the beginning of the recording session. When we first isolated pharmacologically GABA or glycine mediated PSCs and then applied TTX, a major difference between GABA and glycine mediated transmission became apparent. As indicated by the histograms of Fig. 12, pharmacologically isolated glycinergic events did not change in frequency or amplitude after adding TTX. This finding indicates that spontaneous glycine mediated neurotransmission was essentially independent of network activity. Conversely, GABAergic events were slightly, yet significantly, reduced in amplitude and dramatically decreased (by more than 2/3rd) in frequency, outlining the requirement for strong network activity to express spontaneous GABAergic events.

3.4 Modulation of glycinergic and GABAergic PSCs by mGluR activity

3.4.1 Modulation of sPSCs

The broad spectrum mGluR agonist *t*-ACPD was tested on 18 cells, on which it evoked a slowly rising, persistent inward current (on average -20±1 pA) together with a large increase in input resistance (30±10%) and in synaptic activity, despite the presence of kynurenic acid (Fig. 13 A). This observation indicates that the enhancing action of *t*-ACPD on synaptic activity was mainly targeted to Cl^- mediated events. To check whether glycinergic or GABAergic systems were differentially affected by the activation of mGluRs, bicuculline or strychnine was added to separate sPSC.

Fig. 13 B shows typical traces of glycinergic sPSCs (in bicuculline solution) before and after addition of *t*-ACPD while Fig. 13 C depicts similar data with GABAergic sPSCs. In both cases there was a large increase in sPCS frequency. These results are

quantified (Fig. 13 D) in terms of changes in normalized frequency or amplitude for glycinergic (black bars) and GABAergic (white bars) events before and after *t*-ACPD.

3.4.2 Modulation of mPSCs

We next studied the effect of *t*-ACPD on mPSC pharmacologically separated as above. As shown by the representative traces in Fig 14 A, the frequency of glycinergic mPSCs was enhanced in a reversible fashion. On the other hand, the frequency and amplitude of GABAergic mPSCs remained insensitive to *t*-ACPD (Fig. 14 B). The histograms of Fig 14 C provide statistical analysis of average data, confirming that the process of GABA release from pharmacologically isolated GABAergic terminals in the presence of TTX was unaffected by *t*-ACPD while the comparable process of glycine release remained *t*-ACPD-sensitive.

3.5 Characteristics of GABAergic or glycinergic evoked postsynaptic currents (ePSCs)

3.5.1 Low frequency stimulation (0.2 Hz)

ePSCs were evoked by electrical stimuli applied to the lateral reticular formation (ipsilateral to the patched motoneuron; see Borke et al 1983; Travers and Norgren 1983; Umemiya and Berger 1995). Since ePCSs appeared with a relatively short, constant latency after applying weak pulses, it seems probable that these were mainly monosynaptic events. Further support for this notion was obtained in experiments like the one shown in Fig. 15 A, B in which stepwise increments in ePSC amplitude were observed whenever the stimulus strength was increased from one range of intensity to the next. Within each stimulus range the synaptic response remained constant, indicating that there was no gradual recruitment of additional fibres. The constant amplitude of synaptic currents for each range of stimulus intensity is plotted in Fig. 15 B. Using the same pharmacological antagonists employed for testing spontaneous events, we investigated, in isolation, glycinergic (Fig. 16 A) or GABAergic evoked currents (Fig. 16 B). For both types of ePSC the calculated reversal potential was 10±3 mV (Fig. 16 C, D; insets beside I/V curves show sample traces of ePSCs at different values of V_h). ePSCs had average rise and decay times (Fig. 16 E) similar to those of sPSCs and mPSCS. These data thus confirmed the comparatively slow kinetics of all GABAergic synaptic currents.

Although GABA or glycine mediated currents were kinetically distinct, it seemed interesting to find out whether, on the same cell, they might be evoked by the same electrical stimulus and what relative contribution each component might make to the composite Cl^- mediated current. The example of Fig. 16 F shows a cell in which average bicuculline-sensitive or strychnine-sensitive (after bicuculline washout) evoked responses were recorded in kynurenic acid solution. Co-application of these antagonists fully blocked any evoked response. Digital summation of these two components gave a waveform which could be almost completely superimposed to the control event in kynurenic acid solution only (see bottom records in Fig. 16 F). On this same cell the percentage of failure was virtually the same in control solution (42 %) or in the presence of bicuculline (43 %) or strychnine (50 %). These characteristics are compatible with a co-release mechanism of GABA and glycine but were observed in 2 cells only out of a sample of 8 cells which all exhibited distinct evoked GABAergic and glycinergic components. On further 7 cells we observed exclusively one type of electrically evoked events (glycinergic on 5 cells and GABAergic on 2 cells). In summary then, co-release properties of electrically evoked transmission could be found in less than 20% of recorded motoneurons.

3.5.2 High frequency stimulation:

In a separate set of experiments, we compared pharmacologically isolated glycinergic or GABAergic responses evoked by repetitive stimulation at 2, 5, 10 or 20 Hz.

Sample traces of GABAergic currents evoked by a train of 100 stimuli delivered at 5 Hz are shown, at slow chart speed, in Fig. 17 A over the full length of the stimulus train. In Fig 7 B the initial part of the same trace is depicted on a faster time scale. Note that responses to the first few stimuli are of small amplitude but they build up in amplitude with successive pulses. Likewise, Fig.17 C shows the full time course of glycinergic responses to a 5 Hz train with the initial part of them displayed on a faster time base in Fig. 17 D. Note a less intense potentation when compared with GABAergic synapses.

Corresponding responses to at least five trains like the one shown in Fig 17 A were averaged to minimize the intrinsic variability of synaptic responses.

Fig 18 A-D shows actual amplitude of glycinergic ePSCs (averaged as described in METHODS) during a train of 100 stimuli delivered at 2, 5, 10 or 20Hz.

Peak responses to a tetanus were then normalized to the first response, so to assess time dependent changes in ePSCs during the pulse. Fig. 18 E shows normalized

responses versus the number of stimuli. Time course of normalized response was fitted with an exponential function of the form f= a+(1-a)exp(-t/b), where a quantifies the extent of potentiation of the response (namely, the fractional increase in amplitude), and b expresses the time constant of it. The average parameters a, b obtained for the facilitation of response to tetanic stimulations at various frequencies are reported in Fig. 18 F. In general, it was found only a slight facilitation in glycinergic response. As shown in Fig. 18 F the maximum of glycinergic response amplification was obtained for 5 Hz stimulation (a= 4.7±0.1); facilitation reached its saturation very slowly in the case of 2 Hz stimulation, whereas it became faster by increasing the frequency of stimulation.

Furthermore, we investigated the dependence of the extent of glycinergic synaptic facilitation on the extracellular Ca^{2+} concentration ($[Ca]^{2+}_o$). Fig. 19 A shows the time course of the average peak responses to 100 stimuli delivered at 5 Hz for three different $[Ca]^{2+}_o$: 1.5, 2.5 and 5 mM. As summarized in the histograms of the parameters for exponential fitting (Fig. 19 B), the extent of glycinergic synaptic facilitation decreased and reached saturation faster when $[Ca]^{2+}_o$ was raised. Increasing $[Ca]^{2+}_o$ had also another effect, shown in the sample traces (Fig. 19 C) and summarized in the histograms in Fig 19 D: it increased slightly the amplitude (Fig 19 D, top) and significantly the frequency (measured as the inter-event interval between events, Fig 19 D, bottom) of the underlying spontaneous glycinergic events, recorded during the interstimulus recovery periods. Asterisks indicate a significant change with respect to control (namely, extracellular solution with 1.5 mM $[Ca]^{2+}_o$).

A major difference in facilitation of synaptic responses emerged when we analyzed GABAergic events. In fact, as shown in Fig. 20 A, the increase in isolated GABAergic peak responses (during a train of stimuli delivered at 5 Hz) was one order of magnitude higher than the facilitation observed at the same frequency of stimulation for glycinergic responses (compare with Fig. 18, B and F). Facilitation of GABAergic responses was stronger than that observed for glycinergic ones also for when 10 Hz stimulation (although not as marked as in the case of the 5 Hz stimulation, Fig. 20 B). Activation of $GABA_B$Rs by endogenous GABA did not seem to play a major role even during a tetanic stimulation that might have transiently increased the local concentration of GABA to activate $GABA_B$Rs, since bath application of the selective $GABA_B$R antagonist CGP-52432 (10 μM) did not significantly change facilitation of responses to 10 Hz stimulation (Fig. 20 C).

GABAergic responses to 10 Hz stimulation were fully suppressed by 10 μM bicuculline, as shown in Fig. 20 D, confirming that the responses were mediated by activation of $GABA_A$Rs only.

The time course of GABAergic response facilitation is represented in Fig. 20 E for the four different experimental conditions discussed above, while Fig. 20 F summarizes the exponential fit parameters. Facilitation of GABAergic response was very large in response to a 5 Hz tetanus (note that facilitation of glycinergic response at the same frequency was one order of magnitude smaller), and moderate at 10 Hz. Also in this case, as seen for glycinergic events, facilitation reached its saturation faster when the frequency of stimulation was increased.

One possible mechanism that could underly facilitation of GABAergic responses to high frequency stimulation is that consecutive responses were summating, because of the longer decay time of GABAergic events with respect to glycinergic ones. To check this possibility out, we analyzed the decay time of GABAergic responses to high frequency stimulation (5 and 10 Hz). In fact, the tetanic stimulation favoured GABA spillover, thus transiently increasing the local concentration GABA. It is known that such effect could lead to increased receptor desensitization that results in prolongation of the decay time at the level of macroscopic currents (Jones and Westbrook 1995, 1996; Jones et al 1998). Indeed, there was a slight increase in the decay time of GABAergic responses (as shown in Fig 21 A); the histograms in Fig. 21 B represent the average decay time for events evoked in control condition (0.2 Hz), and during tetanic stimuli at 5 Hz and 10 Hz. However, for the 5 Hz stimulation (which determined the stronger facilitation) the decay time increase was not large enough to lead to summation of consecutive responses, as in the sample trace of Fig. 21 C (top): before each new stimulus, the current response came back to the baseline level. Indeed, the bottom trace in the same panel represents part of the upper one on a faster time scale to allow identification of single consecutive responses and their time course. Summation of consecutive events might have played a role in facilitation for the 10 Hz stimulation only; as shown in Fig. 21 D (top; bottom, same trace on different time scale) the current returned to the baseline level at the end of the train pulse only. These data suggest that summation might take place for frequency stimulation $\geq$ 10 Hz; however, it does not seem to be the only mechanism involved in facilitation of GABAergic responses.

DISCUSSION

The principal finding of the present study on hypoglossal motoneurons of the neonatal rat is that they expressed both $GABA_A$ and glycine functional receptors, the activation of which by exogenously applied of agonists inhibited cell excitability (presumably due to a marked reduction in cell input resistance). Despite the presence of both receptor types, spontaneously active glycinergic synapses were more numerous than GABAergic ones.

Glycine mediated events differed from GABAergic ones in terms of frequency, kinetics and sensitivity to TTX or *t*-ACPD. Moreover, preliminary data suggested a major difference between glycinergic and GABAergic synaptic plasticity under conditions of high frequency stimulation. These data do not provide evidence for any substantial co-release of these transmitters at this early postnatal stage and are interpreted in terms of kinetically distinct synaptic roles for glycine and GABA.

1. Effect of activation of GABAA or glycine receptors on motoneuron excitability

Neonatal hypoglossal motoneurons possessed native glycinergic and GABAergic receptors, since application of glycine (100 µM) or GABA (100 µM) to the extracellular solution (containing 2 mM kynurenic acid and 1 µM TTX) elicited robust inward currents accompanied by a comparable fall in cell input resistance (49±7% and 53±7%, respectively). Such a decrease in input resistance presumably operated as a shunting mechanism for motoneuron excitability (Gao and Ziskind-Conhaim 1995). This phenomenon was demonstrated by experiments done under current clamp conditions. In these cases, the action of glycine or GABA was to depolarize hypoglossal motoneurons (as expected in our experimental conditions, since E_{rev} for Cl^- ions was several tens of millivolts above the resting membrane potential)[1] and to depress cell ability to fire action potentials in response to brief current injections. The decreased excitability of cells was not due to inactivation of voltage gated Na^+ channels by depolarization (Zhang and Jackson 1995). In fact, in presence of these agonist, repolarization by DC steady current injection failed to restore motoneuron firing. Gramicidin perforated-patch recordings (Singer et al 1998)

[1] E_{rev} for Cl^- calculated from the Nernst equation for our experimental condition with K methylsulphate electrodes was –37 mV, whereas the resting potential of HMs, in the same conditions, was –70±3 mM.

of glycine evoked currents from neonatal or juvenile hypoglossal motoneurons have revealed a postnatal shift of E_{Cl}^- from –37 to –73 mV. Thus, even if opening of (somatic) Cl^- channels by glycine (or GABA) might lead to depolarization of neonatal cells, the reduction in cell input resistance following channels opening can inhibit excitability.

2. Bicuculline and strychnine receptor selectivity

Some experiments were carried out to establish that strychnine or bicuculline retained their receptor selectivity at the concentrations used in the present study. Since 0.4 μM strychinine did not affect the inward current evoked by 100 μM GABA and 10 μM bicuculline was ineffective against 100 μM glycine, it was concluded that each antagonist retained the expected receptor specificity of action. This should not be surprising, since it has been shown in other preparations that the IC_{50}s of strychnine (or bicuculline) for glycinergic and GABAergic currents differ substantially (Jonas et al 1998; Lewis and Faber 1993). However, since it is not obvious that receptors from neonatal animals possess the same pharmacological characteristics as young or adult ones, and since strychnine and bicuculline have been routinely used in the present study to separate GABAergic and glycinergic mediated currents, it was necessary to assess first their selectivity on receptors of neonatal hypoglossal motoneurons.

