ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

FUNCTION AND MODULATION OF GABAERGIC AND GLYCINERGIC TRANSMISSION IN NEONATAL RAT HYPOGLOSSAL MOTONEURONS

Thesis submitted for the degree of

"Doctor Philosophiae"

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SISSA - SCUOLA NTERNAZIONALE SUPERIORE STUDI AVANZATI

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NOTE

All work reported arises solely from my own experiments and data analysis. Part of the data reported in the present thesis have been published in the following article:

Marchetti, C., **Pagnotta, S.E.**, Donato, R., Nistri, A. (2002). Inhibition of spinal or hypoglossal motoneurons of the newborn rat by glycine or GABA, *Eur. J. Neurosci.*, **15**, 975-983.

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Thanks to my family finally: to my mother and my sister for loving me so much, being always so proud of me, and to Fabio, brave enough to share his life with me.

ABSTRACT

In the present study, I investigated the function and modulation of glycinergic and GABAergic transmission in neonatal rat hypoglossal motoneurons.

Whole cell recording were made from brain stem slice preparation, and kynurenic acid (2 mM), strychnine (0.4 μM) or bicuculline (10 μM) were used as pharmacological tools to block glutamatergic, glycinergic or GABAergic transmissions, respectively.

Current clamp experiments showed that cell firing, elicited by current pulse injection, is inhibited by evoked glycinergic or GABAergic postsynaptic potentials. The mechanism proposed is a shunt inhibition of the membrane excitability, due to the fall in input resistance induced by chloride channel opening.

In voltage clamp experiments, I tested the effect of muscarine application on spontaneous postsynaptic currents (sPSCs), miniature postsynaptic currents (mPSCs), electrically evoked postsynaptic currents (ePSCs) and pressure-pulse evoked postsynaptic currents.

Muscarinic receptor activation strongly reduced amplitude and frequency of spontaneous glycinergic and GABAergic transmission (with no major differences between the two transmitters). Miniature GABAergic currents resulted completely unaffected by muscarine, while glycinergic ones were slightly reduced in amplitude. Evoked glycinergic and GABAergic currents were also reduced in amplitude by muscarine application. Postsynaptically, muscarine depressed the maximal amplitude of both glycinergic and GABAergic dose-response curves.

To identify the muscarinic receptor subtypes involved in the modulatory actions of muscarine, I used pirenzepine, AFDX-116, DAMP or tropicamide as selective antagonists against M_1, M_2, M_3, or M_4 receptor subtypes, respectively. Muscarininc actions on evoked glycinergic or GABAergic transmission were apparently mediated by M_2 or M_1 and M_3 receptors, respectively, while, as far as spontaneous transmission is concerned, the pharmacological pattern of antagonist block was more complex. However, experiments indicated a predominant role for putative, presynaptically-located M_2 receptors. Finally, postsynaptic M_2 and M_3 receptors accounted for muscarine action on postsynaptic glycinergic or GABAergic currents, respectively.

These results suggest that various muscarinic receptors down-regulated action-potential dependent inhibitory transmission via postsynaptic and network-based action.
Moreover, muscarinic modulation of glycine or GABA transmission was due to different receptor subtypes, indicating that these two transmitters are released from different interneurons, and that their co-release from the same synaptic terminal, if present, represents a limited phenomenon only.

INTRODUCTION

"Tomá mate y avivate!"

(M.D. Rosato Siri, argentine proverb)

1. GLYCINE AND γ-AMINOBUTYRIC ACID (GABA) RECEPTORS: LIGAND-GATED ANION CHANNELS

Fast inhibitory neurotransmission in the mammalian central nervous system (CNS) is primarily mediated by the neurotransmitters glycine and GABA, with glycine being predominantly used in the spinal cord and in the brainstem, and GABA prevailing in higher regions of the neuraxis. The binding of these two amino acids to distinct postsynaptic receptors opens membrane chloride channels[1], thus usually generating, in the adult animal, hyperpolarising inhibitory currents[2]. Glycinergic transmission is selectively antagonized by nanomolar concentrations of the convulsive alkaloid strychnine, while GABAergic one, in contrast, is blocked by bicuculline or picrotoxin and potentiated by benzodiazepines and barbiturates. Together with nicotinic acetylcholine receptors (nAChRs), 5-HT_3 receptor and some invertebrate anionic glutamate receptors, glycine and $GABA_A$ receptors belong to the ligand-gated ion channel superfamily (LGICS). Members of the LGIC superfamily have a common structure in which five subunits form an ion channel (fig.1). They share both structural and primary sequence homology and are thought to have evolved from a common ancestral receptor subunit (Betz, 1990). Each subunit consists of a large amino-terminal extracellular domain of ~ 200 amino acids, which contains a conserved motif, called Cys loop, 4 putative transmembrane domains (TM), and a short extracellular carboxy terminus (fig.1a). TM3 and TM4 are linked by a cytosolic loop of variable length. Although there are no three-dimensional crystals available for any of these receptor channels, some structural information can be obtained from low-resolution three-dimensional images of two-dimensional nAChR crystals in a closed channel conformation and in an open conformation (Unwin, 1993, 1995, 1996).

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[1] The effects of GABA can be mediated by the activation of either ionotropic ($GABA_A$, $GABA_C$) or metabotropic receptors ($GABA_B$). This subdivision will be briefly discussed later, even though the present study is focused on the ionotropic $GABA_A$ receptor only.

These images suggest that only TM2 is α-helical, whereas the other three domains are probably β-sheets (Gorne-Tschelnokow *et al.*, 1994). The five α-helical TM2 domains, one from each subunit, kink at the center of the membrane to form the ion channel gate. As a first approximation, these data might be extrapolated to the putative structure of GABA and glycine receptors.

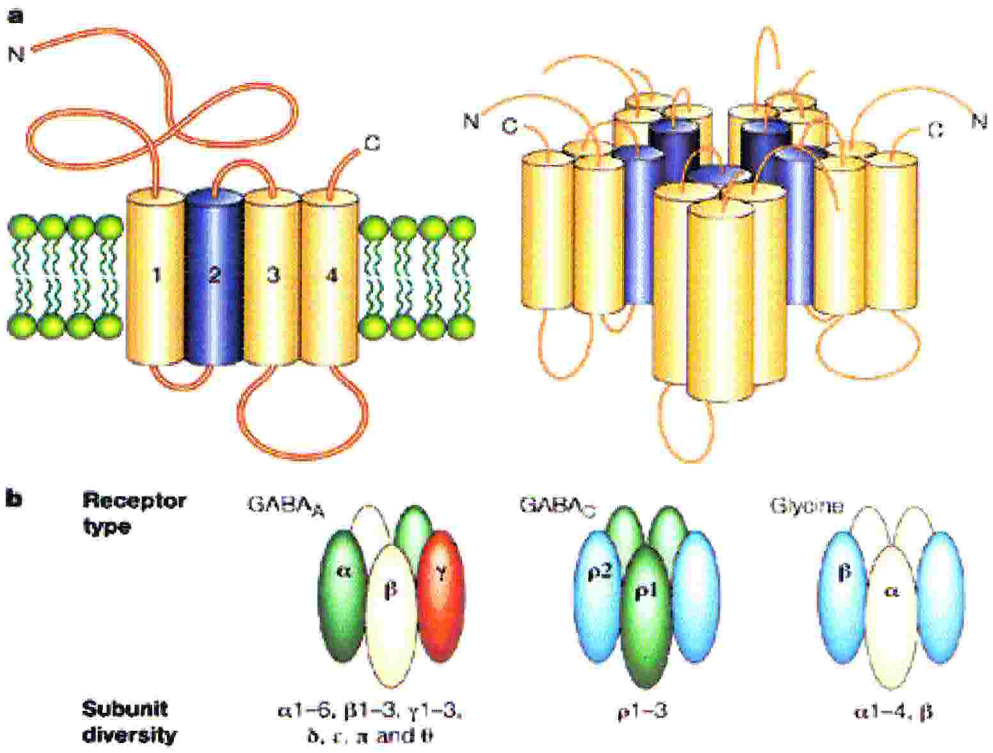

Fig1: Structure and diversity of inhibitory ligand-gated ion channels (taken from: Moss, S.J. & Smart, T.G. *Nat Rev Neurosci*, **2**, 240-250, 2001)

1.1 GLYCINE RECEPTOR

Glycine is the major inhibitory neurotransmitter in the brainstem and spinal cord, where it participates in a variety of motor and sensory functions. This amino acid is also present in the forebrain, where it has recently been shown to function as coagonist at the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor (Johnson & Ascher, 1987; Curras & Pallotta, 1996; Stanley *et al.*, 1997; Paudice *et al.*, 1998), promoting the action of glutamate itself. Thus, glycine subserves both inhibitory and excitatory functions within the adult CNS.

Glycine is formed from serine by the enzyme serine hydroxymethyltransferase. It is released from nerve endings in a Ca^{2+}-dependent fashion, and its actions are

[2] The situation is quite different in the neonatal animal, as it will be discussed later.

terminated primarily by reuptake via Na^+/Cl^--dependent, high-affinity, glycine transporters. The agonists for the glycine receptor (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. 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). The only known agonist that is not an amino acid is cesium (Hughes *et al.*, 1987; Smith *et al.*, 1989). The plant alkaloid strychnine and its derivatives, and the steroid RU5135 as well as 1,5-diphenyl-3,7-diazaadamantan-9-ol, are the only antagonists that act at nanomolar concentrations on the glycine receptor, with an IC_{50} of about 30 nM (Betz, 1990); strychnine blocks the action of glycine but not of GABA on motoneurons and interneurons, and starts becoming non-selective only at very high concentrations (Davidoff *et al.*, 1969; Curtis *et al.*, 1971; Donato & Nistri, 2000).

1.1.1 MOLECULAR STRUCTURE AND SUBUNIT COMPOSITION

The glycine receptor was the first neurotransmitter receptor protein to be isolated from the mammalian CNS, by exploiting its high affinity for the alkaloid strychnine (Pfeiffer & Betz, 1981; Pfeiffer *et al.*, 1982). It can be assembled from two subunit classes, α (four isoforms) and β (one isoform). cDNA of the adult 48-kDa α_1 subunit and the 58-kDa β subunit were first isolated (Grenningloh *et al.*, 1987;Grenningloh *et al.*, 1990); subsequently, cDNA clones corresponding to the embryonic α_2 and the adult α_3 subunits were cloned by homology screening (Grenningloh *et al.*, 1990; Kuhse *et al.*, 1990a; Kuhse *et al.*, 1991; Akagi *et al.*, 1991a). A fourth α subunit has been identified in mouse and chick (Matzenbach *et al.*, 1994).

Adult glycine-receptor isoforms are generally assumed to be α3/β2 heteromers, where eight key residues close to the N terminus are critical for receptor assembly and form the intersubunit contact surface (Griffon *et al.*, 1999). On the other hand, fetal glycine receptors are probably homomers of α_2 subunits, as primary cultures of rat or mouse fetal spinal cord express predominantly α_2 (Hoch *et al.*, 1989). Indeed, functional properties of recombinant homomeric α_2 channels resemble those of native fetal glycine receptors rather than those of adults (Takahashi *et al.*, 1992).