3. Electrophysiological characteristics of spontaneous synaptic events

In the present investigation synaptic currents were always studied during pharmacological block of glutamatergic ionotropic receptors, and were found to reverse near 0 mV, in accordance with the value predicted by the Nernst equation for Cl^- mediated responses. Indeed, GABA and glycine receptors, besides Cl^-, are permeable also to another anion, namely HCO_3^- (Bormann et al 1987). The presence of this anion in normal physiological conditions implies that it should be considered when calculating the reversal potential (V_{rev}) of agonist-activated currents in accordance with the Goldman-Hodgkin-Katz equation:

$$V_{rev} = -\frac{RT}{F} \cdot \ln \frac{[Cl^-]_o + (P_A/P_{Cl}) \cdot [A^-]_o}{[Cl^-]_i + (P_A/P_{Cl}) \cdot [A^-]_i}$$

where R is the gas constant (1,987 cal K^{-1} mol $^{-1}$), F is the Faraday's constant ($9.648 \cdot 10^4$ C mol^{-1}), T is the absolute temperature, the subscripts o and i denote external and internal ion species, respectively, A is the anion considered and P_A/P_{Cl} the permeability ratio between the anion A and Cl^-. However, in our experimental conditions HCO_3^- fluxes through GABA or glycine receptors were negligible, since the buffer HEPES was always used in the intracellular and external solution instead of the CO_2/HCO_3^- complex. Steady state I-V relations for GABA and glycine mediated currents did not show any evident rectification (Bormann et al 1987), at least within the range of V_h tested (Fig 7 and 8 F for mPSCs, and Fig.16 C, D for ePSCs).

Pharmacological separation of glycine-mediated events from those mediated by $GABA_A$ receptors allowed us to ascertain the relative contribution of each transmitter to the observed activity. It should also be noted that co-application of bicuculline and strychnine (after pharmacological block of ionotropic glutamatergic transmission) fully suppressed any synaptic activity, indicating that the synaptic currents measured under the present conditions were only mediated by glycine or $GABA_A$ receptor activation.

In general, glycinergic events were significantly faster than GABAergic events and occurred at higher frequency. The effect of TTX on glycinergic or GABAergic events was dramatically different. In fact, the frequency of glycinergic events remained substantially unchanged (and their amplitude was not significantly depressed) whereas GABAergic events were reduced in amplitude and, especially, in frequency. This observation indicated that asynchronous release of GABA largely depended on network activity while the release of glycine did not.

The difference in kinetics between glycinergic and GABAergic events was also found when mPSCs were analyzed. In fact, GABAergic events were significantly slower, smaller and occurred at lower frequency than their glycinergic counterparts.

In some cases 0.4 μM strychnine completely suppressed any Cl^- mediated synaptic events even if exogenously applied GABA was still able to elicit robust inward currents.

The contribution of any action potential-independent release of GABA to the overall synaptic activity of resting hypoglossal motoneurons must therefore have been quite small even though $GABA_A$ receptors on these cells were functional.

3.1 Can differences in kinetics between glycinergic and GABAergic events be due to spatial segregation of their receptors?

The time course of GABAergic sPCSs, mPSCs and ePSCs was always slower than the one of glycine-mediated responses. The slow decay of $GABA_A$ mediated events (see also Banks and Pearce 2000; Banks et al. 1998; Rossi and Hamman 1998) was unlikely due to electrotonic filtering of remotely generated responses as there was no apparent correlation between amplitude and rise time (or decay) of GABAergic events (Soltesz et al. 1995; Ulrich and Lüscher 1993; Spruston et al 1993). Although the synaptic location of these receptors on hypoglossal motoneurons has not yet been proven with ultrastructural studies, $GABA_A$ receptors containing the α_2 subunit (essential to confer them functional properties; Fritschy et al. 1997) were readily found on the soma of these cells by our immunocytochemical studies. This subunit is a major constituent of developing $GABA_A$ receptors while the $\alpha 1$ subunit, which could not be found in the present experiments, is indeed typical of adult $GABA_A$ receptors (Fritshy and Mohler 1995; Fritschy et al. 1994; Mohler et al. 1996). The same subunit has also been detected on motoneuron soma in the adult rat (Fritschy and Mohler 1995). Likewise, glycine receptors are found on the cell body of neonatal (Singer et al. 1998) and adult (Racca et al. 1998) hypoglossal motoneurons. These results collectively suggest that GABA and glycine receptors have dendritic as well as somatic location. In the light of these considerations it seems that the slow kinetics of GABAergic events perhaps require a different interpretation.

It seems unlikely that some intrinsic properties of $GABA_A$ receptors were responsible for this slow kinetics as $\alpha 2$ subunit containing receptors generate fast onset responses (Lavoie et al. 1997; McCellan and Twyman 1999). In particular, the time to peak is at least twice faster for GABA currents mediated by activation of $\alpha 2\beta 1\gamma 2$ receptor than for the $\alpha 1\beta 1\gamma 2$ type (0.5 ms versus 1.0 ms, respectively), and several fold smaller than the one measured in our study for $GABA_A$ mediated currents. It seems also unlikely that the measure of such a slow kinetics could be due to an intrinsic time resolution limit of our experimental recording apparatus, since glycinergic currents, measured in similar experimental conditions, displayed significantly faster time courses.

3.2 GABA spillover?

Another possibility to account for slow kinetics and in particular for such a slow activation, is that $GABA_A$ receptors spread out beyond the subsynaptic area and were thus activated by transmitter spillover (Barbour and Hausser 1997; Brickley et al 1996; Faber and Korn 1988; Isaacson et al. 1993; Kullmann and Asztely 1998; Rossi and Hamman 1998). The slow rise time of GABA mediated events might then represent the time integral required to activate a sparse population of receptors (Clements 1996; Jones et al. 1998; Kruk et al. 1997; Maconochie et al. 1994; Uteshev and Pennefather 1996, 1997). In conjunction with this hypothesis, the small amplitude of GABA events may indicate a low density of subsynaptic $GABA_A$ receptors. This proposal is in accordance with the mechanisms of GABAergic transmission suggested to operate in the dorsal horn of the spinal cord (Chery and De Konick 1999) or in the hippocampus (Banks and Pearce 2000; Banks et al. 1998), where most $GABA_A$ receptors are extrasynaptic and are activated mainly by GABA spillover, and GABAergic spontaneous currents have very slow rise time. The *en passant* varicosities made by GABAergic fibres on hypoglossal motoneurones (Takasu and Hashimoto 1988) might represent the structures involved in this form of synaptic transmission in the hypoglossus nucleus.

The possibility of GABA spilling over onto hypoglossal motoneurons is also suggest by the observation that the majority of cells tested with bicuculline (in the presence of the glutamate antagonist and TTX) displayed an outward shift in mean baseline current and a reduction in baseline noise. A similar phenomenon has been described to occur on granule cells of rat cerebellum (Kaneda et al 1995; Brickley et al 1996; Wall and Usowicz 1997). Here, these authors have demonstrated that the effect of bicuculline is to block a tonic conductance that arises from persistent activation of presumably extrasynaptic $GABA_A$ receptors by GABA escaping from neighboring synapses (cerebellar granule cells display also $GABA_AR$ mediated PSCs), in analogy with the tonic, non-quantal release of acetylcholine at the neuromuscolar junction (Katz and Miledi, 1977). In the cerebellum, the characteristic geometry of the glomerular structure (in which the GABAergic synapse between granule and Golgi cells is enveloped) is presumably the morphological support for the tonic activation of receptors, since the glomerulus is enclosed within a glial sheath, and synaptically released GABA may linger to activate synaptic as well as extrasynaptic $GABA_A$ receptors (Kaneda et al 1995). Such a tonic receptor activation might provide a

shunting inhibition capable of dynamically modulating granule cell excitability to maintain reliance on synchronized input from independent mossy fibers, even at high input frequency (Gabbiani et al 1994; D'Angelo et al 1995).

In the hypoglossal nucleus GABA spillover might result, at macroscopic level, both in slow rising events, due to "almost synchronous" activation of extrasynaptic, sparse $GABA_A$ receptors by synaptically released GABA, and in a background noise which would contribute to the leak current, due to asynchronous persistent (tonic) activation of extrasynaptic $GABA_A$ receptors. Investigation of this possibility would however require further experiments, targeted for example to the assessment of the role of GABA uptake system.

3.3 Difference in kinetics: is it enough to detect co-released mPSCs?

It is interesting that a small fraction of mPSCs displayed complex decay kinetics which might have suggested simultaneous activation of $GABA_A$ and glycine receptors by their synaptic co-release (Jonas et al. 1998; O'Brien and Berger 1999). Nevertheless, subsequent application of either bicuculline or strychnine failed to abolish this subpopulation of mPSCs: this result suggests that a high order of mPCS decay is not *per se* indicative of co-release mechanism. Complex decay might reflect spatial segregation of synaptic receptors or heterogenous receptors for the same transmitters (Lewis and Faber 1996a,b). Another possibility is that receptors possess intrinsically complicated kinetics, with high and low affinity states producing non desensitizing and desensitizing components of the macroscopic current, in analogy with those exhibited by nicotinic AChRs (Boyd 1987; Neubig et al 1982; Sine and Taylor 1979).

The low occurrence of GABAergic mPSCs makes it difficult to identify their contribution to the common properties of average mPSCs (such as distribution of decay – or rise – times). Although the kinetics of GABAergic currents were significantly slower, the mean parameters of averaged mPSCs were dominated by the glycinergic mPSC characteristics. This is evident from the histogram of mPSC decay time (Fig. 9B), in which the decay time distribution is shaped by the glycinergic events. The difficulty in distinguishing GABAergic events among all the occurring mPSCs persisted even after enhancing their kinetics with pentobarbital. The contribution due to GABAergic mPSCs was clearly manifested only when GABAergic events were recorded in isolation after glycinergic transmission block.

4. Differences between GABAergic and glycinergic inputs

The large reduction in GABAergic events brought about by acute application of TTX suggests that, even in the absence of glutamate-mediated synaptic transmission, GABAergic interneurons, contained in the brainstem slice, were spontaneously active because they either received a non-glutamatergic drive or fired spikes due to the operation of their intrinsic conductances. In our recording conditions glycine-releasing neurons were not spontaneously active. Although a similar phenomenon has been observed in a different model (ganglion cells of rat retinal slices, Protti et al 1997), full elucidation of the mechanisms underlying such a different behaviour is not currently available. However, one likely explanation is that glycinergic interneurons might have had their somata severed during the slice preparation. In this case glycine release would have merely reflected spontaneous quantal discharge of this neurotransmitter, a process undoubtedly endowed with either a high probability of release or a high density of active synaptic sites (Singer and Berger 1999) in view of the large, frequent events detected in the present study. *Vice versa*, a substantial number of functional GABAergic neurons might have been preserved in the slice preparation: their collective network behaviour would have been thus responsible for their strong sensitivity to TTX action. However, immunocytochemical studies show that GABAergic and glycinergic premotoneurons are distributed in several areas of the brainstem and are often not spatially segregated, although their relative preponderance in each projection pathway is unclear (Li et al. 1997). Another possibility would be that glycinergic boutons had a much higher probability of release than GABAergic ones, either because glycinergic terminals possess a more efficient release machinery or because locally released GABA inhibits its own release (Lim et al. 2000).

5. Differential modulation of glycine or GABA mediated transmission by t-ACPD

We tested the effect of upregulating Cl^- dependent synaptic transmission with *t*-ACDP, which is known to modulate glycine or GABA mediated synaptic transmission on other neurons (Bond and Lodge 1995; Chu and Hablitz 1998; Miles and Poncer 1993).

In analogy with other studies (Dong et al. 1996; Schoppa and Westbrook 1997), *t*-ACPD induced an inward current, associated with a large increase in the frequency of

spontaneous PSCs. The action of *t*-ACPD is complex and exerted at pre and postsynaptic level via distinct receptor subclasses (Nakanishi 1994). Recently, Schwartz and Alford (2000) have demonstrated that, at the vestibulospinal-reticulospinal (VS-RS) synapse in the lamprey brain stem, synaptic activation of presynaptic Group I mGluRs leads to both an increase in intracellular Ca^{2+} and an enhancement of spontaneous and evoked transmitter release. It seems likely that on hypoglossal motoneurons the effects of *t*-ACPD on spontaneous events were chiefly generated at presynaptic level since the main change was in frequency rather than amplitude. In the case of mPSCs *t*-ACPD increased the frequency of glycinergic events only, presumably via a presynaptic site of action. There is no current evidence for selective upregulation of glycine receptors by metabotropic receptor activation at postsynaptic level. The differential action by *t*-ACDP on glycinergic versus GABAergic mPSCs suggests that the action of this substance was not a mere epiphenomenon of a rise in cell input resistance which enabled detection of electrotonically remote events.

The present observations also help to clarify the question of co-release of GABA and glycine from the same presynaptic cell. The fact that *t*-ACPD enhanced glycine release without affecting GABA release in TTX solution suggests that co-release did not apparently take place.

6. Electrophysiological characteristics of stimulus-evoked synaptic currents

Minimal stimulation of afferent inputs is supposed to activate one or very few presynaptic inputs to the recorded cell (Raastad 1995). In the present experiments this stimulation protocol was applied to the fibres originating from the lateral reticular formation (Umemiya and Berger 1995). When examining GABA or glycine mediated responses separately, their overall properties like rise and decay times were similar to those of spontaneous PCSs mediated by glycine or GABA, respectively.

On 8 cells recording stability was sufficiently long to allow studying the probability of failures first in control solution, then in the presence of bicuculline, and, finally, in the presence of strychnine (after bicuculline washout). On two cells only an approximately equivalent number of failures was found under all three experimental conditions, an observation compatible with co-release of glycine and GABA in this limited number (<20%) of cases. However, other data (mainly a differential modulation of glycinergic and GABAergic mPSCs by *t*-ACPD, but also differential

TTX sensitivity, persistence of complex decay mPCSs during antagonist application) did not support the co-release hypothesis. Further clarification of this issue might be provided by pair recording from a single presynaptic fibre and its postsynaptic cell, a hardly achievable aim within a slice preparation. Co-release might have taken place in limited instances when some inputs were electrically stimulated although another possibility is that even "minimal stimuli" might have activated a larger number of fibres than anticipated. In the latter case summation and/or occlusion of inputs would have been likely. These data thus suggest that caution is necessary when considering the possibility of co-release on the basis of minimal stimulation experiments in which unequivocal control over presynaptic inputs is lacking.

7. Short-term synaptic plasticity

Preliminary experiments were addressed to investigate some properties of short-term synaptic plasticity of glycinergic and GABAergic synapses, namely by testing how synaptic responses evolve during high frequency electrical stimulation. From these experiments a major difference between glycinergic and GABAergic synapses emerged. Glycinergic synapses showed slight facilitation only, whereas GABAergic ones exhibited large facilitation. In particular, in response to 5 Hz stimulation, GABAergic facilitation was one order of magnitude higher than the glycinergic one.

Synaptic information can be integrated over a period of time (10-20 ms), depending on the location and time course of synaptic inputs, the morphological structure of the neuron, and the effective time constant of motoneuron membrane. Another way to integrate information might be through discrete changes in synaptic efficacy. As the amount of transmitter released varies with different frequencies and patterns of action potential discharge, the postsynaptic response becomes a function of the frequency of stimulation. Changes in synaptic efficacy can result, for example, from changes in calcium entry or sequestration, in the number (or position) of synaptic vesicles, in the number of active release sites or the factors that lead to the fusion of synaptic vesicles with the plasma membrane, or in the number of functional postsynaptic receptors (Magleby 1987). It is known that Ca^{2+} is involved in the induction of all forms of short (and long) term plasticity (Zucker 1989). One hypothesis is that residual Ca^{2+} left over from prior activity simply added to the increments in $[Ca^{2+}]_i$ at active zones to yield higher peaks of $[Ca^{2+}]_i$ to each successive action potential, and therefore more transmitter release (Katz and Miledi 1968; Rahamimoff and Yaari 1973). In our

experiment on glycinergic responses to 5 Hz stimuli we found that, when $[Ca^{2+}]_o$ was raised, facilitation was decreased. However, raising $[Ca^{2+}]_o$ determined also an increase in the underlying spontaneous activity recorded during the interstimulus periods. It is thus possible that, at least in the case of glycinergic transmission, two contrasting phenomena limited the degree of synaptic facilitation. On the one hand, high background release might have reduced the number of vesicles available for stimulus-induced release. On the other hand, repetitive stimuli tended to raise free $[Ca^{2+}]_i$ and to promote facilitation of subsequent responses in a train. It is interesting to note that depletion is usually related to synaptic depression, a phenomenon that, within the range and duration of stimuli applied in our study, we have never observed. Depression can be generally related to a reduction in the number of quanta released (Bailey and Chen 1983), or to desensitization of postsynaptic receptors (Gardner and Kandel 1977), and might have a physiological corrispondence in adaptation or habituation (Zucker 1989). Further experiments will be necessary to examine the action of raised $[Ca^{2+}]_o$ on background and stimulus-evoked GABAergic transmission.

Although plasticity phenomena may have a pre or a post-synaptic origin, there is a certain agreement on the presynaptic origin of short-term changes in synaptic activity (Fisher et al 1997). Although we cannot exclude that some postsynaptic change had occurred, the facilitation we observed developed in few seconds and seems compatible with a presynaptic mechanism. One of the possible presynaptic mechanisms responsible for differential properties in facilitation between glycinergic and GABAergic synapses might be a difference in the distribution/type of calcium channels or in the homeostasis of calcium in the presynaptic terminal. In favour to this hypothesis is also the difference of glycinergic versus GABAergic transmission in sensitivity to TTX. Such a difference, in fact, might be postulated to be due to a different regulation of calcium influx into the presynaptic terminals so that, even without action potential depolarization, calcium ions can flow into and trigger neurotransmitter release from the presynaptic glycinergic terminal.
Note, moreover, that once again this scenario makes it unlikely the possibility that GABA and glycine are released from the same presynaptic terminal, since the presynaptic mechanisms leading to the release of the two neurotransmitters would seem to be substantially different.

Another possibility to explain the difference in the extent of facilitation for glycinergic and GABAergic synapses is that in the latter case, due to the slower kinetics of GABA mediated events, responses were summating. In fact, we previously characterized the time course of GABAergic responses evoked at low frequency (0.5 Hz, Fig 16 E), and found the decay time constant of GABAergic events to be 30±3 ms. This means that, for a frequency of stimulation ≥ 10 Hz, summation should occur, since the duration of each single event is approximately 100 ms. However, during a tetanic stimulation the local concentration of GABA might be transiently increased due to a massive release from the presynaptic terminal, a phenomenon known (Jones and Westbrook, 1995, 1996; Jones et al 1998) to affect the kinetics of $GABA_A$ receptors. In particular, an increase in receptor desensitization due to a higher or longer presence of neurotransmitter might lead to an increase in decay time of the macroscopic $GABA_AR$ mediated currents. To explore further such a possibility, we studied the decay time of GABAergic responses during the tetanus. Indeed, we found that the decay time of responses had a tendency to increase with the frequency of stimulation, but in the case of 5 Hz stimulation such increase was not enough to determine summation of consecutive responses. However, summation occurred at frequency of stimulation ≥ 10 Hz. Thus, mechanisms involved in the potentiation of GABAergic responses are multiple and may have both pre- and post- synaptic origin.
It is interesting to observe that addition of CGP52432, a potent and selective $GABA_BR$ antagonist, had no significant effect on GABAergic responses (evoked at 10 Hz), indicating that, at least at an early stage of development, the presence (or the activity) of $GABA_B$ receptors is negligible. This result confirms at the electrophysiological level what has been found with immunocytochemical studies on the rat hindbrain (McDermott et al 1999): McDermott and collegues did not find any staining for the two known isoforms of $GABA_B$ receptors within the hypoglossal nucleus, whereas other neighbouring regions (nucleus of the tractus solitarius, dorsal motor nucleus of vagus, raphe obscurus and magnus) were intensely labelled. However, developmental changes in the expression of $GABA_B$ receptors in the hypoglossal nucleus are presently unknown.

There are many mechanisms underlying short-term plasticity, and further work should clarify those involved in the facilitation observed at GABAergic (and, to a less extent,

glycinergic) synapses on hypoglossal motoneurons. Nevertheless, the striking difference found in the temporal information processing of GABAergic and glycinergic synapses opens the possibility for a markedly different functional role for the two neurotransmitters. A possible suggestion comes from developmental studies on mouse neurons *in vivo* and *in vitro* by Paton and Richter (1995). They investigated the role of GABAergic and glycinergic inputs from the respiratory neurons to hypoglossal motoneurons taking advantage of a brainstem tilted sagittal-slice preparation (600-700 μm thick). In such a preparation the respiratory network is preserved and it is possible to record rhythmic, respiratory-driven activity from hypoglossal rootlets. Paton and Richter have found that, whereas antagonism of neonatal glycinergic receptors does not affect the respiratory rhythm generator (although in the adult low doses of strychnine severely disrupt respiratory rhythmic activity), bicuculline dramatically affects the respiratory-driven rhythm recorded from hypoglossal motoneurons, both in neonatal and in adult animals. In the neonatal animal, in particular, bicuculline increases the burst duration and cycle length of respiratory-driven rhythmic activity and induces a sustained depolarization of the motoneuron membrane. Thus, in neonatal animals, GABA mediated activity has a foundamental role in keeping low (at physiological levels, namely ≈ 0.2 Hz) the respiratory-driven rhythmic activity and in moulding the firing pattern, whereas, at the same stage of development, glycine seems not to be involved in regulation of the respiratory rhythm. In adult animals, both GABA and glycine are involved in the control of respiratory rhythm; in this case even relatively low doses of strychnine induce disruption and loss of respiratory rhythmic activity, and it is known that in mature animals glycine mediated inhibition plays a major role in the switch-off mechanisms between the different phases of the respiratory cycle (Champagnat et al 1982; Ballantyne and Richter 1984; Klages et al 1993). Interestingly, the time course for the increase in sensitivity of the respiratory rhythm generator to strychnine is strictly correlated with the developmental changes in the molecular subunit structure of the glycine receptor (Kuhse et al 1991; Singer et al 1998) from α_2 to α_1, known to accelerate the kinetics of the evoked chloride currents (Akagi and Miledi 1988; Takahashi et al 1992) and its clustering (St John and Stephens 1993).

So far, although our preparation excludes any respiratory input and hence any rhythmic activity, functional properties of GABAergic (or glycinergic) synapses are

preserved and the substantial difference in their temporal coding might underlie, at the cellular level, the different functional role that GABAergic and glycinergic trasnmission have on neonatal respiratory rhythmic activity.

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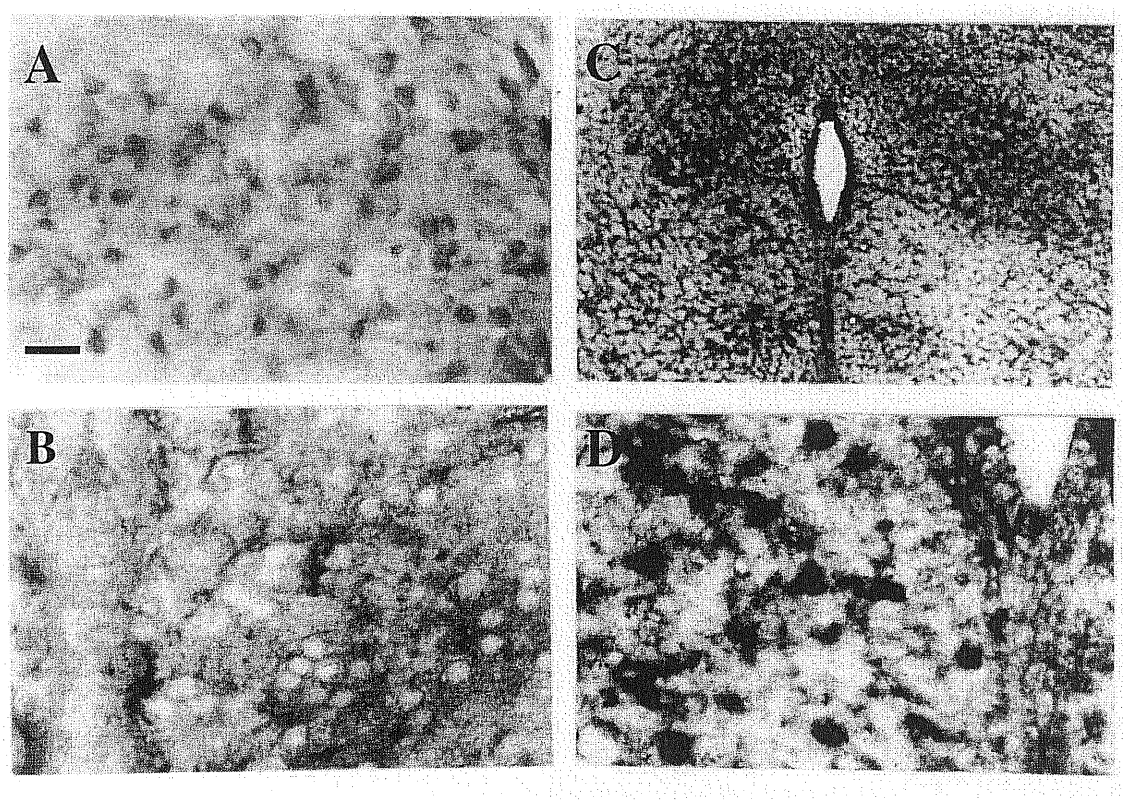

Figure 1. **Immunocytochemical markers of hypoglossal neurons. A:** choline acetyltransferase immunoreactivity of motoneurons in a 200 μm thick slice of the hypoglossus nucleus. Note that stained cells have large diameter, various somatic shapes (ovoidal, triangular, multipolar). Proximal dendrites are also visible. **B:** hypoglossal motoneurons (20 μm thick sections) are immunostained-negative for the α1 subunit of $GABA_A$ receptors. **C, D:** immunostaining for the α2 subunit of $GABA_A$ receptors at low (C) and high (D) magnification. Note intense staining of motoneuron somata. Scale bar: A, B, D = 100 μM; C= 400 μM.

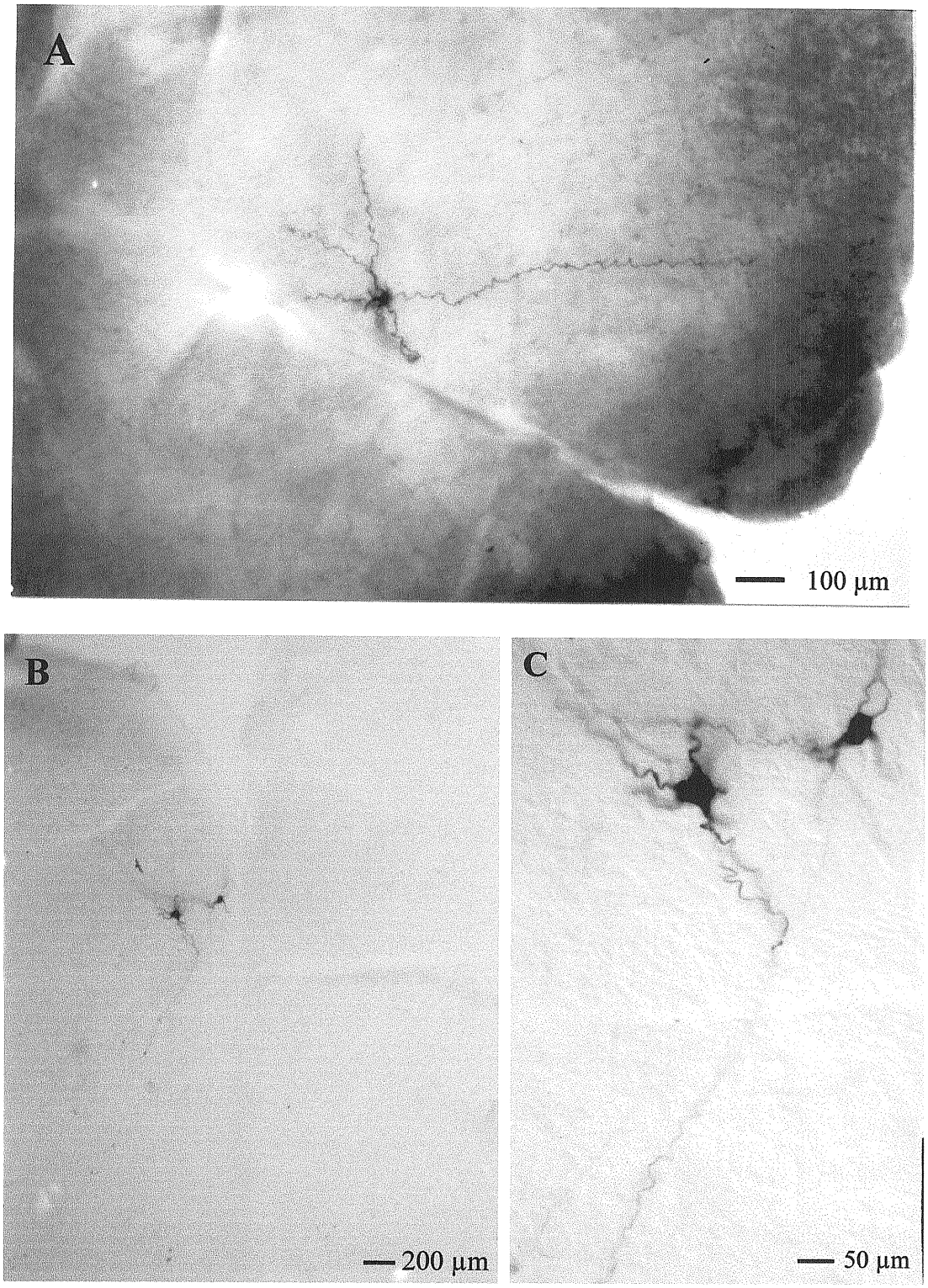

Figure 2. **Hypoglossal motoneurons injected with neurobiotin. A:** motoneuron in a 200 µm thick slice injected with neurobiotin; note the long process on the slice plane running along the direction of exit of hypoglossal axons. Major processes are only partially visible because of slice thickness. Scale bar: 100 µM. **B :** another example of two motoneurons injected during electrophysiological recording with neurobiotin. The middle line of the figure shows the central canal, and, dorsally to the stained motoneurons there are the boundaries between hypoglossal and vagus nuclei. Scale bar: 200 µM. **C:** higher magnification of the same microphotograph as in B at an (scale bar: 50 µM).