While homomeric α5 receptor complexes, as well as other mixed α/β heteromers, are functional, pure β5 complexes do not form functional ion channels (Kingsmore *et al.*, 1994; Mulhardt *et al.*, 1994). Absence or presence of the β subunit in glycine receptors gives rise to subtle changes in single-channel properties, with lower conductance levels being predominantly present in α_1/β heteromeric receptors as compared to $\alpha_1 5$ homomers (Bormann *et al.*, 1993). However, β subunits exert only a minor influence on macroscopic parameters of glycine-receptor function, such as whole-cell current amplitudes or receptor desensitisation. Presence of the β subunit reduces picrotoxin sensitivity of recombinant glycine receptors (Pribilla *et al.*, 1992). This picrotoxin insensitivity has been found to depend on a single residue within TM 2, namely α_1(T258), which corresponds to β(F282).

β subunits are also associated with gephyrin, a 93-kDa peripheral membrane protein copurified with the glycine receptor (Pfeiffer *et al.*, 1982; Schmitt *et al.*, 1987; Prior *et al.*, 1992).

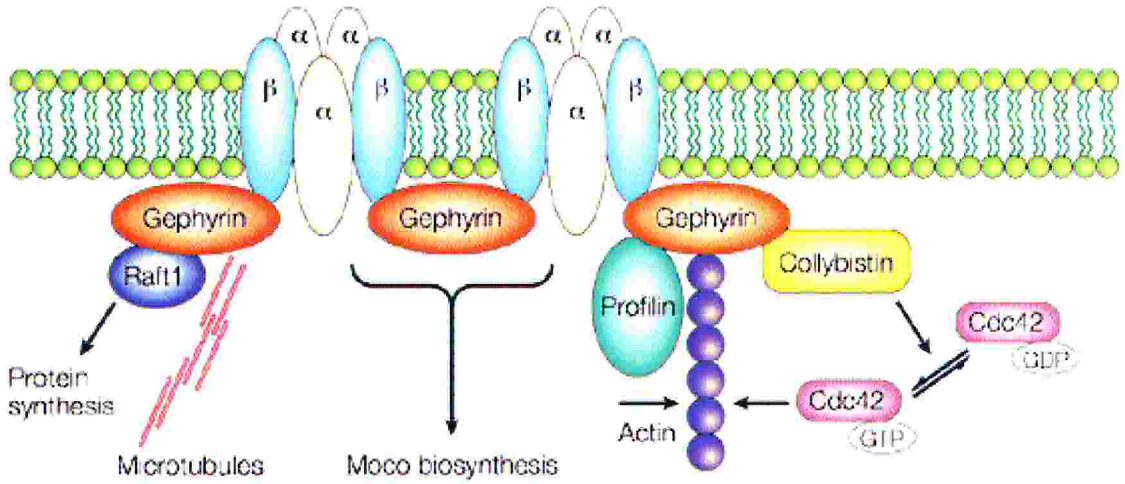

Fig2: The synaptic localization of glycine receptors is controlled by gephyrin (taken from: Owens, D.F., Kriegstein, A.R. *Nat Rev Neurosci*, **3**, 715-727, 2002).

Gephyrin binds to the large cytoplasmic loop of the β-subunit via an amphipathic sequence (Meyer *et al.*, 1995; Kneussel *et al.*, 1999), and also shows high affinity for polymerized tubulin (Kirsch *et al.*, 1991). For this reason gephyrin is postulated to anchors and immobilize the glycine receptor on the subsynaptic cytoskeleton (Kirsch & Betz, 1995). Indeed, inhibition of gephyrin expression with antisense oligonucleotide prevents formation of glycine receptor clusters (Kirsch *et al.*, 1993),

altering the expression level of the receptor itself. So, regulation of GlyR packing density by gephyrin could modulate the functional properties of the receptor, and thus synaptic efficacy. It has also been shown that gephyrin is required for clustering and postsynaptic localization of $GABA_A$ receptors (Cabot *et al.*, 1995).

1.1.2 DISTRIBUTION IN CNS

The distribution of individual α and β subunit isoforms has been examinated by *in situ* hybridization, immunohistochemical studies (using monoclonal antibodies against different receptor subunits), and autoradiography (using [^{3}H]strychnine and [^{3}H]glycine binding), revealing both temporal and spatial variation in their expression within the CNS (Young & Snyder, 1973; Zarbin *et al.*, 1981; Triller *et al.*, 1985; Basbaum, 1988; van den Pol & Gorcs, 1988; White *et al.*, 1990; Malosio *et al.*, 1991). Glycine receptors are principally expressed in the spinal cord, with a marked rostro-caudal gradient, and the medulla. Lower levels are found in midbrain and hypothalamus, but they are virtually absent in the forebrain. Moreover, glycine and GABA receptors often coexist in the same spinal cord neuron (Furuyama *et al.*, 1992; Bohlhalter *et al.*, 1994), and they can be both released from spinal interneurons to activate functionally distinct receptors on their postsynaptic target cells (Jonas *et al.*, 1998). In addition to their expression in the CNS, glycine receptors have also been found in the retina, adrenal gland, kidney, liver, and sperm (Greferath *et al.*, 1994; Miller & Schnellmann, 1994; Yadid *et al.*, 1995; Ikejima *et al.*, 1997; Meizel, 1997).

1.1.3 FUNCTIONAL PROPERTIES

Single-channel recordings of glycine receptors of neurons in primary culture revealed a relative permeability sequence of $SCN^- > NO_3^- > I^- > Br^- > Cl^- > F^-$, whereas the relative conductance states were $Cl^- > Br^- > NO_3^- > I^- > SCN^- > F^-$ (Bormann *et al.*, 1987; Fatima-Shad & Barry, 1992). Glycine receptors have multiple conductance states: the predominant conduction levels of homomeric (α_1, α_2, α_3) receptors are significantly higher than those of heteromeric ($\alpha_1\beta$, $\alpha_2\beta$, $\alpha_3\beta$) and native glycine receptors. Frequency distribution histograms suggested the existence of at least three different open states. The mean open time of α_1 homomeric channels was much shorter than those of α_2 homomeric channels, consistent with a reduction in channel open times during development (Takahashi *et al.*, 1992). Raising glycine concentrations did not

affect the open time constants but increased the channel open frequency (Twyman & Macdonald, 1991). Glycine receptors were reported to strongly rectify at voltages more negative than –50 mV both in cultured neurons and upon expression in *Xenopus* oocytes (Gundersen *et al.*, 1984; Akagi *et al.*, 1991b; sMorales *et al.*, 1994). Others however reported that rectification was absent in rat homomeric or heteromeric glycine receptors recombinantly expressed in *Xenopus* oocytes or HEK293 cells (Sontheimer *et al.*, 1989; Kuhse *et al.*, 1990b; Bormann *et al.*, 1993; Rajendra *et al.*, 1994). In some cases, a difference in rectification between whole cell and single channel recordings was observed (Bormann *et al.*, 1987; Morales *et al.*, 1994). Glycine receptors desensitize with time, resulting in a transient signal upon agonist binding. Decay time constants generally decreased with increasing agonist concentration, with the shorter time constants corresponding to the decay time constant of glycinergic inhibitory postsynaptic potentials. The receptors recovered completely from desensitization within 60 s. The time constant varies widely from 10 ms to > 10s (Akaike & Kaneda, 1989; Lewis *et al.*, 1991).

1.1.4 RECEPTOR MODULATION

Cellular environment: There are several intracellular factors that can affect glycine-receptor function (Breitinger & Becker, 1998), and, among them, phosphorylation constitutes an important mechanism to modulate their response properties (Huganir & Greengard, 1990). In cultured brain stem neurons, stimulation of protein kinase A enhances glycine response (Song & Huang, 1990). Similarly, in oocytes injected with mRNA from rat spinal cord, glycine-activated currents increase after cAMP treatment (Vaello *et al.*, 1994). On the other hand, an opposite effect is produced by phosphorylation by protein kinase C: decreased currents elicited by glycine were recorded from oocytes injected with mRNA, after stimulation of protein kinase C (Vaello *et al.*, 1994). Moreover, protein kinase C dependent phosphorylation of serine at position 391 of the GlyR α_1 subunit was demonstrated biochemically (Vaello *et al.*, 1994). Thus, multiple phosphorylation events may regulate GlyR function *in vivo*, allowing crosstalk of intracellular signalling pathways with glycinergic neurotransmission.

Modulation of glycine-receptor function by a Ca^{2+} binding factor was proposed from a detailed study on rat neurons and recombinant receptors (Fucile *et al.*, 2000).

Receptor density has also been shown to affect receptor properties in oocytes (Maammar *et al.*, 1997). Yet it is not known whether this last effect is due to interactions between neighbouring receptors or to other protein modifications.

Despite the amount of information obtained with all the previously mentioned studies, it is important to stress that functional differences between receptors from neurons and recombinant systems have been observed with glycine receptors. For example, expression of glycine-receptor α_1 (R271Q) subunits in HEK-293 cells results in higher ligand affinity of mutant glycine receptors than expression in rat dorsal horn neurons, a fact attributed to differences in posttranslational modification in the host cells (Kung *et al.*, 2001). Moreover, two widely used expression systems, *Xenopus* oocytes and HEK-293 cells, both supply a background of functional receptor modulation that is difficult to control and may vary from cell to cell. In the case of homomeric recombinant α_1 or α_2 glycine receptors from zebrafish (*Danio rerio*), more than tenfold differences were measured in EC_{50} values for the same receptor from one oocyte to the next and also from one HEK-293 cell to the next (De Saint *et al.*, 2001). Although these differences could be attributed to alterations in channel gating, the underlying modification is not known.

Modulatory compounds: Plasticity of neurotransmission is considered a prerequisite of higher brain functions, and, therefore, modulatory ligands of neurotransmitter receptors have gained increasing attention. A fundamental example of a physiological modulatory ligand is zinc, a bivalent metal ion stored in synaptic vescicles and co-released with neurotransmitters upon stimulation (Assaf & Chung, 1984). Zinc ions strongly modulate glycine-activated currents in cultured spinal neurons or *Xenopus* oocytes expressing recombinant glycine receptors (Bloomenthal *et al.*, 1994; Laube *et al.*, 1995), and in particular low concentrations (1-10 μM) potentiate the response about threefold, whereas higher concentrations (≥100 μM) cause inhibition. Several residues that are critical for zinc binding have been identified with site-directed mutagenesis studies and analysis of chimeric glycine receptors (Bloomenthal *et al.*, 1994; Laube *et al.*, 1995, 2000; Suwa *et al.*, 2001). Other important modulators of receptor function, such as alcohols and anaesthetics, have been intensively studied in recent years (Harrison *et al.*, 1993; Mascia *et al.*, 1996; Krasowski & Harrison, 2000; Yamakura *et al.*, 2000; Yamakura & Harris, 2000) These studies led to the

identification of individual residues and modifications that are critical for alcohol potentiation of glycine receptors (Mascia *et al.*, 1996, 1998, 2000; Yamakura *et al.*, 1999).

Glycine receptors as secondary targets: Several pharmacologically relevant compounds, that have other primary targets, also interact with glycine receptors. Such cross-reactivity is frequently observed with $GABA_A$ receptor ligands (Breitinger & Becker, 1998; Grudt & Henderson, 1998; Kohno *et al.*, 1999). Calcium-channel antagonists, such as dihydropyridines and verapamil, were found to be direct blockers of the glycine-receptor channel (Chesnoy-Marchais & Cathala, 2001). Several agonists and antagonists of the $5\text{-}HT_3$ receptor were able to displace [^{3}H]strychnine from glycine receptors with micromolar affinity, that is, an affinity similar to glycine itself (Maksay, 1998). Tropisetron and atropine were found to be inhibitors, while a number of $5\text{-}HT_3$ receptor antagonists potentiate glycine receptor currents (Maksay *et al.*, 1999; Chesnoy-Marchais *et al.*, 2000). Potentiation of $\alpha_1(5)$ glycine receptor currents by ICS-205,930 is more pronounced than with α_2 homomers; moreover this potentiation for both subunits is sensitive to the presence or absence of β subunits, a fact indicating participation of the β subunits in the allosteric potentiation site (Supplisson & Chesnoy-Marchais, 2000). Substance P shows both indirect as well as direct potentiation of glycine-receptor responses (Wang *et al.*, 1999; Wang *et al.*, 2001). Forskolin, in addition to its activation of protein kinase A (PKA), appears to bind directly to $GABA_A$ and glycine receptors in carp amacrine cells where it accelerates current desensitisation (Li & Yang, 2001). Direct binding of the neuroprotective drug riluzole induces fast desensitisation of glycine receptors (Mohammadi *et al.*, 2001). The anthelmintic ivermectin has recently been identified as a novel glycine-receptor agonist which binds to the receptor and induces channel opening from a site different from the glycine and strychnine binding site. Absence of cross-desensitisation suggests an altogether different mechanism of glycine-receptor activation by ivermectine (Shan *et al.*, 2001). While such compounds are of great interest for the delineation of the mechanisms of glycine-receptor function, their multiple physiological effects render a therapeutical application rather unlikely.

1.2 GABA RECEPTOR

Moving from spinal cord and brain stem to higher regions of the neuraxis of the adult animal, γ-aminobutyric acid (GABA) becomes the major inhibitory neurotransmitter.

It is a small amino acid derived from glutamate, with a reaction that is catalysed by two glutamic acid decarboxylase (GAD) enzymes, GAD65 and GAD67 (Erlander *et al.*, 1991). GABA is then loaded into synaptic vesicles by a vesicular neurotransmitter transporter (VGAT, Fon & Edwards, 2001) and, like glycine, is liberated from nerve terminals by calcium-dependent exocytosis. However, non-vesicular forms of GABA secretion (for example, by reverse transporter action) have also been described and might be particularly important during development (Taylor & Gordon-Weeks, 1991; Attwell *et al.*, 1993). Once released, GABA can bind to different types of GABA receptor, identified on the basis of their pharmacology and electrophysiology: $GABA_A$ and $GABA_C$ receptors are Cl^- permeable channels (Bormann *et al.*, 1987; Polenzani *et al.*, 1991), whereas $GABA_B$ receptors are G-protein coupled receptors (Kaupmann *et al.*, 1997) [3].

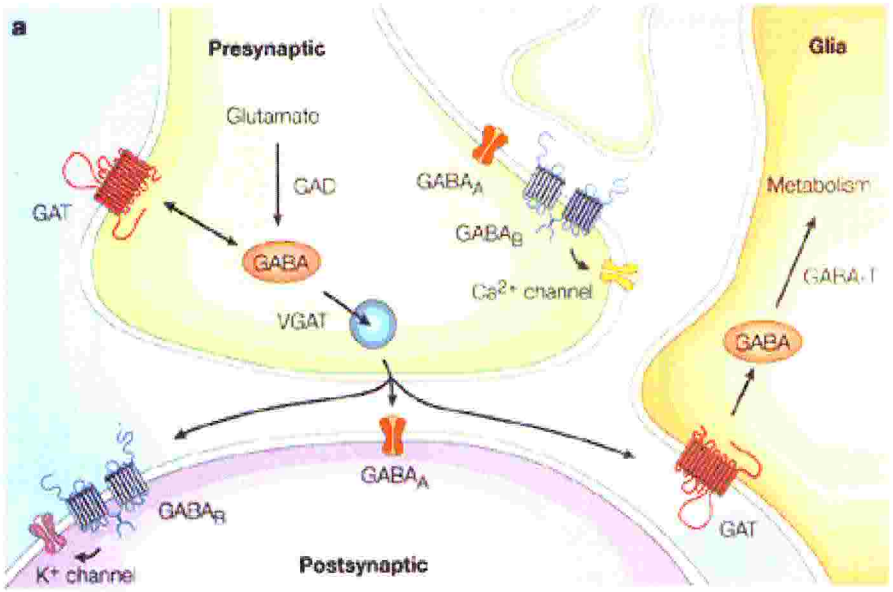

Fig.3: Components of the GABA signalling pathway (taken from: Owens, D.F., Kriegstein, A.R. *Nat Rev Neurosci*, 3, 715-727, 2002).

$GABA_A$ receptors can be activated by a number of compounds such as muscimol, isoguvacine, 3-aminopropane sulphonic acid and piperidine-4-sulphonic acid (Krogsgraard-Larsen et al., 1984). They are also inhibited by the convulsant alkaloid bicuculline in a competitive manner. This pharmacological behavior distinguishes $GABA_A$ from $GABA_B$ receptors, which are stimulated by (-)baclofen and inhibited by phaclofen (Bowery, 1989, 1993; Kerr & Ong, 1995; Misgeld *et al.*, 1995), and from the $GABA_C$ receptor that is insensitive to bicuculline. GABA signals are terminated by

[3] $GABA_B$ and $GABA_C$ receptors will be briefly discussed in this paragraph with no further reference to them.

reuptake of the neurotransmitter into nerve terminals and/or into surrounding glial cells by a class of plasma-membrane GABA transporters, namely GATs (Cherubini & Conti, 2001); thereafter, GABA is metabolized by a transamination reaction catalyzed by GABA transaminase GABA-T (Roberts, 1988).

1.2.1 MOLECULAR STRUCTURE AND SUBUNIT COMPOSITION

$GABA_A$ receptors are believed to be heteropentameric proteins, with molecular mass of 230-270 kDa, constructed from subunits derived from several related genes or gene families (Macdonald & Olsen, 1994). Starting from α_1 and β_1 subunits, originally cloned by Schofield in 1987, six α, three β, three γ, one δ, one ε, one π, and one θ subunits have been identified (Schofield *et al.*, 1987; Macdonald & Olsen, 1994; Mehta & Ticku, 1999). A fourth β and a fourth γ subunit were also identified in chicken, but have not yet been described in mammals (Bateson *et al.*, 1991; Harvey *et al.*, 1994). This multiplicity provides a daunting number of potential subunit combinations, even if it is very unlikely that all these receptors have distinct biological functions. Although all subunits except ε (Davies *et al.*, 1997a) may form homomeric receptors (Pritchett *et al.*, 1988; Blair *et al.*, 1988; Shivers *et al.*, 1989), in vitro studies indicate that certain heteromeric combinations are preferred. In particular, native receptors contain at least one α, one β and one γ subunit, with the δ, ε, π and θ ones able to substitute for the γ subunit (McKernan & Whiting, 1996). In addition, receptors with different compositions seem to be distributed to different cellular locations, where they are positioned to mediate primarily synaptic or extrasynaptic signalling (Mody, 2001).

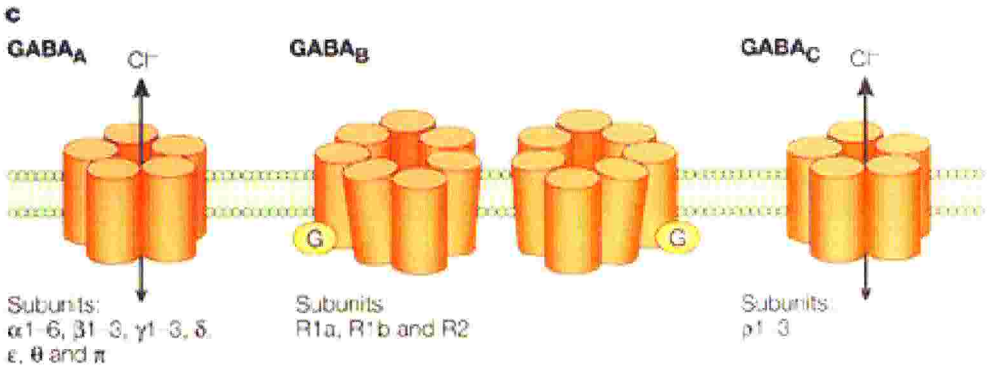

Fig.4: GABA receptors differ in subunit composition and assembly (taken from: Owens, D.F., Kriegstein, A.R. *Nat Rev Neurosci*, 3, 715-727, 2002).

As previously described for glycine receptors, it is now known that GABA receptors colocalize with gephyrin at some synapses (Todd *et al.*, 1996). Immunocytochemistry

revealed intense gephyrin immunoreactivity at GABAergic synapses in the spinal cord (Bohlhalter *et al.*, 1994; Cabot *et al.*, 1995; Todd *et al.*, 1996), retina (Sassoe-Pognetto *et al.*, 1995), and olfactory bulb (Giustetto *et al.*, 1998) as well as in cultured hippocampal (Craig *et al.*, 1996) and cortical neurons (Essrich *et al.*, 1998). A major unresolved problem is whether the interaction between gephyrin and $GABA_A$ receptors is direct or it rather involves other proteins (Kneussel & Betz, 2000). Gephyrin does not bind $GABA_A$ receptor subunits in overlay assays (Meyer *et al.*, 1995), and it is missing from purified $GABA_A$ receptor preparations (Kannenberg *et al.*, 1997). On the other hand, in γ_2 deficient mice, both the postsynaptic expression of gephyrin and of $GABA_A$ receptors is reduced (Essrich *et al.*, 1998). Postsynaptic expression could be restored by the transgenic expression of the γ_3 subunit (Baer *et al.*, 1999). In gephyrin KO mice, $GABA_A$ receptor subunits γ_2 and α_2 no longer cluster postsynaptically (Kneussel *et al.*, 1999). Thus the stabilization of $GABA_A$ receptor complexes at postsynaptic sites probably depends on their subunit composition.