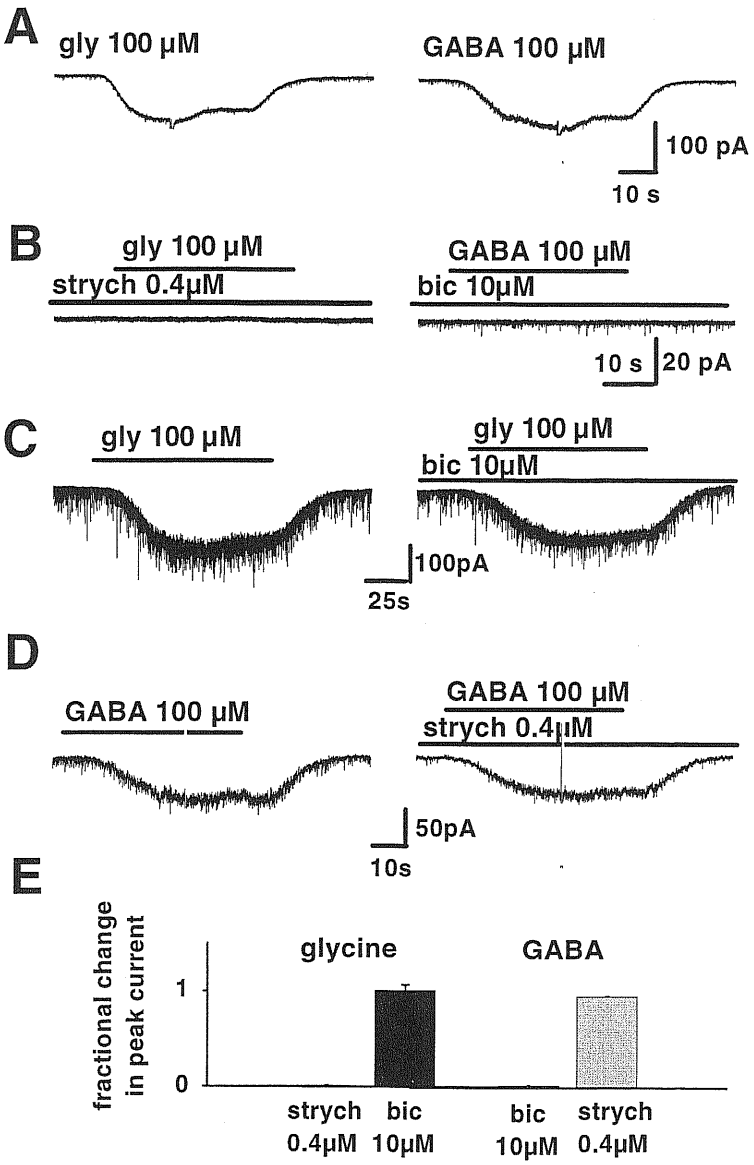

Figure 3. **Hypoglossal motoneurons possess both GlyRs and $GABA_A$Rs. A:** Current responses to glycine (100 µM; left) or GABA (100 µM; right) bath applied to the same motoneuron. Blanked out artifacts in the middle of responses are ramp tests. **B:** The response evoked by 100 µM glycine was fully blocked by 0.4 µM strychnine (left); likewise the GABA evoked current was completely antagonized by 10 µM bicuculline. **C:** glycine evoked current (left) was not blocked by 10 µM bicuculline (right); **D:** GABA evoked current was not blocked by 0.4 µM strychnine; **E:** histograms of depression of glycine (left) or GABA (right) effects by pharmacological blockers.

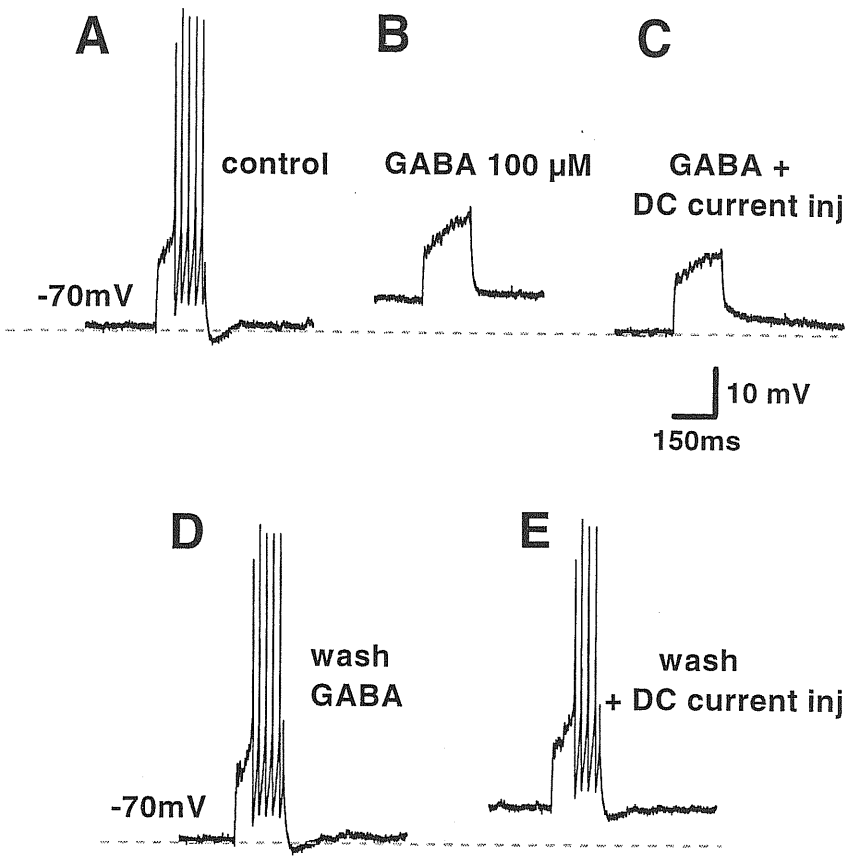

Figure 4. **GABA inhibits motoneuron firing. A:** hypoglossal motoneuron firing in response to small current step (ΔI= 200pA, Δt=160ms). **B:** after adding GABA (200 µM) cell membrane potential was slightly depolarized (~7 mV), and firing in response to the same current step was inhibited. **C:** even after repolarizing cell membrane potential with a DC current injection, in the continuous presence of GABA motoneuron, did not fire in response to the current step. **D:** after washing out GABA, cell injected with the current step fired again as in control. **E:** inhibition of firing did not depend merely on cell membrane depolarization following GABA application, since, when membrane potential was brought to –63mV, the cell fired in response to the current step.

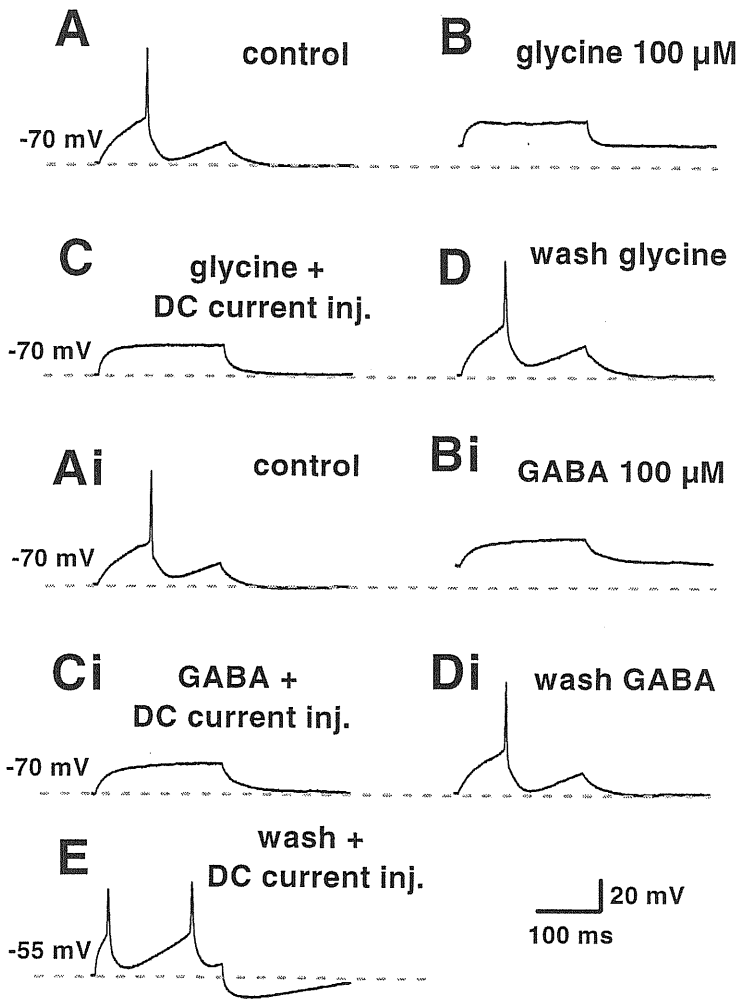

Figure 5. **Glycine or GABA have the same effect on motoneuron excitability. A:** motoneuron fired with a small current (ΔI= 100pA, Δt= 200ms) injection; **B:** glycine (100 µM) inhibited firing and depolarized cell membrane potential (~15 mV); **C:** inhibition of firing in presence of glycine even after repolarizing cell membrane potential with a DC current injection. **D:** inhibition of firing and membrane depolarization were reversible upon washing out glycine. **Ai-Di:** the same described in A-D was repeated onto the same motoneuron except that GABA (100 µM) was used instead of glycine. 100 µM GABA had an inhibitory effect on the excitability of this motoneuron comparable to the one observed after applictaion of 100 µM glycine. **E:** firing of the same motoneuron in control solution after its membrane potential was manually rised to the level reached in GABA (or glycine) solutions.

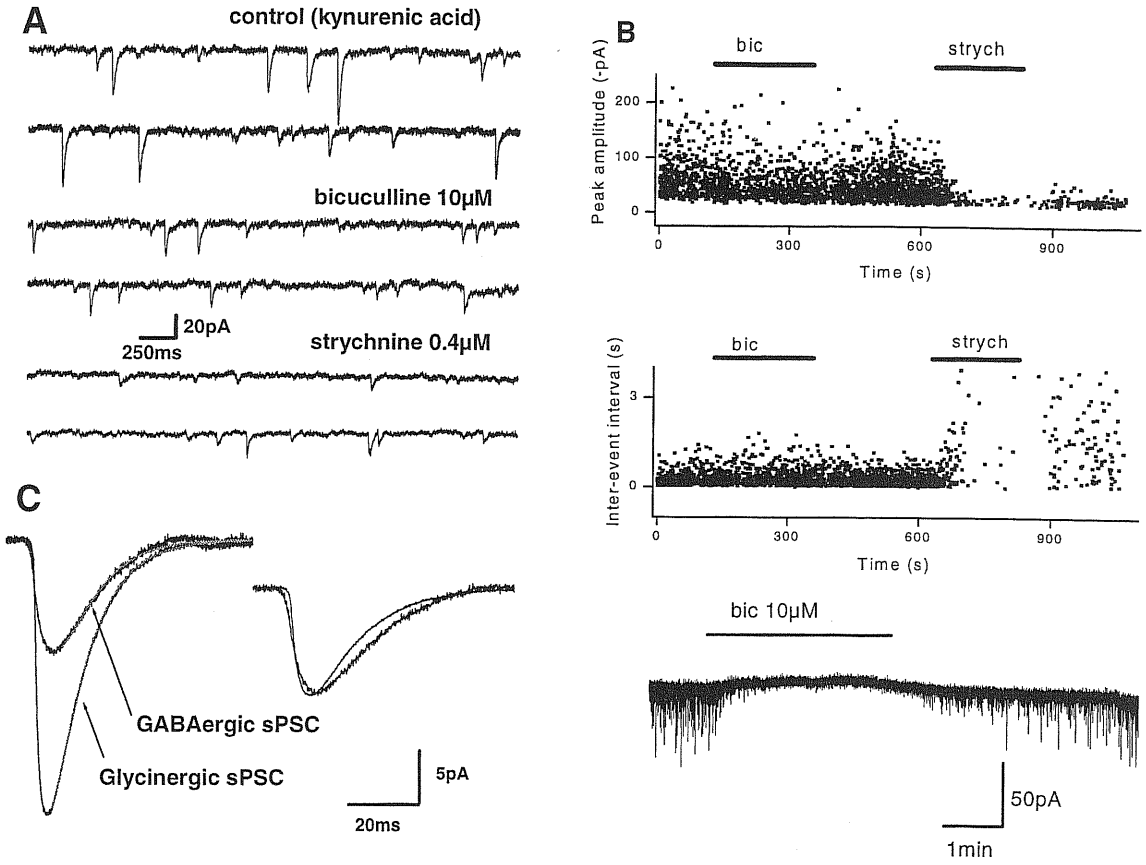

Figure 6. **Spontaneous synaptic currents in kynurenic acid solution comprise glycine or GABA mediated events**. **A:** top, inward currents recorded at –70 V_h in the presence of kynurenic acid (2 mM). Middle, in the presence of bicuculline a number of spontaneous events remain. Bottom, after bicuculline washout and application of strychnine spontaneous events are also detected. **B:** top and middle, time profiles of event peak amplitude and inter-event interval. As in A, antagonists were added to the control solution containing kynurenic acid. Note that, in contrast to the effect of strychnine, the effect of bicuculline on event peak amplitude and frequency was fully reversible. Bottom: bicuculline (10 μM) reversibly suppressed sPSCs recorded in a solution containing kynurenic (2mM) acid and strychnine (0.4μM). Length of bars in the panel corresponds to time duration of drug application.**C:** left, average synaptic current due to activation of glycine or $GABA_A$ receptors (561 or 102 events, respectively); right, after normalizing the amplitude of glycine or GABA-mediated currents the different time course of these responses becomes apparent. The fitting function overlaps almost completely the average records. All data are from the same cell.