1.2.2 DISTRIBUTION IN CNS

$GABA_A$ receptors have a wide, almost ubiquitous distribution on neuronal and certain glial cells, and are expressed both in the central and peripheral nervous systems. They are found also in nonneuronal tissue where their function is often not clear. Even if $GABA_A$ receptor expression generally changes during development (Luddens *et al.*, 1995), the distribution of major subunits has been investigated in various regions of the CNS (Laurie *et al.*, 1992a, 1992b; Wisden *et al.*, 1992a), and discussed in various reviews (Hevers & Luddens, 1998; Mehta & Ticku, 1999; Whiting *et al.*, 1999). The most comprehensive immunochemical mapping was carried out by Fritschy and Mohler (, 1995).

Each $GABA_A$ receptor subunit mRNA has a distinct pattern of expression. With the exceptions of the α_6 subunit mRNA, which is localized almost exclusively to mature cerebellar granule cells (Luddens *et al.*, 1990), and the ρ subunits, which are found predominantly in the retina, different $GABA_A$ receptor subunit mRNAs have heterogeneous distribution across many different cell types in adult brain. Briefly, the α_1 subunit is the most abundant CNS subunit (Benke *et al.*, 1991; Laurie *et al.*, 1992c; Fritschy & Mohler, 1995), with a predominant expression in the cerebellum, and it often colocalizes with the β_2 subunit. The $\alpha_1\beta_2$ complex, in turn, colocalizes frequently

with the γ_2 subunit, found in nearly all brain regions, but with different abundance (Gutierrez *et al.*, 1994). Hence, the most abundant receptor may consist of α_1, β_2, and γ_2 subunits. In general, α variants, other than α_1, show more limited distributions. α_2 to α_5 subunits are predominantly expressed in hippocampus, whereas the cerebellum seems to lack these subunits. The α_1-α_4 subunits are expressed at intermediate levels in the cerebral cortex, which expresses only low levels of α_5. The α_6 subunit appears to be almost exclusively expressed on cerebellar granule cells, but traces are also found in the dorsal cochlea (Varecka *et al.*, 1994). α_4 and α_6 subunits often colocalize with the δ subunit (Wisden *et al.*, 1991). In particular, the α_6 KO mouse suggested that the stability of the δ subunit depends on its interaction with the α_6 subunit, because this mouse also lacked δ subunit expression in cerebellar granule cells (Jones *et al.*, 1997), where the δ subunit is normally predominantly expressed. δ subunit are also expressed, in minor amount, in part of the cerebral cortex, the thalamus and the olfactory bulb (Shivers *et al.*, 1989; Laurie *et al.*, 1992d).

The role and distribution of β subunits have been studied in less detail. β_1 mRNA signals are the strongest in hippocampus, less pronounced in part of the basal nuclei and septum, and only weak in the amygdala and hypothalamus. On the other hand, the β_2 subunit shows a more generalized distribution. It is weaker where strong β_1 and β_3 concentration are found, such as in the hypothalamus and in certain regions of the hippocampus (Lolait *et al.*, 1989; Zhang *et al.*, 1991; Wisden *et al.*, 1992b). The β_3 subunit is strong in the hippocampus, in the olfactory bulb, cortex, part of the basal nuclei, and hypothalamus. Weak signals are found in the amygdala and thalamus (Lolait *et al.*, 1989; Zhang *et al.*, 1991; Wisden *et al.*, 1992b). The γ_2 subunit is the most abundant of the γ subunit mRNAs followed by γ_1, while the γ_3 subunit is rare (Herb *et al.*, 1992; Wisden *et al.*, 1992b).

Overall, the most ubiquitously expressed subunits are the α_1, α_2, α_3, β_2, β_3 and γ_2 subunits, while other isoforms show more restricted patterns of distribution. For example, ε is enriched in the subthalamic nucleus and the π subunit in the uterus (Davies *et al.*, 1997b). Further, it is clear that some cell types (probably the most studied being the cerebellar granule cell) express more than five different $GABA_A$ receptor subunit transcripts, implying that there is more than one receptor type per cell. Similar general correlates can be made from immunochemical studies. Some of

these have been extended to the subcellular distribution and even quantification of certain receptor subunits. Enrichment but not an exclusive localization at synaptic areas is found.

1.2.3 FUNCTIONAL PROPERTIES

The biophysical properties of $GABA_A$ receptors have been investigated in native tissues and heterologous expression systems, and it was found that the permeability sequence was $SCN^- > I^- > Br^- > Cl^- >> F^-$. They are also permeable to bicarbonate ions, with a permeability ~ 20% of that of Cl^-. The permeability ratio of K^+ to Cl^- (P_K/P_{Cl}) is <0.05. Because relatively large polyatomic molecules can permeate the pore of $GABA_A$ receptors, a pore diameter of 5.6 Å is suggested (Bormann *et al.*, 1987).

GABA receptors exhibit multiple conductance levels, with conductances of 12, 17-20, and 27-30 pS (Bormann *et al.*, 1987), and three different open states of 0.5, 2.6, and 7.6 ms duration (Macdonald *et al.*, 1989). With increasing GABA concentrations, the relative contributions are shifted toward the longer states (Macdonald *et al.*, 1989; Twyman *et al.*, 1990). The open frequency and mean open time is reduced by the competitive antagonist bicuculline, whereas the noncompetetive antagonist picrotoxin reduces channel open times (Macdonald *et al.*, 1989). GABA receptors can also be blocked by penicillin that, at high concentrations, increases the open frequency and shortens the open times (Twyman *et al.*, 1992), without affecting the single-channel conductance.

1.2.4 RECEPTOR MODULATION

Cellular environment: As previously described for glycinergic receptors, $GABA_A$ receptor function can be modulated by several intracellular factors.

Many of the $GABA_A$ receptor subunits contain, within their respective intracellular loops, sites for phosphorylation by the serine/threonine protein kinases protein kinase A, protein kinase C, cGMP-dependent protein kinase and CaM kinase II, as well as sites for phosphorylation by the tyrosine kinases, PDGF receptor and src (Moss & Smart, 1996). The intracellular loops expressed as fusion proteins in *Escherichia coli* are phosphorylated *in vitro* by the appropriate kinases. Sometimes the subunit is phosphorylated at multiple sites by the same kinase. The phosphorylation sites of functional cloned receptors have been mapped. In some cases, the residues

phosphorylated in the cytoplasmic loop are not always phosphorylated in assembled receptors, e.g. β_1, β_2 and β_3 intracellular loops are all phosphorylated by protein kinase A but only β_1 and β_3 subunits are phosphorylated in whole receptors. Modification of $GABA_A$ receptor subunits by phosphorylation has many different effects on receptor function, dependent on the kinase type. Acute effects include both a time-dependent enhancement and decrease in GABA-induced currents and modulation of receptor desensitization. These have been described for both cloned receptors and a variety of neuronal preparations. Presumably, the net effect of phosphorylation will depend upon the $GABA_A$ receptor subunits expressed at a particular developmental stage in a certain cell. Chronic activation of protein kinase A results in the enhanced assembly of $GABA_A$ receptors.

Also intracellular calcium concentration and association with cytoskeletal anchoring proteins have been shown to alter ligand affinity, peak current size and the rate of desensitization of $GABA_A$ receptors (Moss & Smart, 1996; Mozrzymas & Cherubini, 1998; Chen *et al.*, 2000).

Modulatory compounds: Modulation of $GABA_A$ receptor (and its pharmacological profile) is a rather complex phenomenon influenced by the receptor subunit composition (see for a review:Hevers & Luddens, 1998).

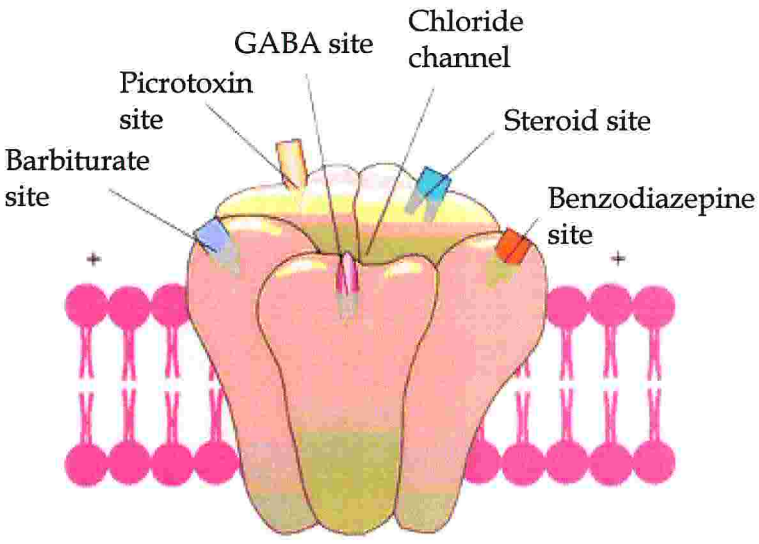

Fig.5: Schematic illustration of $GABA_A$ receptor with its main extracellular binding sites.

In particular, the pharmacological and electrophysiological characterization of defined receptor isoforms has been possible using heterologous expression of

different subunit combinations. These profiles may also be used to differentiate between subunit combinations in vivo and to identify native receptor isoforms.

Drugs that interact with GABA receptors can be divided into antagonists and potentiators. The former act as convulsants, while the latter depress the CNS and may be clinically useful as sedatives, anesthetics, and anticonvulsants. As previously mentioned, $GABA_A$ receptors are selectively activated by muscimol and isoguvacine, and, unlike glycine receptors, their activity may be potentiated by benzodiazepines, barbiturates, anesthetics, alcohol, and some steroids. Additional binding sites for a number of substances, e.g., loreclezole, avermectin, zinc, or lanthanum, are also present (Sieghart, 1995). All these modulators work through at least six different binding sites, allosterically interacting with each other, and they act mainly by increasing the channel open time and/or by enhancing the frequency of the channel opening. Picrotoxin, a mixture of picrotin and picrotoxinin, is a noncompetitive inhibitor that reduces channel activity. It binds to a site different from the GABA-binding site. The mechanism of inhibition by picrotoxin is not completely understood. In contrast, bicuculline acts as a competitive antagonist by binding to the GABA-binding site without opening the channel.

2. GLYCINE AND GABA: CO-LOCALIZATION AND CO-RELEASE

Even if GABA is the main inhibitory transmitter in the forebrain while glycine is predominant in brain stem and spinal cord, often, in various regions of CNS, the two transmitter systems overlap. The extent of this overlapping is a recurrent theme in studies of inhibitory transmission.

In some regions of CNS, for example in the spinal cord, there is strong anatomical evidence for the co-localization of $GABA_A$ and glycine receptors at single postsynaptic densities (Triller *et al.*, 1987; Bohlhalter *et al.*, 1994; Todd *et al.*, 1996) and for GABA and glycine co-localization in presynaptic terminals (Ornung *et al.*, 1994; Todd *et al.*, 1996; Ornung *et al.*, 1998). Moreover, electrophysiological measurements seem to indicate that both GABA and glycine contribute to IPSP/C in cranial motoneurons (Kolta, 1997), as well as to recurrent (Schneider & Fyffe, 1992), afferent and descending inhibitory inputs to spinal motoneurons (Wu *et al.*, 1992; Stuart & Redman, 1992; Pinco & Lev-Tov, 1994; Gao & Ziskind-Conhaim, 1995; Gao *et al.*,

1998a). In particular, it has been recently shown that in the spinal cord GABA and glycine can be co-released from the same presynaptic vesicle (Jonas *et al.*, 1998), even if the postsynaptic complement of receptors, is not constant between synapses. Analysis of evoked and mIPSC suggests three types of inhibitory synapses on spinal motoneurons: GABA only, glycine only, and mixed synapses comprising 15, 41, and 44% of the total input, respectively (Jonas *et al.*, 1998). GABA and glycine co-release seems to take place also in some brain stem nuclei, the abducens nucleus (Russier *et al.*, 2002) and the hypoglossal one (O'Brien & Berger, 1999), even if for the last nucleus data remain controversial (Donato & Nistri, 2000).

When evaluating the possible extent of the co-release phenomenon throughout the CNS, an important fact to consider is the different synaptic distribution of GABA and glycine receptors (Triller *et al.*, 1990). In general, glycine receptors are concentrated opposite to presynaptic terminals, while $GABA_A$ are more diffusely distributed. This situation has been observed in the cerebellum of the cat, rat and monkey (Somogyi *et al.*, 1989). Moreover, in hippocampal pyramidal cells and in the lamina I neurons of adult rat spinal cord, the presence of extrasynaptic $GABA_A$ receptors seems to be responsible for the generation of slow inhibitory post synaptic currents (Chery & de Koninck, 1999; Banks & Pearce, 2000).

These data raise a large number of important questions regarding how a nerve terminal type is determined, how transmitters are packaged, and how postsynaptic densities are constructed to match terminal type (Nicoll & Malenka, 1998). The functional significance of this co-release to synaptic integration and motoneuron excitability is also uncertain.

3. GLYCINE AND GABA MODULATION OF MOTONEURONAL EXCITABILITY[4]

Inhibitory control of motoneuronal excitability is elaborated mainly by GABA and glycine receptor diversity, heterogeneous spatial distribution of receptors, and co-localization of GABA and glycine. The primary difference between GABAergic and glycinergic receptors lies in their response kinetics. In particular, $GABA_A$ receptor mediated responses decay more slowly and show greater desensitization with respect

[4] See for a review: Rekling, J.C. *et al.* , 2000.

to glycinergic ones, e.g., time constants of decay for $GABA_A$ and glycine component in spinal motoneurones are ~59 and ~16 ms, respectively (Gao & Ziskind-Conhaim, 1995; Jonas *et al.*, 1998). The physiological significance to motoneuronal excitability of the potential diversity of GABA and glycine receptors conferred by multiple subtypes, subunits, and posttranscriptional modification remains one of the major unanswered question of amino acid transmission.

3.1 ROLE OF GLYCINE AND GABA RECEPTOR DIVERSITY

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 $GABA_A$ receptors varies from 0.3 to 15 μM (Ducic *et al.*, 1995). Even if the importance of varing affinity of GABA receptors for the control of motoneuron excitability is not clear, it is postulated that, in hippocampal and cortical pyramidal neurons, it is critical for synchronizing the firing rate and coordinating neuronal interactions in columnary cortical activity (Costa, 1998).

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 the heterogeneity in the voltage dependence and desensitization properties of glycine responses (Rajendra *et al.*, 1997). The most obvious change in glycine receptor 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 significant α_2 subunit.

As reported in the previous section, the open time of recombinant α_2 receptors is much greater than for homomeric α_1 and native adult receptors (Takahashi *et al.*, 1992; Singer *et al.*, 1998). 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.

3.2 GLYCINE AND GABA IN NEONATAL RAT CNS: INHIBITORY OR EXCITATORY TRANSMITTERS?

As stated from the beginning of this chapter, in the adult mammalian CNS, glycine and GABA have been associated primarily with mediation of synaptic inhibition (Curtis & Johnston, 1974; Krnjevic, 1974). However, there are several ways in which synaptic inhibition can be produced, and this has lead to some confusion in the classification of GABA and glycine receptor actions on immature neurons.

The first intracellular recordings in the vertebrate CNS showed that activation of inhibitory neurons resulted in membrane hyperpolarization, leading to the suggestion that *inhibition resulted by driving the membrane potential further from the action potential threshold* (Coombs *et al.*, 1955). On the other hand, experiments in the crustacean nervous system showed that *inhibition could be produced also by an increase in membrane conductance that was associated with either no change in membrane potential or even depolarization* (Fatt & Katz, 1953; Kuffler, 1960; Takeuchi & Takeuchi, 1965). This kind of behavior has been observed also in adult cortical neurons where the activation of $GABA_A$ receptors can result in hyperpolarization, depolarization or no change in membrane potential, depending on the experimental conditions (Krnjevic & Schwartz, 1967; Dreifuss *et al.*, 1969; Connors *et al.*, 1988; McCormick, 1989), however producing, in nearly all cases, inhibition. Collectively, these observations indicate that *the direction of membrane potential change might not be the most important factor for the inhibitory process, but that the key element in synaptic inhibition might be the increase in membrane conductance.* This, in fact, will act to shunt the ability of excitatory potentials to depolarize the membrane to spike threshold, providing that the inhibitory equilibrium potential is below this value. It has to be stressed, however, that the depolarization induced by $GABA_A$ or glycine receptor activation sometimes can exert also an excitatory action. For example, in the newborn rat hippocampus, GABA is considered to be one major excitatory neurotransmitter (Cherubini *et al.*, 1991) acting synergistically with glutamate (Ben Ari *et al.*, 1997).

So, the role of GABA and glycine during early neuronal development is still debatable. In particular if we try to answer experimentally to the question "is this early depolarizing effect by GABA (or glycine) exerting an excitatory or inhibitory action on neonatal neurons?" we can found that data are controversial even from the

same region. As previously mentioned, in the newborn rat hippocampus GABA exerts a clear excitatory effect. Furthermore, GABA-evoked excitation can even be neurotoxic (Lukasiuk & Pitkanen, 2000). Conversely, on hippocampal pyramidal cells at postnatal (P) days 0-2 (P0-P2), depolarizing $GABA_A$ receptor-mediated transmission is thought to be strongly inhibitory on glutamatergic transmission (Lamsa *et al.*, 2000). On developing hypothalamic neurons the action of GABA is biphasic with early inhibition followed by facilitation of excitatory events (Gao *et al.*, 1998a). GABA-induced excitation is regarded as the primary process to drive action potentials (Gao & van den Pol, 2001). In the spinal cord GABA or glycine depolarizes P1-P3 motoneurons and cultured dorsal horn neurons (Reichling *et al.*, 1994; Wang *et al.*, 1994; Gao & Ziskind-Conhaim, 1995). Despite the depolarizing nature of these responses, they are thought to be inhibitory because of shunting of excitatory currents by the Cl^- conductance increase (Wu *et al.*, 1992; Gao & Ziskind-Conhaim, 1995; Singer *et al.*, 1998). Furthermore, GABA-mediated depolarization of primary afferent terminals is responsible for presynaptic inhibition (Eccles *et al.*, 1963). Even in the case of the spinal cord there are, however, reports of an excitatory role of GABA or glycine (Nishimaru *et al.*, 1996; Chub & O'Donovan, 1998). On young mouse spinal neurons, GABA apparently contributes more than glutamate to induction of action potentials (Gao & van den Pol, 2001). On brainstem motoneurons the effect of glycine or GABA shifts from depolarization to hyperpolarization during the first 2-3 postnatal weeks, together with changes in their receptor structure and associated channel kinetics (Singer & Berger, 2000; Rekling *et al.*, 2000). Parallel to these alterations, the reversal potential of glycinergic currents becomes very negative at P18 (Singer *et al.*, 1998). It is however uncertain if GABA or glycine can actually inhibit firing of brainstem motoneurons.

The shift from depolarizing to hyperpolarizing direction of GABA or glycine-mediated currents is related to the transmembrane Cl gradient, as it changes during development due to maturation of Cl transporters (Rivera *et al.*, 1999). In particular, in neonatal animals, the intense depolarizing action of $GABA_A$ or glycine receptor activation is due to the relatively high intracellular chloride concentration ($[Cl]_i$) of the immature neurons, that makes the reversal potential for this ion (E_{Cl}) significantly

more positive than the resting membrane potentials (Ben Ari *et al.*, 1989; Owens *et al.*, 1996; Brickley *et al.*, 1996; Chen *et al.*, 1996; Rohrbough & Spitzer, 1996).

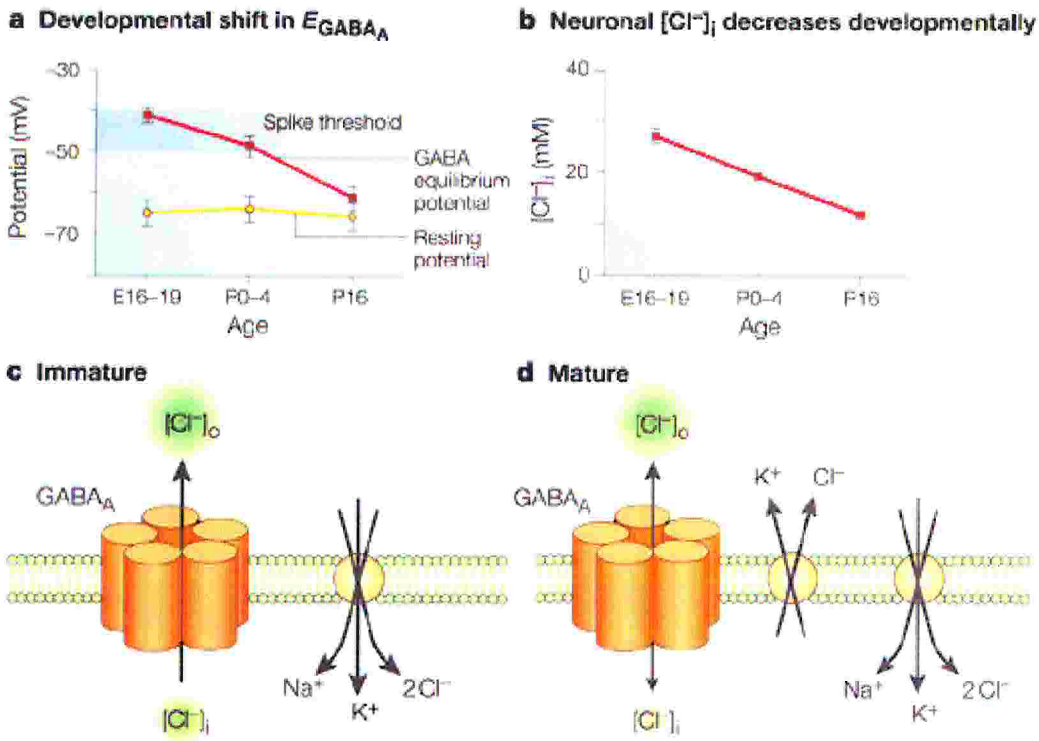

Fig.6: Developmental shift in GABA action due to a change in intracellular chloride concentration (taken from: Owens, D.F., Kriegstein, A.R. *Nat Rev Neurosci*, **3**, 715-727, 2002)

As development proceeds, neuronal $[Cl^-]_i$ decreases and the reversal potential for chloride becomes more negative, allowing the effect of GABA or glycine to become progressively hyperpolarizing (Owens *et al.*, 1996). Finally, it has to be again stressed that depolarization and excitation are not necessarily equivalent. Even when, for example, $GABA_A$ receptor activation can depolarize the membrane potential above spike threshold and excite a cell, it can be still able to shunt and inhibit other inputs (Gao *et al.*, 1998b; Lu & Trussell, 2001). Therefore, in the embryonic and early postnatal brain, when $GABA_A$ receptor activation can, by itself, be excitatory, the resulting change in conductance can modulate other excitatory inputs as well, by either inhibiting or facilitating them depending on the timing (Gao *et al.*, 1998b).