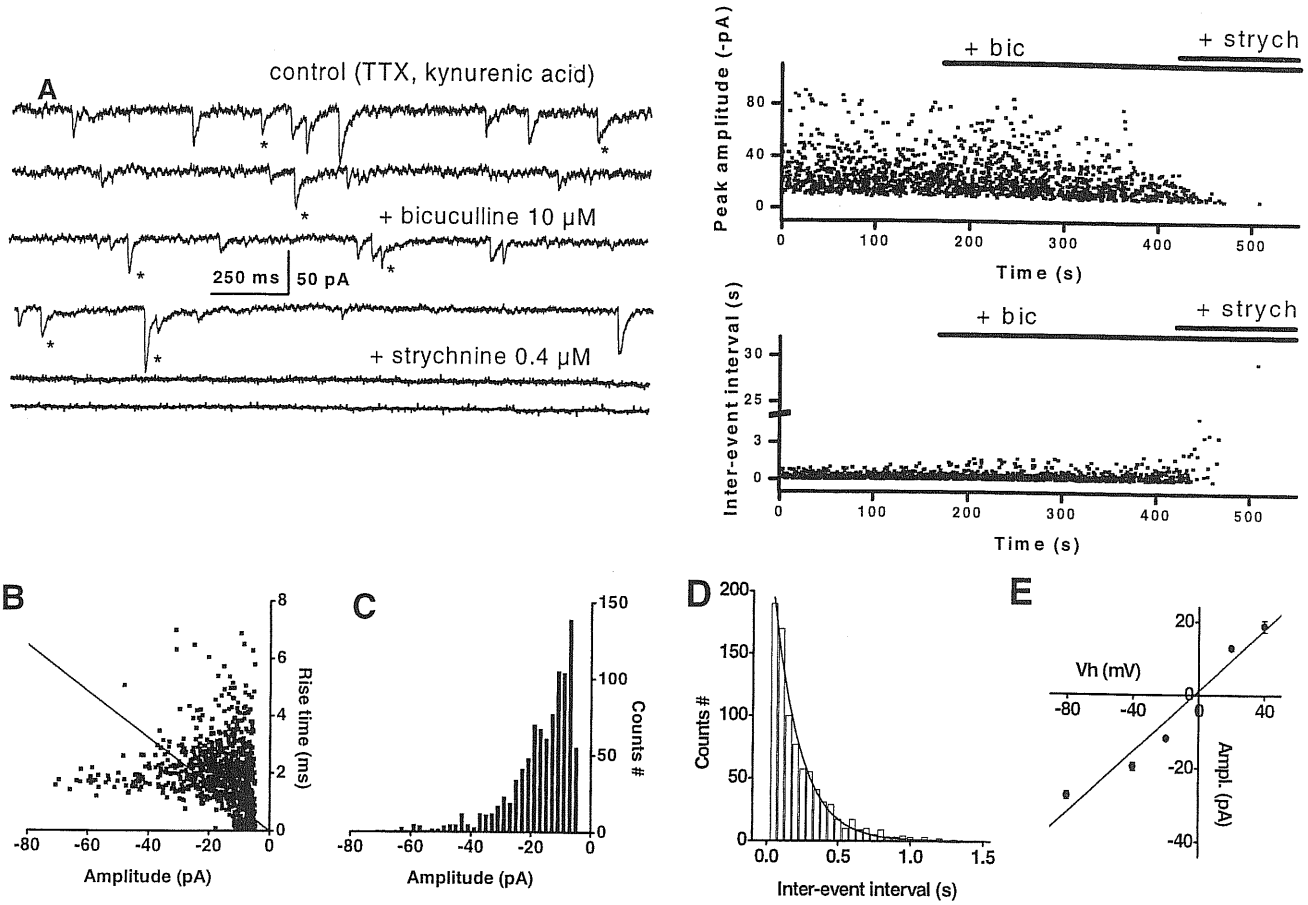

Figure 7. **Properties of glycinergic miniature postsynaptic currents (mPSCs).** **A**: left panel (top), two consecutive traces of mPSCs in control solution. Asterisks indicate events with complex decay. Left (middle): after adding bicuculline glycinergic mPCSs can be recorded, some of which with complex decay (see asterisks). Left (bottom): further application of strychnine leads to full suppression of synaptic activity. Right panel: sample scatter plots of event peak amplitude (top) and inter-event interval (bottom) during antagonists addition to the control solution (1µM TTX and 2mM kynurenic acid). **B:** plots of glycinergic event amplitude against corresponding rise-time. The slope value is –0.081±0.002 ms/pA while the correlation coefficient *r* is –0.085. The probability that the correlation coefficient is significantly different from 1 is <0.0001. **C:** amplitude distribution histograms for glycinergic mPCSs. Most events are grouped between –10 and –30 pA. **D:** histograms of distribution of inter-event intervals for responses shown in B and C. A single exponential function (time constant=180±10 ms) can adequately fit the interval distribution. **E:** current/voltage relation for glycinergic mPCSc. The calculated reversal potential is -3±5 mV.

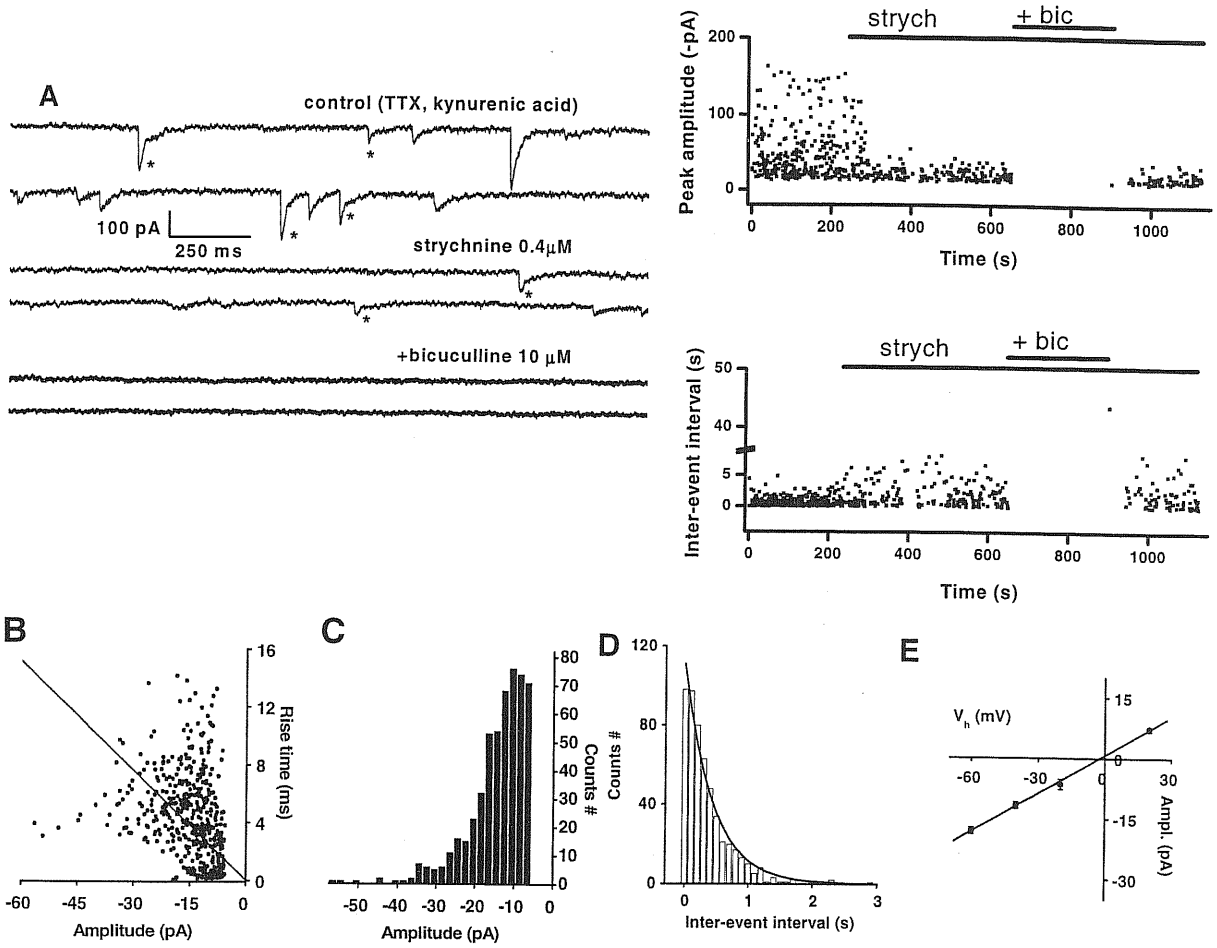

Figure 8. **Properties of GABAergic mPCSs. A:** Left (top), two consecutive traces of mPSCs in control solution. Asterisks indicate events with complex decay. Left (middle): after adding strychnine (0.4 μM) GABAergic mPSCs can be recorded in isolation, some of which with complex decay (see asterisks). Left (bottom): further application of bicuculline (10 μM) leads to full suppression of synaptic activity. Right: time profiles of the effect, on mPSCs amplitude (top) and inter-event interval (bottom), of antagonists application (same concentration as in the left panel). **B:** plots of GABAergic event amplitude against corresponding rise-time. The slope value is –0.252±0.009 ms/pA while the correlation coefficient *r* is –0.272. The probability that the correlation coefficient is significantly different from 1 is <0.0001. **C:** amplitude distribution histograms for GABAergic mPCSs. Most events are grouped between –5 and –20 pA. **D:** histograms of distribution of inter-event intervals for responses shown in B and C. A single exponential function (time constant=430±20 ms) can adequately fit the interval distribution. **E:** current/voltage relation for GABAergic mPCSc. The calculated reversal potential is -2±1 mV.

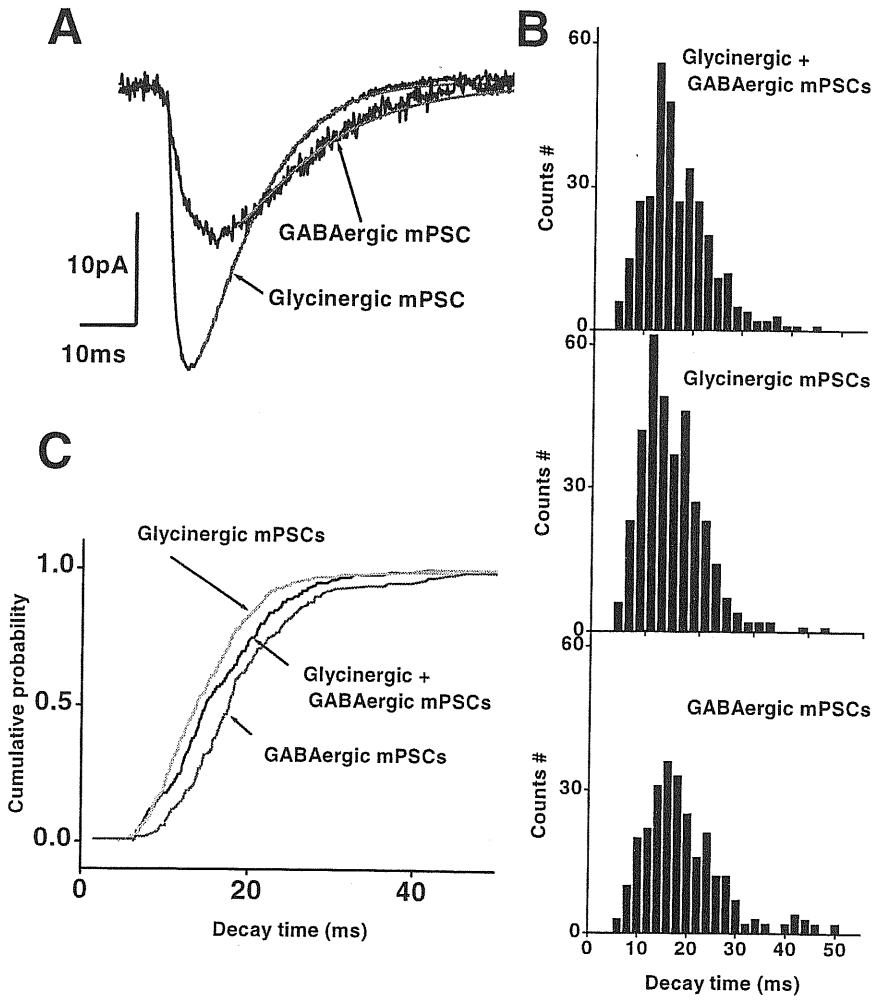

Figure 9. **Contrasting properties of glycinergic and GABAergic synaptic events. A:** average mPSCs recorded from the same cell in the presence of strychnine (GABAergic events; n=37) or in the presence of bicuculline (glycinergic events; n=526) in kynurenic acid and TTX solution. Note slower time course of GABAergic mPSCs (fitting functions are virtually superimposed on average records). **B:** Histograms show, for the same cell, the distribution of decay values for all mPSC in control solution, glycinergic mPCSs and GABAergic mPSCs. Note that in order to maximize the capture of GABAergic events (which occurred at low frequency) records in the bottom panel were analysed for 10 min instead of the customary 5 min used for the top and middle panels. **C:** cumulative probability distribution for decay time values claculated in control, and after dissection of glycinergic first and then GABAergic mPSCs (same cell than in panel B), showing that the three distribution were significantly different.