3.3 FUNCTIONAL ROLE OF GLYCINE AND GABA

Activation of postsynaptic inhibitory receptors via local interneurons within or near cranial and spinal motoneuron pools (Waldvogel *et al.*, 1990; Friedland *et al.*, 1995a; Friedland *et al.*, 1995b; Rampon *et al.*, 1996; Li *et al.*, 1997), in conjunction with

inhibitory projection neurons from the brain stem (Holstege, 1991; Holstege & Bongers, 1991), exerts various functions . First of all, it controls motoneuron responses to mono and polysynaptic afferent and descending inputs, second, it helps to establish motoneuron recruitment order and gain, and third, it shapes temporal and spatial patterns of activity in different motoneuron pools during reflexive and rhythmic behaviors.

The contribution of postsynaptic $GABA_A$ and glycine receptors to afferent reflex and descending inhibitory control of motoneuron activity, has been demonstrated in spinal motoneurons by stimulating dorsal roots (Jiang *et al.*, 1990; Wu *et al.*, 1992), ventral funiculi (Pinco & Lev-Tov, 1994), single afferent fibers, and premoto/interneurons (Jonas *et al.*, 1998). Also cranial motoneurons are subject to similar inhibitory control (Nakamura *et al.*, 1978;Takata, 1993;Kolta, 1997). However, considerable heterogeneity is apparent in these control systems, and, moreover, the relative contribution of GABA and glycine varies regionally between motoneuron pools and input pathways.

GABA and glycine mediated postsynaptic inhibition of motoneuron activity also contributes significantly to the production of rhythmic behaviors, including respiration (Merrill & Fedorko, 1984; Feldman, 1986), locomotion (Cazalets *et al.*, 1996; Kjaerulff & Kiehn, 1997), chewing (Chandler *et al.*, 1985; Inoue *et al.*, 1994), and swallowing (Bieger, 1991). These motoneuronal activities are characterized by sequential phases of excitation and inhibition. Inhibitory synaptic control of excitability during the active phase will affect the timing of burst onset and offset (affecting onset of muscle contraction and relaxation), firing pattern, and recruitment order (Robertson & Stein, 1988; Withington-Wray *et al.*, 1988; Parkis *et al.*, 1999). Inhibitory inputs during the quiescent phase in a motoneuron, e.g., during the expiratory phase in an inspiratory neuron, will reduce the probability of spurious, and inappropriate activation (Robertson & Stein, 1988).

Inhibition may also provide phase-specific control of motoneuron gain, as suggested by the observation of a phasic inspiratory inhibition of phrenic motoneurons that matches the shape and time course of their inspiratory excitatory drive (Parkis *et al.*, 1999). Because phrenic motoneurons (Grelot *et al.*, 1992), like most motoneurons,

participate in multiple behaviors, such a mechanism may contribute to the optimization of motoneuron excitability for different motor tasks.

Glycinergic inhibition of most spinal and some cranial motoneurons is also responsible for the atonia of rapid-eye-movement (REM) sleep (Chase & Morales, 1990; Kohyama *et al.*, 1994; Hishikawa & Shimizu, 1995). Among cranial motoneurons, the mechanism of inhibition varies during REM sleep. For example, in trigeminal motoneurons (Rampon *et al.*, 1996; Kohlmeier *et al.*, 1997), inhibition is glycinergic, whereas the inhibition of hypoglossal motoneuron activity results primarily from disfacilitation (Kubin *et al.*, 1993; Kubin *et al.*, 1996). The mechanisms underlying this differential control are incompletely understood, but they remain of considerable interest because of their potential involvement in conditions such as cataplexy, REM behavior disorder, and state-dependent respiratory disorders such as obstructive sleep apnea and perhaps sudden infant death syndrome.

4. MODULATION OF GLYCINERGIC AND GABAERGIC TRANSMISSION BY OTHER NEUROTRANSMITTERS

The release and action of glycinergic or GABAergic are controlled by various factors, among which neuromodulators have a predominant role. Neuromodulators can act both pre and postsynaptically via ionotropic or metabotropic receptors, operating through various pathways to fine tune inhibitory transmission.

Presynaptically, the Ca^{2+}-dependent release process of neurotransmitters can be modulated through auto or heteroreceptors, the first being activated by the neuron's own transmitter, the second by co-transmitter neuropeptides, transmitters released from adjacent terminals or locally produced factors. In the hippocampus, for example, it is known that kainate receptors modulate directly glutamate release (Chittajallu *et al.*, 1996), while in the basolateral amygdala they control the release of GABA (Braga *et al.*, 2003). We can find also several examples of transmitter release modulation via metabotropic pathways. In the neostriatum, in fact, presynaptic activation of muscarinic acetylcholine receptors (mAChRs) decreases the endogenous release of acetylcholine (Weiler *et al.*, 1984), while on dopamine neurons of the rat ventral tegmental area, agonists for metabotropic glutamate and muscarinic receptors act presynaptically to reduce the NMDA component of excitatory synaptic transmissions

(Zheng & Johnson, 2003). Postsynaptically, the modulation of neurotransmission takes place mainly through intracellular pathways (as described in section 1.1.4 and 1.2.4, for glycine and GABA receptors, respectively). These pathways can be triggered by metabotropic receptor activation, like in prefrontal cortex pyramidal neurons, where serotonine receptor activation inhibits GABA currents through receptor phosphorylation (Feng *et al.*, 2001).

On brain stem motoneurons, the model system used in the present study, neuromodulation by metabotropic receptors has been observed both pre and postsynaptically. In particular in the hypoglossus, it is known that serotonin (5-HT), acting presynaptically via 5-HT_{1B} receptors, inhibits excitatory glutamatergic synaptic currents (Singer *et al.*, 1996), and that *t*-ACPD, the broad spectrum agonist of metabotropic glutamate receptors (mGluRs), differentially modulates glycinergic and GABAergic transmission (Donato & Nistri, 2000). Also the muscarinic receptor family plays a role in this nucleus, as mAChRs depress excitatory synaptic inputs to hypoglossal motoneurons, via a presynaptic mechanism (Bellingham & Berger, 1996). On the other hand, in other systems like the chick lateral spiriform nucleus, mAChRs can act both pre and postsynaptically enhancing spontaneous GABA release and inhibiting electrically evoked GABAergic synaptic transmission (Guo & Chiappinelli, 2001).

Previous studies have shown that mAChR activation strongly effects spike firing on hypoglossal motoneurons by modulating certain K^+ conductances responsible for spike frequency accomodation (Viana *et al.*, 1993; Lape & Nistri, 2000). Since mAChR activity reduces excitatory inputs to HMs (Bellingham & Berger, 1996) and yet it facilitates firing, it is apparent that, against these two contrasting effects, inhibitory transmission becomes crucial to bias the overall result of mAChR activity towards excitation or inhibition. The present project, thus, investigated the potential modulation of inhibitory synaptic mechanisms by mAChRs on hypoglossal motoneurons.

5. MODULATION OF GLYCINERGIC AND GABAERGIC TRANSMISSION: THE ROLE OF MUSCARINIC RECEPTORS

Muscarinic receptors mediate most of the inhibitory and excitatory effects of acetylcholine (ACh) on central neurons (Krnjevic, 1974; Buckley & Caulfield, 1992). In general, receptors for acetylcholine were first divided into two pharmacological classes by Sir Henry Dale in 1914. Some responses to ACh could be mimicked by the alkaloid muscarine and were termed muscarinic, whereas other responses were mimiked by nicotine and were termed nicotinic. It is now recognized that nicotinic and muscarinic receptors represent two different gene families which are members of two different receptor superfamilies with different mechanisms of action, and which probably evolved independently to bind the same neurotransmitter. The action of acetylcholine at the muscarinic acetylcholine receptor (mAChR) was responsible for the identification of Ach as the first neurotransmitter. In the 1920s Loewi demonstrated that stimulation of the vagus nerve caused the release of a substance that, when put on an isolated heart, resulted in a decreased heart rate. This substance had the biochemical and pharmacological properties expected of ACh.

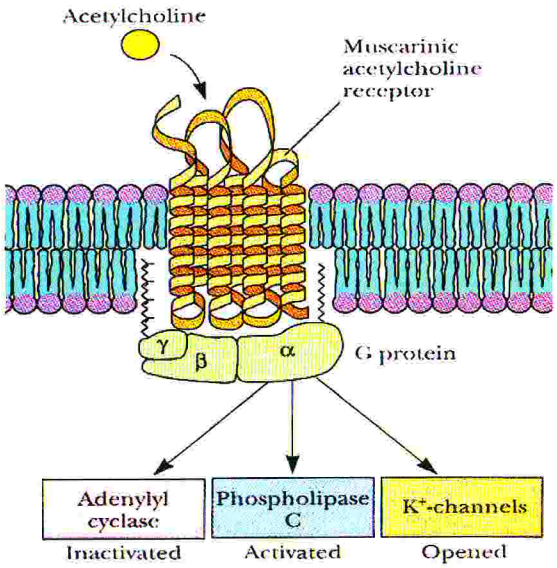

Fig.7: Schematic representation of muscarinic receptor.

Unlike nicotinic ACh receptors, which belong to the LGICS, muscarinic ACh receptors (mAChRs) are structurally classified as members of the G-protein coupled receptor superfamily (GPCRS). These receptors, when activated by an agonist, exert their effect by coupling to a G-protein, thereby activating GDP-bound G_α subunits by

promoting loss of GDP and binding of GTP (with uncoupling of receptor/β-γ complex and dissociation of agonist). The activated G_α subunits then initiate the response, either by stimulating an enzyme to generate a "second messenger", or producing a response by interacting more directly with an effector, such as an ion channel (for a review see: Birnbaumer *et al.*, 1990).

Like other G-protein coupled receptors, the peptide sequence of mAChRs has seven putative transmembrane domains (TM1-TM7), constituted largely of hydrophobic amino acids, and three hydrophilic intracellular loops (i1-i3), the last of which (i3) is at least in part involved in coupling of the receptor to the G-protein (Hulme *et al.*, 1990; Jones *et al.*, 1992).