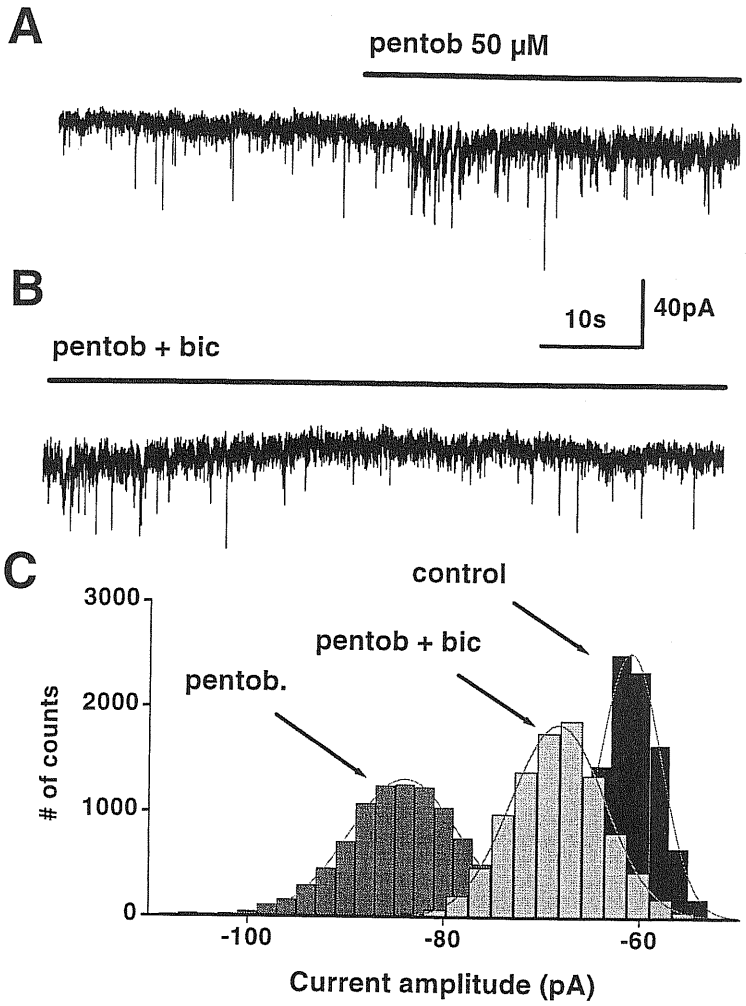

Figure 10. **Effect of 50 µM pentobarbital on mPSCs. A:** pentobarbital (50 µM) added to the extracellular control solution (containing 1 µM TTX and 2 mM kynurenic acid) evoked an inward current and increased synaptic noise; this effect was reversed by application of 10 µM bicuculline (**B**); **C:** histograms of baseline noise in control, after addition of 50 µM pentobarbital and after further addition of 10 µM bicuculline.

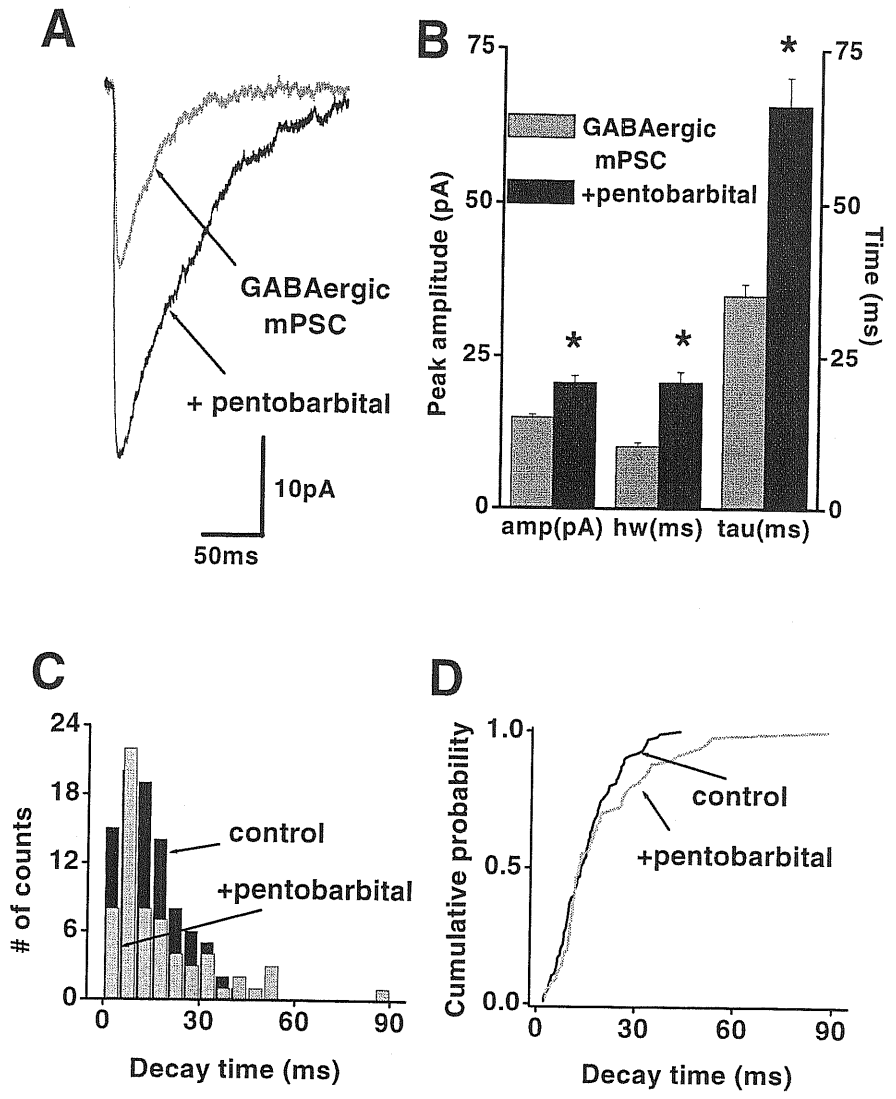

Figure 11. **Effect of 15μM pentobarbital on GABAergic mPSCs. A:** sample trace of pharmacologically isolated GABAergic mPSC before and after addition of 15 μM pentobarbital; **B:** summary of pentobarbital effect on GABAergic mPSC kinetics. **C**: histogram of decay time of all mPSCs in control and after addition of pentobarbital. **D:** cumulative distributions of decay time of mPSCs in control or pentobarbital solution: the two distributions were not significantly different (p=0.396, D=0.15, Kolmogorov-Smirnov test).

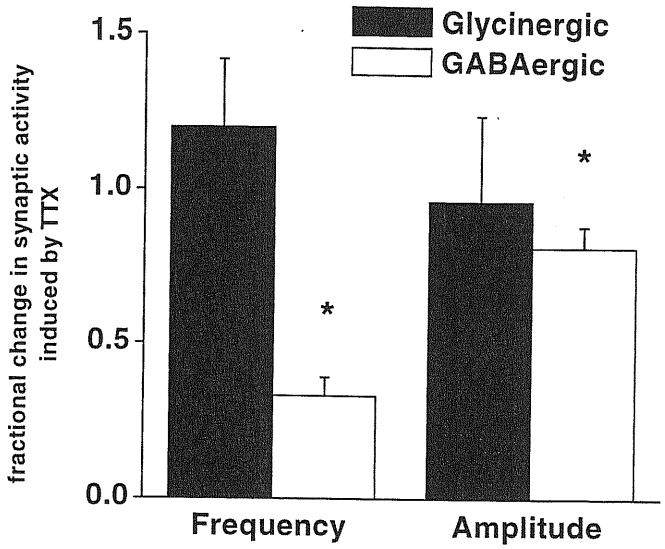

Figure 12. **Cl^- mediated sPSCs sensitivity to TTX.** Histograms depicting the sensitivity of glycinergic or GABAergic synaptic responses to TTX. Application of TTX brings about a large fall in the frequency of GABA-mediated events and a smaller, albeit significant, reduction in their mean amplitude (n=6). Glycine mediated events are essentially unchanged by TTX (n=4). Asterisks indicate $p<0.05$.

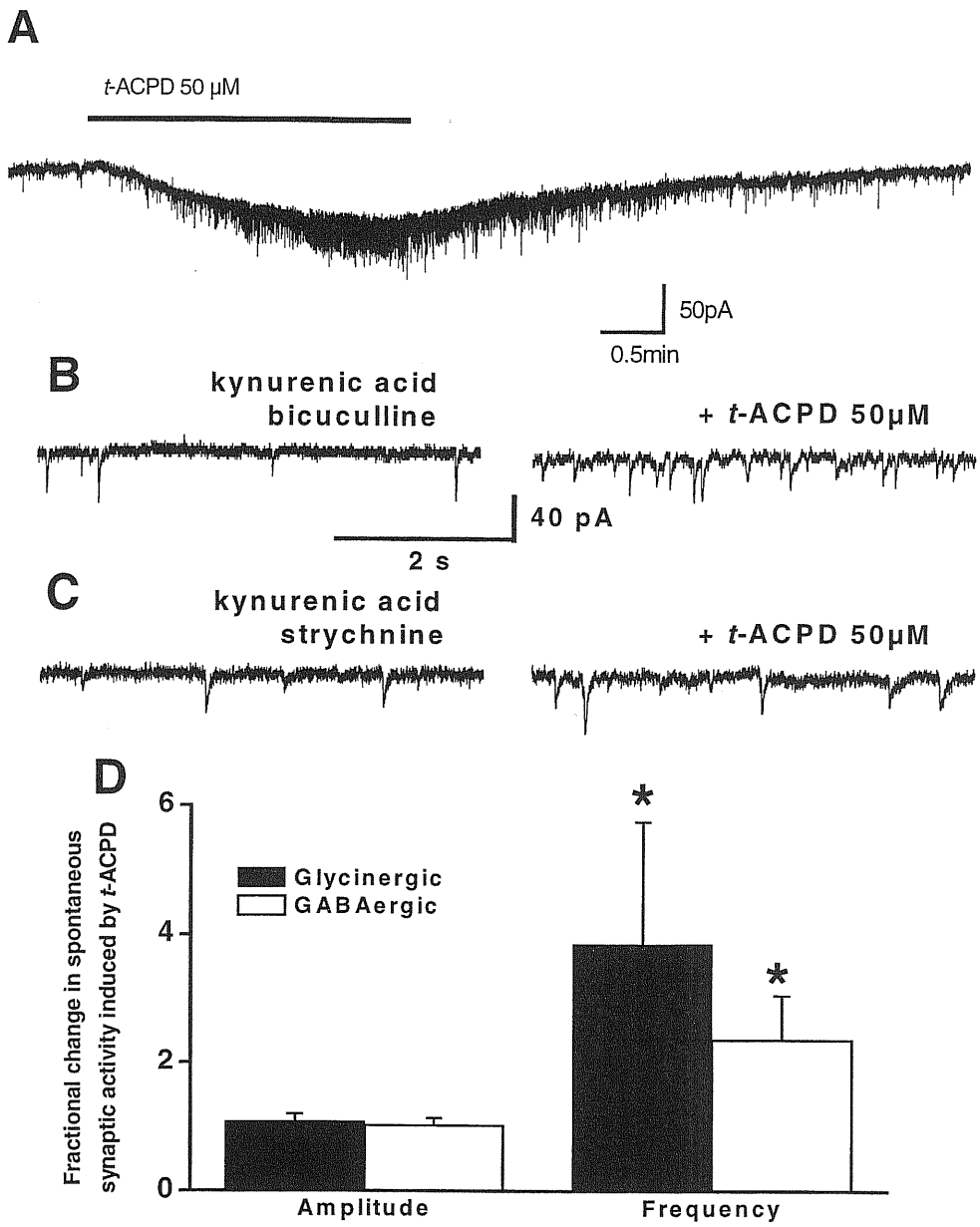

Figure 13. **Frequency upregulation by *t*-ACPD (50 µM) of spontaneous postsynaptic currents (sPSCs).** All records are in kynurenic acid solution. **A**: *t*-ACPD induced a slowly rising, persistent inward current and a large increase in synaptic activity; after washout, the baseline current and sPSCs frequency returned to the control value. **B**: effect of *t*-ACPD on glycinergic currents in the presence of bicuculline. **C:** effect of *t*-ACPD on GABAergic currents in the presence of strychnine. **D:** histograms of fractional changes in sPSC amplitude or frequency in the presence of *t*-ACPD. Note significant increase in frequency but not in amplitude (glycine: n=3, GABA: n=5; asterisks indicate $p<0.05$).

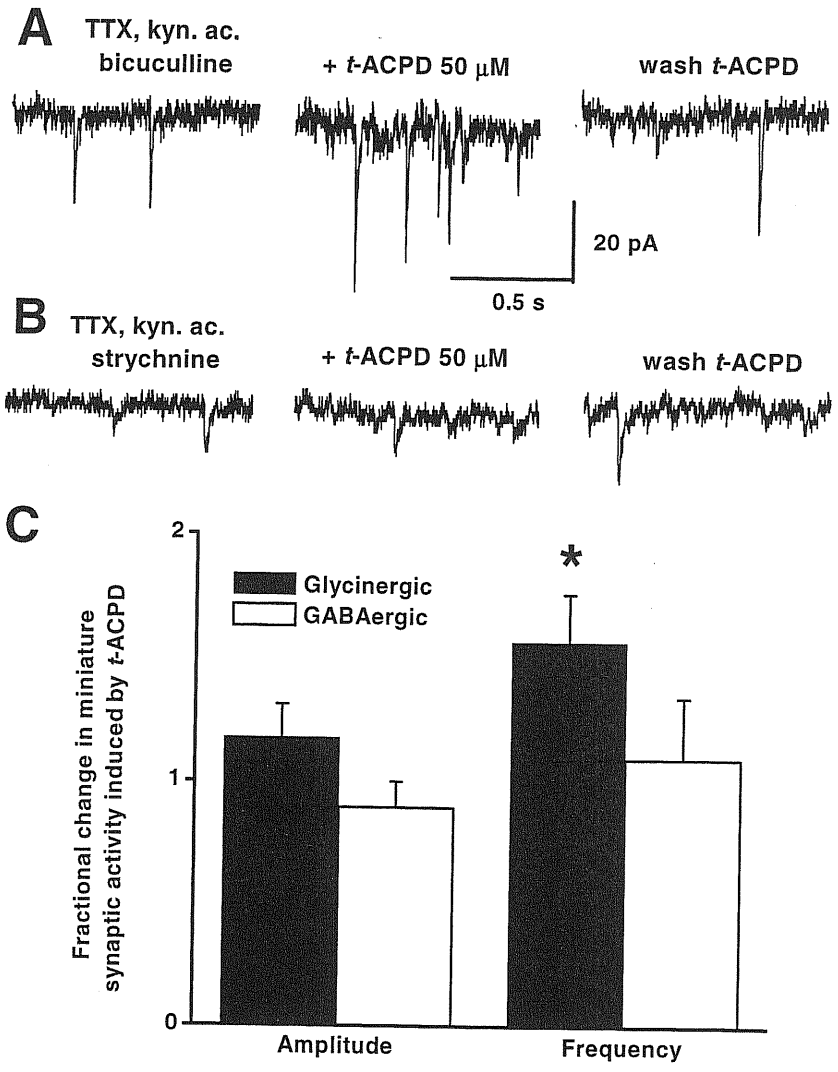

Figure 14. **Differential modulation of GABAergic or glycinergic mPSCs by *t*-ACPD** All records are in kynurenic acid (kyn. ac.) solution. **A:** glycinergic miniature events in control (left), *t*-ACPD (middle) or washout of *t*-ACPD. Note large and reversible increase in frequency. **B:** GABAergic miniature events in control (left), *t*-ACPD (middle) or washout of *t*-ACPD. Note lack of effect of *t*-ACPD. **C**: histograms of fractional changes in amplitude or frequency of mPSCs mediated by glycine (n=4) or GABA (n=10). Note significant increase in frequency (but not in amplitude) of glycinergic events only (asterisk indicates $p<0.05$).

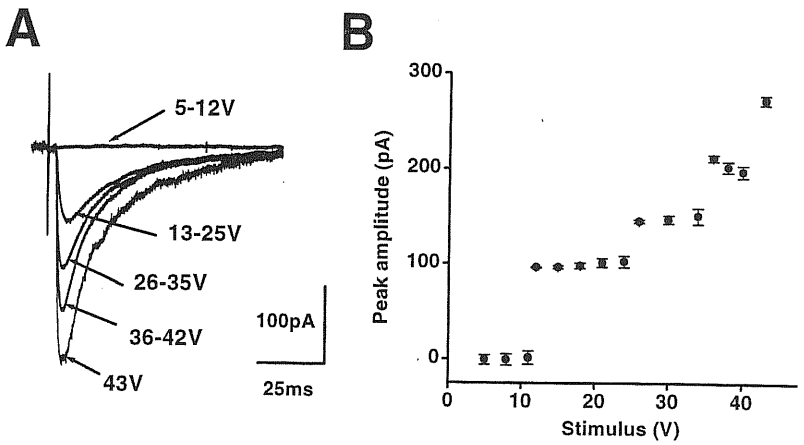

Figure 15. **Examples of ePSCs induced by minimal stimulation. A:** average records of synaptic currents induced by ranges of stimulus strength (V; indicated by arrows). **B:** plot of ePSC peak amplitude versus stimulus strength (V). All data are from the same cell in kynurenic acid solution.

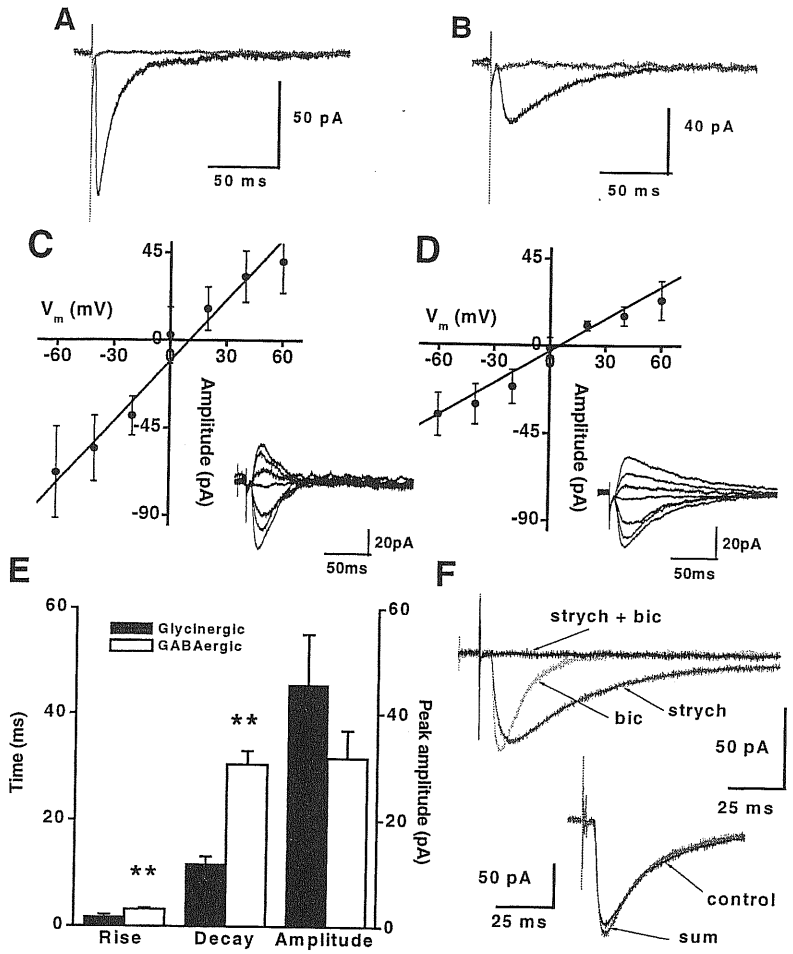

Figure 16. **Characteristics of electrically evoked postsynaptic currents (ePSCs).**
All records are in kynurenic acid solution following weak electrical stimulation of the reticular formation adjacent to the recorded cell. **A**: average (22 responses) of glycinergic ePSCs (in 10 μM bicuculline solution) and subsequent full block by 0.4 μM strychnine. **B:** average (20 responses) of GABAergic ePSCs (in 0.4 μM strychnine solution) and subsequent full block by 10 μM bicuculline. **C, D:** current/voltage plots for responses shown in A, B. Note similar reversal potential. Insets beside I/V curves show sample traces of ePSCs at different V_h, starting from –60 mV to +60 mV at increments of 20 mV. Traces for each value of V_h are the average over 10 responses. **E:** histograms depicting mean rise and decay time values (left ordinate) or amplitude (right ordinate) for glycinergic or GABAergic ePSCs. Data are from 8 or 10 cells (glycinergic or GABAergic events, respectively). Double asterisks indicate $p<0.001$. F: top, example of a cell in which glycinergic and GABAergic ePCSs are recorded after applying bicuculline (bic) or strychnine (stryc), respectively. Combined application of these antagonists (bic + stryc) fully suppresses synaptic transmission. Bottom, control indicates the ePSCs recorded in kynurenic acid solution, which is almost the same as the response reconstructed by digital summation of the average traces shown above.

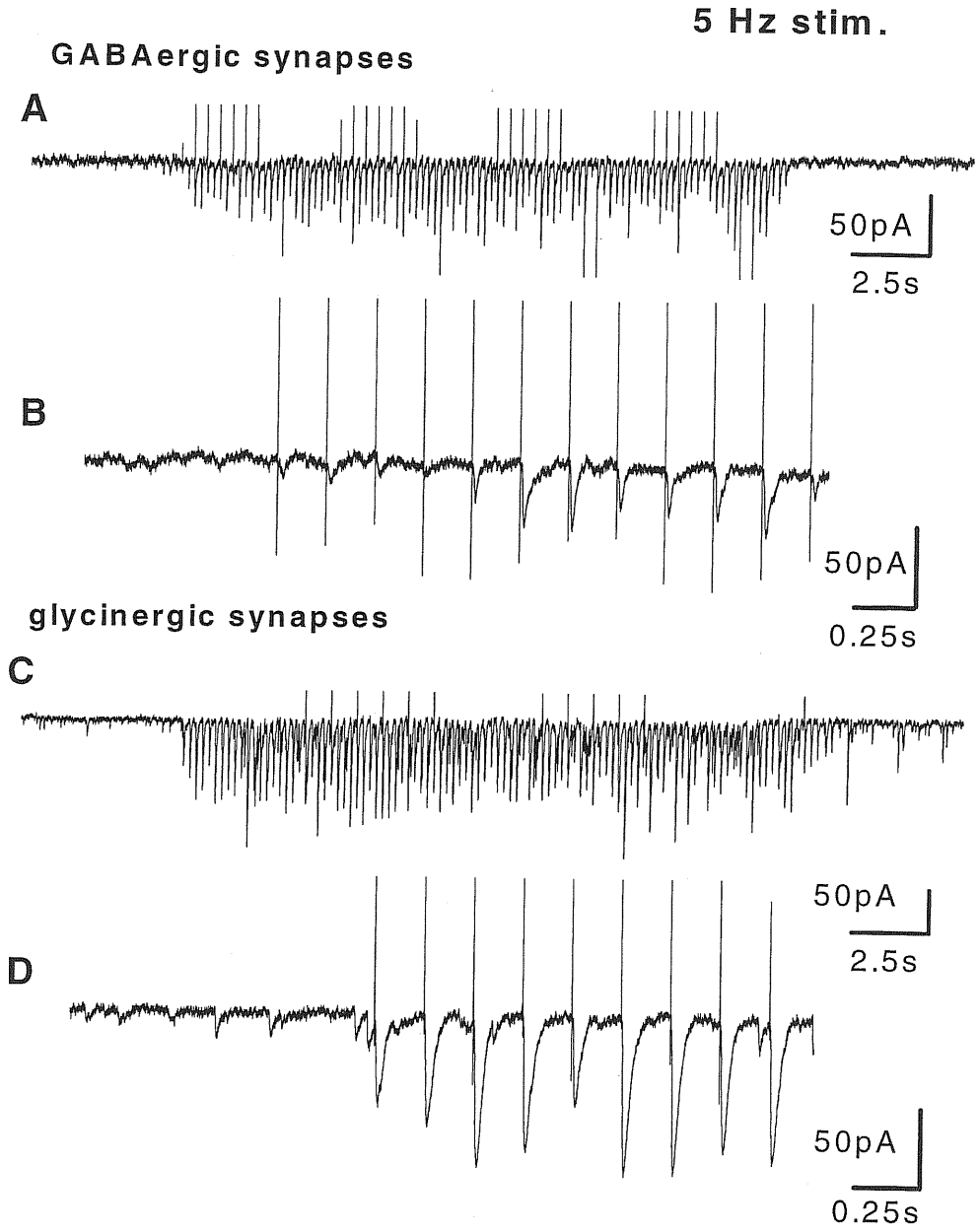

Figure 17. **Glycinergic and GABAergic responses at 5 Hz train tetani.** **A**: GABAergic responses to a 100 stimuli train at 5 Hz. Upward deflections are stimulus artefacts which have been clipped by the sampling rate of the data analysis system. **B**: initial part of the same trace at a faster time scale, to enhance the time course of the increase in the amplitude of synaptic responses. **C, D**: as in A, B for glycinergic responses.

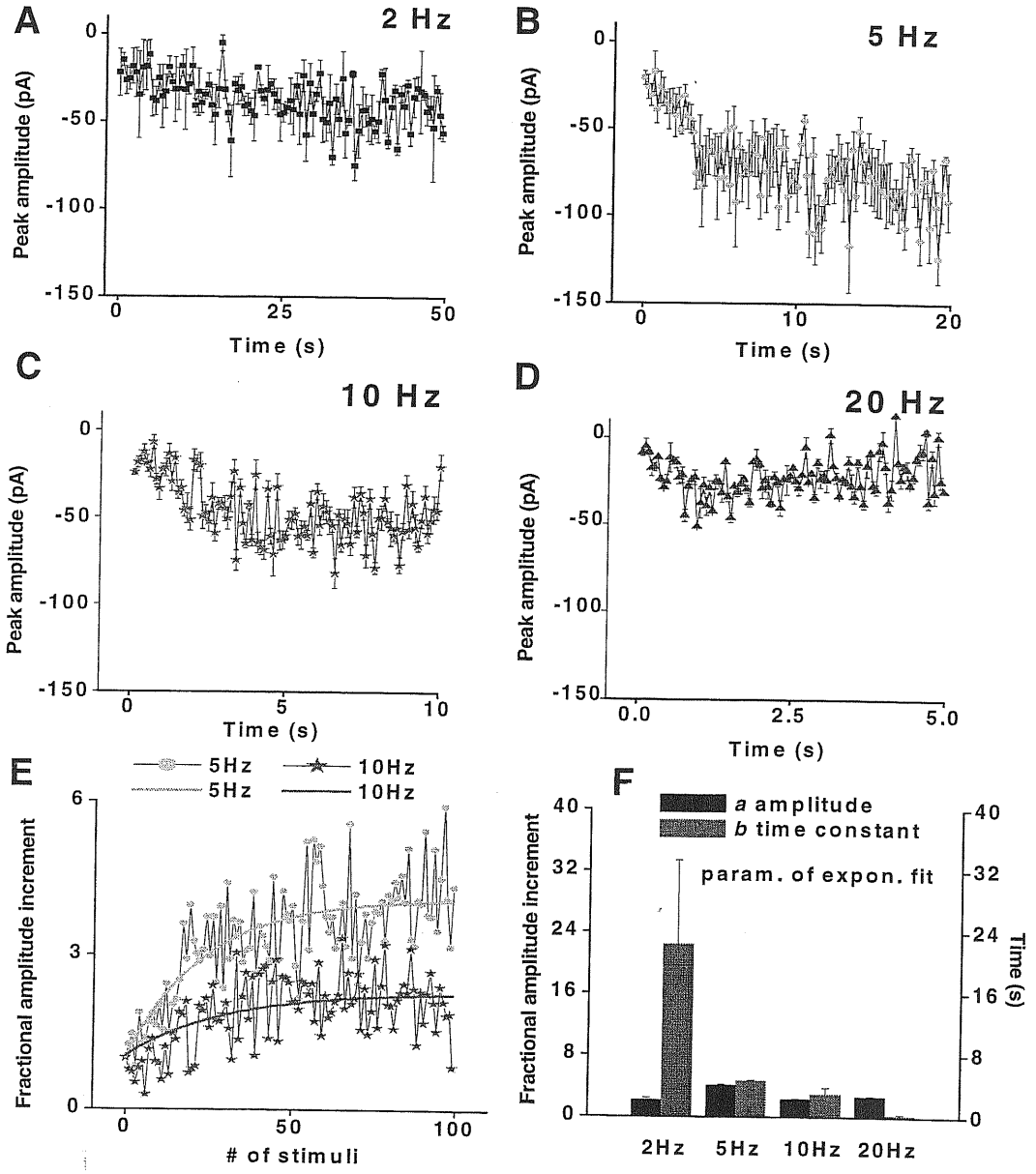

Figure 18. **High frequency stimulation of glycinergic responses. A-D:** average glycinergic peak responses (n=2 cells) to 100 stimuli delivered at 2 Hz (squares), 5 Hz (circles), 10 Hz (stars), 20 Hz (triangles), respectively. **E:** peak responses to 100 stimuli normalized to the first one (for 5 and 10 Hz stimulation, same symbols than in B, C) to represent time course of facilitation. **F:** parameters of the exponential fit of time course of facilitation: a=amplitude, b=time constant (fitted function: a+exp(-t/b)(1-a), see text).