5.1 MUSCARINIC RECEPTOR SUBTYPES AND COUPLING MECHANISMS

For many years after Dale's initial studies, it was thought that, despite the diverse actions mediated by muscarinic receptors, only a single type of mAChRs existed. The existence of multiple subtypes of these receptors was not generally accepted until the beginning of 1980s, when it was demonstrated that the antagonist pirenzepine had a higher affinity for a subset of mAChR in the nervous system than it did for mAChR in the heart or other tissues (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). More detailed studies have subsequently shown the existence of additional, pharmacologically distinct forms of mAChRs, and with the application of molecular biology techniques, at least five different genes coding for putative muscarinic receptors were identified in humans and other mammals (reviewed in: Hulme *et al.*, 1990).

Subtype	No. of amino acids (human/rat)	G-protein family	Tissue with high expression
M_1	460/460	G_q	Cortex, striatum
M_2	466/466	G_i/G_o	Heart, cerebellum
M_3	590/589	G_q	Exocrine glands, smooth muscle
M_4	479/478	G_i/G_o	Striatum
M_5	532/531	G_q	Substantia nigra

Table1 : Subtypes of muscarinic receptors and the G-protein to which each subtyper preferentially couples.

These subtypes are termed M_1, M_2, M_3, M_4 and M_5, and they can be divided into two distinct subgroups with respect to their sequence similarity, consistent with their differences in receptor signalling. The first group comprises M_2 and M_4 receptors, which couple preferentially to the pertussis toxin-sensitive G_i/G_o family of G proteins leading to inhibition of adenylyl cyclase (AC, fig. 8B). In addition, in certain cell types, coupling of the M_2 and M_4 receptors to G_i family members results in the activation of some phospholipase C (PLC) β isoforms and adenylyl cyclase isoforms by βγ-mediated mechanisms. The second group is formed by M_1, M_3, and M_5 receptors, which couple preferentially to the pertussis toxin-insensitive G_q family of G proteins, leading to the preferential activation of other subtypes of PLC-β by an α subunit-mediated mechanism (fig. 8A). In addition, when expressed at high levels, both classes of mAChR can couple to G_s and cause activation of AC.

The activation of PLC leads to the production of diacylglycerol (DAG), which can activate protein kinase C (PKC), and inositol trisphosphate (IP_3), which causes the release of calcium from intracellular stores. In turn, the increase in intracellular calcium concentration can have many diverse actions: activation of calmodulin-dependent protein kinases, activation of calmodulin-dependent adenylyl cyclases, activation of calmodulin-dependent phosphodiesterases, and activation of nitric oxide synthase. Nitric oxide, in turn, can act both on the cell by which it is produced and can diffuse to neighbouring cells to activate guanylyl cyclase[5].

An important feature of mAChRs is their ability to regulate of ion channel activity by both second messenger-dependent and independent pathways. For example, activation of M_2 receptors in the heart can activate GIRK (G protein-coupled inward rectifying) potassium channels by a βγ-mediated mechanism and produce inhibition of both a hyperpolarization-activated cation current and calcium channels by cAMP-dependent mechanisms. In sympathetic ganglion neurons, both M_1 and M_4 receptors can cause inhibition of calcium channel activity. The M_4 receptors utilize a second messenger-independent pathway, due to the interaction of the channels with βγ subunits, while M_1 receptors use an as yet unidentified second messenger.

[5] See for a review on muscarinic coupling mechanisms: Caulfield, M.P. (1993). Muscarinic Receptors – Characterization, Coupling and Function, *Pharmac. Ther.* **58**, 319-379. Brown, D.A. *et al.* (1997). Muscarinic mechanisms in nerve cells, *Life Sci.* **60** (13/14), 1137-1144.

Muscarinic receptors, like other GPCRs, can activate additional signal transduction pathways. Both G_q and G_i coupled receptors can, through a series of adapter proteins and the protooncogene p21 *ras*, activate the mitogen-activated protein (MAP) kinase cascade. Muscarinic receptors can also activate the stress-activated protein (SAP) kinase pathway. Both the MAP kinase and the SAP kinase pathways result in the phosphorylation and activation of various transcription factors and the regulation of cell growth, apoptotic and differentiation pathways (Caulfield, 1993).

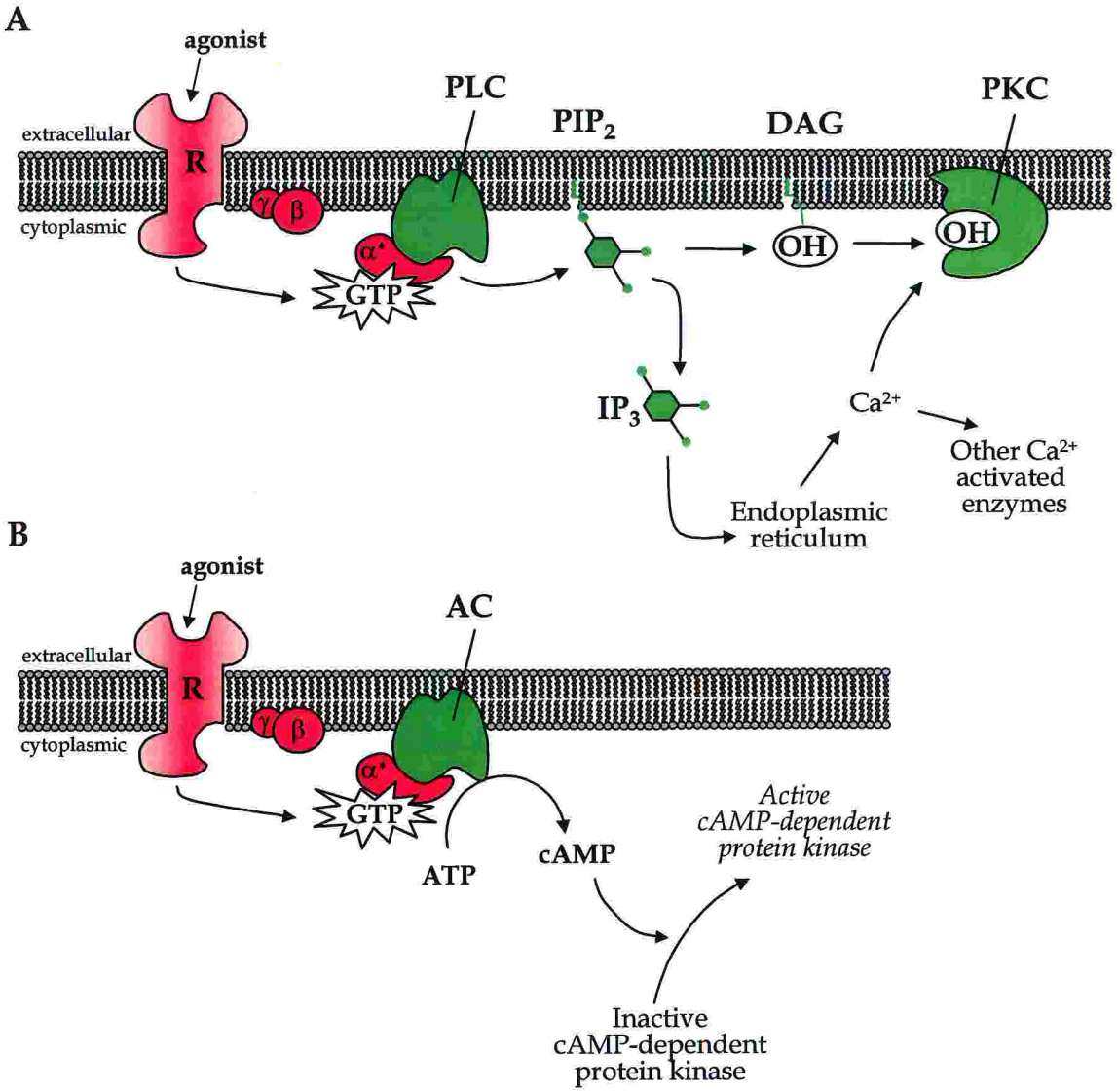

Fig.8: Schematic representation of two G-protein activated pathways (adapted from: Nicholls J.G., Martin A.R., Wallace B.G. (1992) From neuron to brain, 3rd ed. Sinauer)

Muscarinic receptors can also activate a variety of nonreceptor tyrosine kinases, such as members of the Src family of protein kinases, and cause phosphorylation and activation of receptor tyrosine kinases such as the epidermal growth factor receptor

(Felsch *et al.*, 1998); this pathway has been implicated in the regulation of ion channel function and may also affect cell growth and differentiation.

5.2 ACTIONS OF MUSCARINIC RECEPTORS IN THE CNS

Muscarinic receptors have been implicated in many processes in the CNS: various paradigms involving memory and learning, such as passive avoidance learning, active avoidance, and representational and spatial learning. Administration of muscarinic antagonists can interfere with many cognitive tasks. Pharmacological studies suggest that the M_1 receptor is particularly important in learning and memory (Messer, Jr. *et al.*, 1990). A defect in cholinergic transmission at mAChRs is thought to play a major role in the cognitive deficits seen in patients with Alzheimer's disease (Levey, 1996).

Muscarinic receptor subtypes are distributed across brain stem respiratory regions including the ventrolateral medulla (Kinney, 1995b; Mallios *et al.* 1995), and they are involved in central respiratory control (Burton, 1994, 1995; Gillis 1988; Murakoshi 1985; Nattie and Li 1990; Weinstock *et al.* 1981). mAChRs have potent excitatory effects on medullary respiratory neurones and respiratory motoneurones, and are likely to contribute to changes in the central chemosensitive drive to the respiratory control system (Bellingham & Berger, 2002). In particular, defects in the ventral medullary muscarinic system may play a role in disorders of respiratory control such as sudden infant death syndrome (SIDS) (Kinney *et al.*, 1995a). Central cholinergic mechanisms contribute to respiratory failure caused by organophosphate poisoning (Lotti 1991). Perturbations of ACh synthesis, release, degradation, or activation of ACh receptors in the brain stem result in perturbations of respiratory pattern both in vivo (Foutz *et al.* 1987; Gillis *et al.* 1988; Nattie and Li 1990) and in vitro in an en bloc brain stem-spinal cord preparation from neonatal rats (Burton *et al.* 1994, 1995; Monteau *et al.* 1990; Murakoshi *et al.* 1985).

Muscarinic receptors have been implicated in the induction of rapid eye movement (REM) sleep (Jones, 1991), and they may also regulate circadian rhythms (Liu *et al.*, 1997).