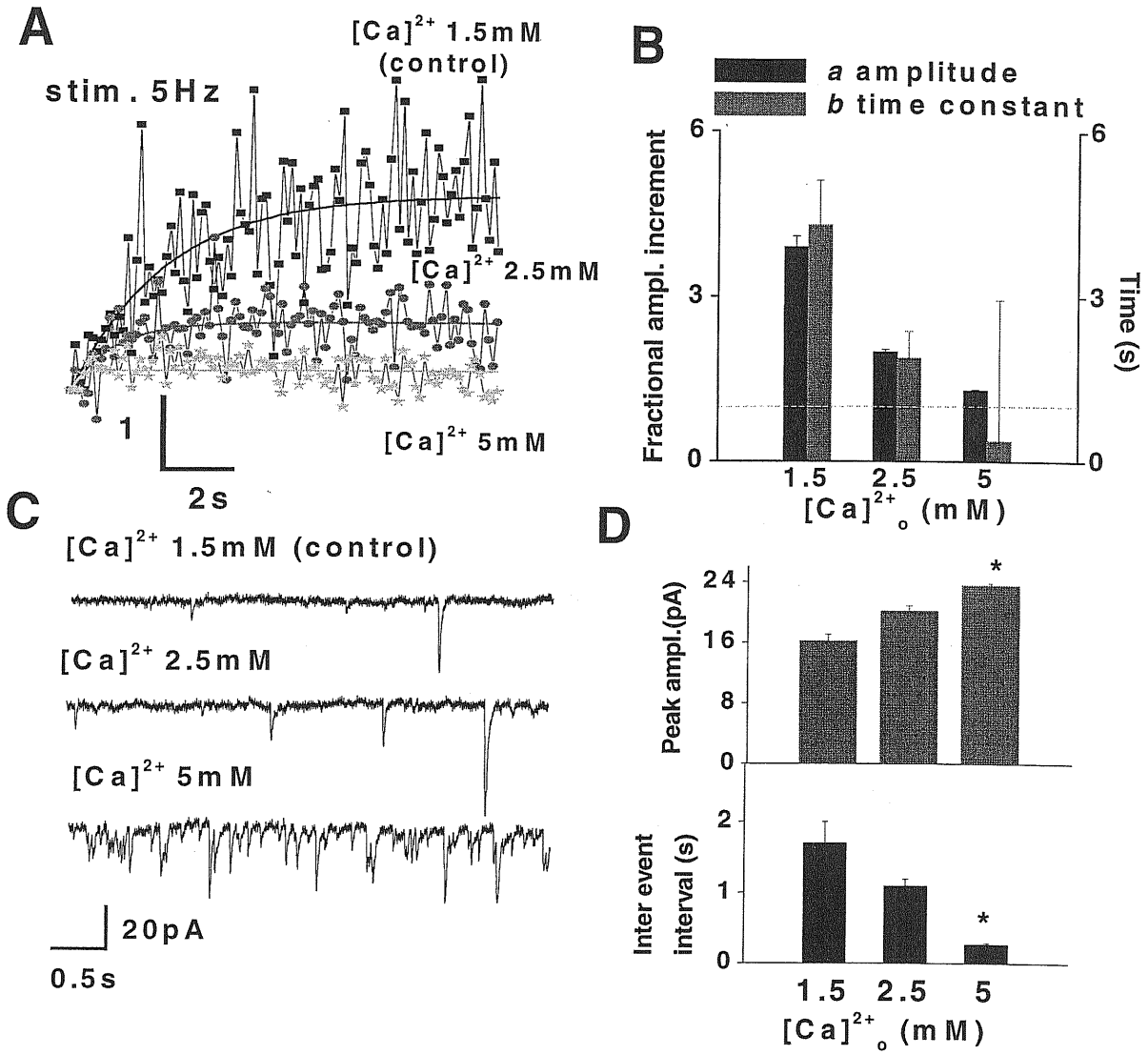

Figure 19. **Dependence of glycinergic facilitation on $[Ca]^{2+}_o$.** **A**: normalized response to 5 Hz tetanus (100 stimuli) at different $[Ca]^{2+}_o$ concentration (1.5 mM, 2.5 mM, 5 mM). **B:** parameters of the exponential fit for the time course of glycinergic facilitation at different $[Ca]^{2+}_o$. Facilitation decreased while time needed to reach maximal response decreased when $[Ca]^{2+}_o$ was raised. **C:** sample records of glycinergic spontaneous currents at different $[Ca]^{2+}_o$; **D:** statistics of amplitude (gray bars; top) and inter event interval (black bars; bottom) of glycinergic spontaneous events at different $[Ca]^{2+}_o$ (n=2; asterisks indicate a significant difference respect to the control, $p<0.001$).

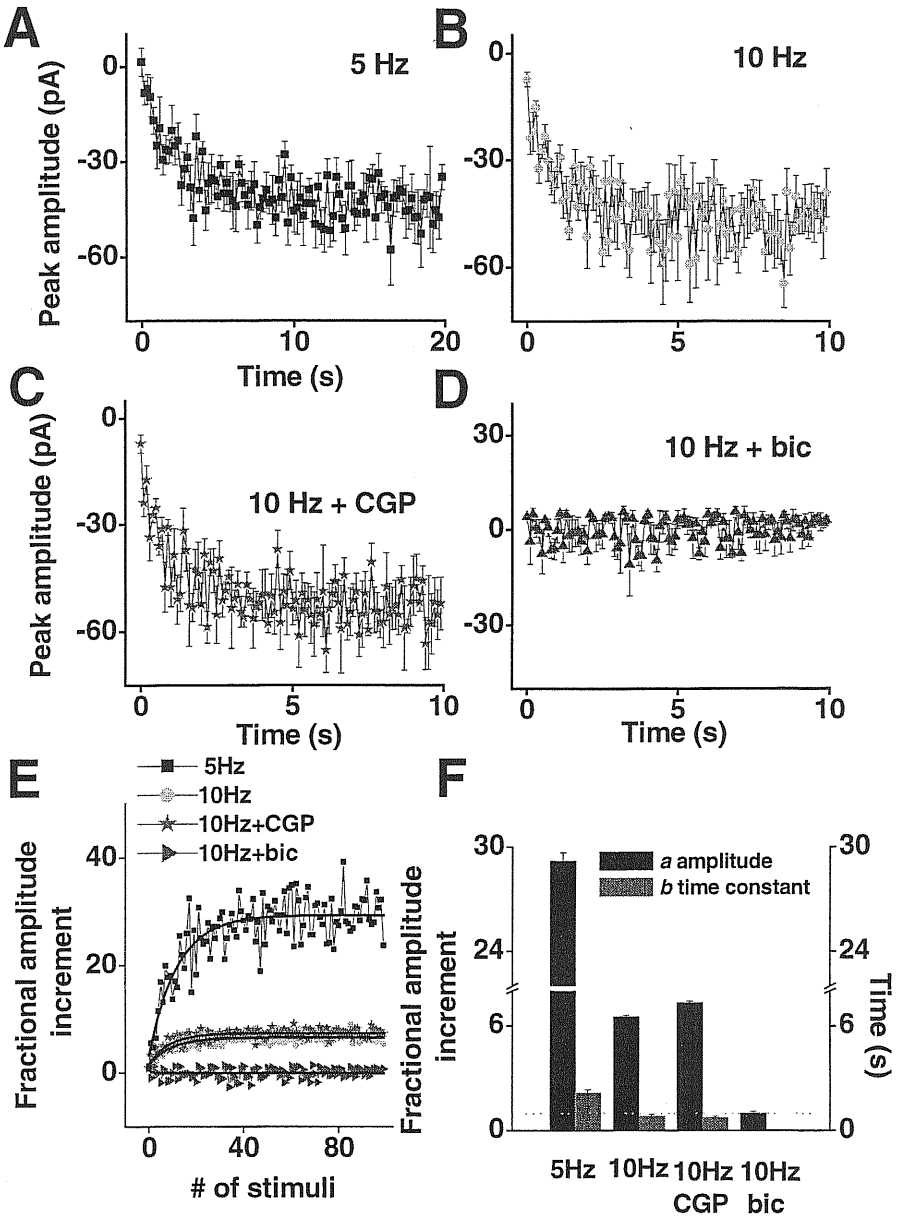

Figure 20. **High frequency stimulation of GABAergic responses. A, B:** average GABAergic peak responses (n=2 cells) to 100 stimuli delivered at 5 Hz (squares, A) and 10 Hz (circles, B); **C:** average peak GABAerigc responses to 10 Hz tetanus in presence of CGP 52432 10 μM; **D:** bicuculline (10 μM) fully blocked GABAergic responses to a 10 Hz tetanus. **E:** peak responses to 100 stimuli normalized to the first one (same symbols as in A-D); **F:** parameters of the exponential fit for GABAergic facilitation (fitted function; a+exp(-t/b(1-a), a=amplitude, b= time constant).

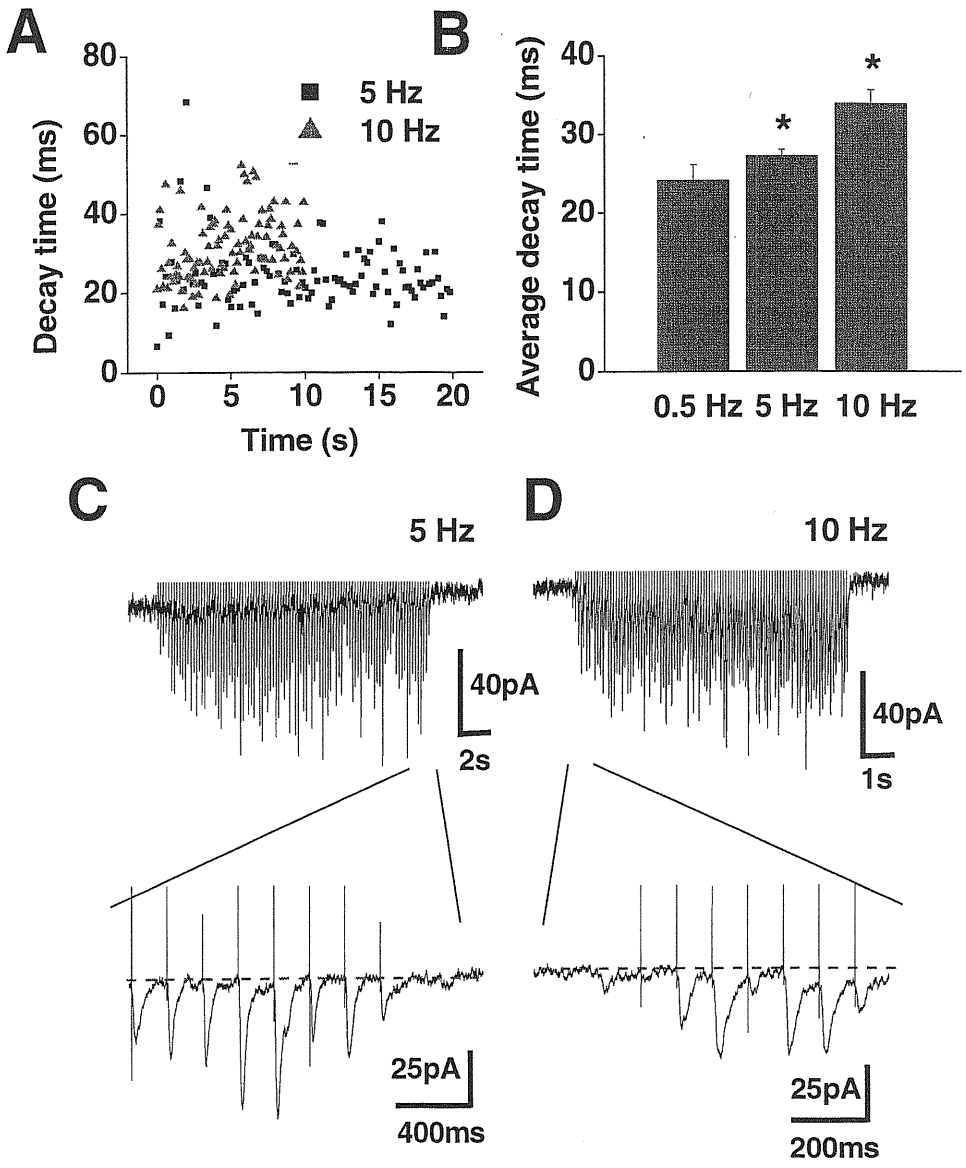

Figure 21. **Dependence of decay time of GABAergic responses on frequency of stimulation. A:** scatter plot of the decay time of GABAergic responses to 5 Hz (squares) or 10 Hz (triangles) tetanic stimulation as function of time. **B:** average decay time for each frequency stimulation; asterisks indicate statistically significant difference respect to the control, i.e. low frequency stimulation at 0.5 Hz. **C:** record of a single trial at 5 Hz (slow time scale, top, and fast time scale, bottom). **D:** as in C for 10 Hz stimulation.