Muscarinic receptors also act both in the spinal cord and at supraspinal sites to modulate nociception. Muscarinic receptors play an important role in the regulation of function of the basal ganglia. M_1 and M_4 receptors on medium spiny neurons

modulate dopaminergic transmission in the striatum (Di Chiara *et al.*, 1994). For example, muscarinic antagonists inhibit the catalepsy (loss of voluntary movement) induced by administration of dopamine D_2 receptor antagonists such as haloperidol. Muscarinic receptors have also been implicated in a wide variety of other processes, including central nervous system-mediated hypotension and bradycardia, generation of epileptic seizures, and drinking behaviour. The recent availability of mice deficient in specific mAChR subtypes (Hamilton *et al.*, 1997) should facilitate elucidation of the role of the various receptor subtypes in specific physiological and behavioural responses.

6. A MODEL SYSTEM FOR INHIBITORY TRANSMISSION: MOTONEURONS OF THE HYPOGLOSSAL NUCLEUS.

Since the first studies by Sherrington (reviewed in: Sherrington, 1947), motoneurons were the paradigmatic neurons of the brain. Many fundamental and general properties of neurons and synaptic transmission were first identified in motoneurons, e.g., quantal release, inhibitory transmission, and the consequent conclusion that chemical neurotransmission is the principal form of interneuronal communication. In the past decade, however, interest in motoneurons has decreased, while, on the other hand, intense investigation into other regions of the brain has lead to an encyclopedic identification of neuronal properties not typically associated with motor control (for example long-term potentiation observed in hippocampal neurons and proposed to be a component of learning).

One difficulty in properly understanding all this information is that many of these well-characterized properties have been identified in neurons in the absence of data concerning how these neurons process signals in actual behavior. In this sense, motoneurons have a special advantage, since we know precisely the information coding of their output signals (contraction of the innervated muscle fibers) and, in many cases, their activity during complex behaviors (either indirectly by observing movements or recording muscle activity, or directly by recording from their axons or their cell bodies). All these features provide a unique context for understanding and interpreting data.

A suitable model system in which it became interesting to study GABAergic and glycinergic chloride-mediated transmission, is the hypoglossal nucleus. This nucleus, which is made up to 90% by motoneurones, is a brain stem structure, located in the lower medulla. It innervates the tongue muscle, and thus plays a role in vital physiological functions like swallowing, suckling, or mastication (Lowe, 1980). Moreover, due to the critical position of tongue in upper airways, hypoglossal motoneurons (HMs) are also important for respiration.

6.1 NUCLEUS OF HYPOGLOSSUS

Most of the motoneurons innervating the lingual musculature originate from the hypoglossal nucleus and send their axons to the hypoglossal nerve (XII cranial nerve). These muscles can be both extrinsic and intrinsic. The first type comprises the genioglossus (GG), the styloglossus (STY), the hyoglossus (HY), the geniohyoid (GH) and the palatoglossus. Vertical, transverse, superior longitudinal, and inferior longitudinal musculature form the second type.

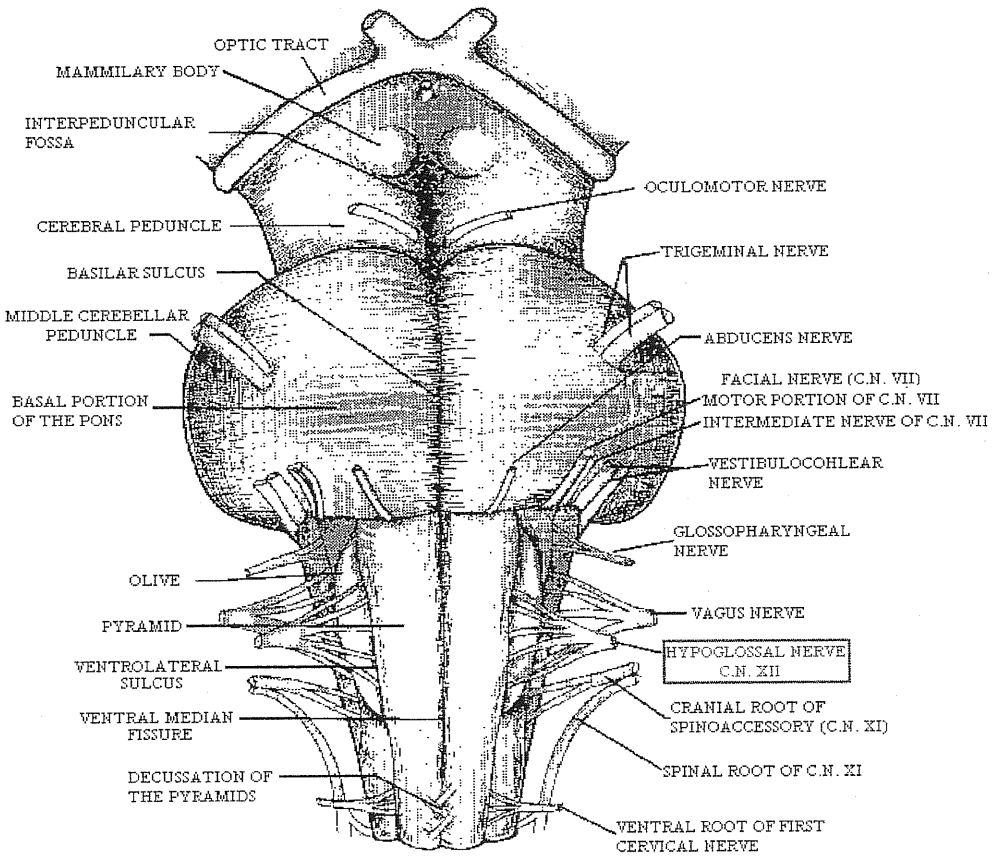

Fig.9: Ventral view of the brain stem, with the cranial nerves.

Each hypoglossal nucleus (nXII) contains approximately 3500 neurons (Lewis *et al.*, 1971). Most cells (>90%) within the nXII are motoneurons but there is anatomical evidence for a small population of interneurons (Boone & Aldes, 1984;Takasu & Hashimoto, 1988). Motoneurons can vary their cytoarchitectonic characteristics of size, shape and dendritic orientation, in different regions of the hypoglossal nucleus (Cooper, 1981; Kitamura *et al.*, 1983; Boone & Aldes, 1984). In particular, cells in the dorsal division appear fusiform, oriented along the medio-lateral axis, and range from 17 to 40 μm; cells in the central region of the nucleus are more globular in shape (17-30 μm in diameter), while those of the ventral division are multipolar and somewhat larger (35-50 μm). Looking at interneurons in the hypoglossal nucleus, they appear smaller (10-18 μm) than motoneurons, have fewer dendritic processes, and are confined to the ventrolateral or dorsolateral borders of the nucleus (Boone & Aldes, 1984).

The hypoglossal nucleus receives afferent projection from several regions. Although there are no direct cortical projections to it (Travers & Norgren, 1983), cells in the midbrain, pons and medulla send their axons to HMs. These projections originate both from well defined cell groups in identified brain stem nuclei and from more widely distributed, poorly defined regions of the reticular formation (Cooper & Fritz, 1981; Travers & Norgren, 1983; Borke *et al.*, 1983).

AIMS

The present study aimed at addressing the following issues:

- The effect of endogenously released glycine and GABA, and exogenously applied glutamate, on the excitability of neonatal hypoglossal motoneurons.
- The modulatory role exerted by mAChRs on glycinergic and GABAergic spontaneous and evoked transmission.
- The pre or postsynaptic localization of muscarinic receptors involved in modulation of inhibitory transmission.
- The muscarinic receptor subtypes involved in the above mentioned effect.

METHODS

"Lasciate fare a me... spesso dove la violenza e la forza bruta falliscono possono vincere l'astuzia e l'intelligenza!
Puo' darsi Rat! Ma non abbiamo proprio il tempo di chiamare qualcun altro... "

(Rat Man, "Rat Man contro il ragno")

1. SLICE PREPARATION AND IDENTIFICATION OF HMs

Experiments were performed on preparations isolated from neonatal Wistar rats (0-5 days old) terminally anesthetized with 0.2 ml urethane (10% i.p. injection). The entire procedure, described earlier by Viana *et al.* (1994), is in accordance with the regulations of the Italian Animal Welfare Act and approved by the local authority veterinary service. After animal decapitation the brainstem was quickly removed and placed in modified ice-cold Krebs solution (see below). A tissue block containing the lower medulla was then fixed (with insect pins) onto an agar block inside a Vibratome chamber (series 1000, TPI, St. Louis USA) filled with ice-cold Krebs solution (bubbled with 95% O_2 5% CO_2), to cut 200μm thick slices.

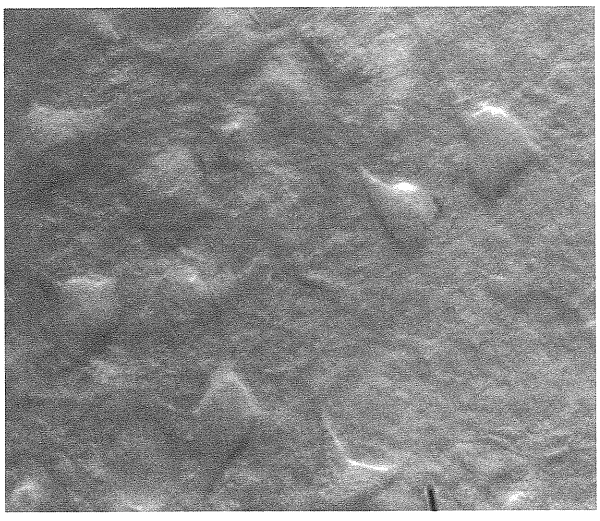

Fig.10: Hypoglossal motoneurons from P2 rat brain stem slice.

Slices were first transferred in to an incubation chamber for 1 hour at 32°C under continuos bubbling and subsequently maintained at room temperature for at least 1

hour before use. After this regeneration period brainstem slices were placed in a small recording chamber, continuously superfused (2-5 ml/min) with the recording Krebs solution (see below) and viewed with a Zeiss Axioscope microscope connected to an infrared video camera, in order to identify single HMs within the nXII. Under x40 magnification HMs were clearly visible and showed a fusiform large soma (30-60 μm diameter) with 1-3 major dendrites (fig.10).

2. SOLUTIONS AND DRUGS

- SLICE CUTTING AND MAINTENANCE. For slice preparation and maintenance the solution contained (in mM): NaCl 130, KCl 3, Na_2HPO_4 1.5, $CaCl_2$ 1, $MgCl_2$ 5, $NaHCO_3$ 25, glucose 10 (pH 7.4, 290-310 mOsm).
- CURRENT CLAMP RECORDINGS. For current clamp recordings the extracellular solution contained (in mM): NaCl 130, KCl 3, Na_2HPO_4 1.5, $CaCl_2$ 1.5, $MgCl_2$ 1, $NaHCO_3$ 25, glucose 10 (pH 7.4 with NaOH, 300-320 mOsm). Patch pipette solution contained (in mM): K-CH_3-SO_4 110, KCl 20, NaCl 7, $MgCl_2$ 2, HEPES 10, BAPTA 10, ATP-MG 2, sucrose 2 (pH 7.2 with KOH, 280-300 mOsm).
- VOLTAGE CLAMP RECORDINGS. The same extracellular solution used for current clamp experiments was employed. Patch pipettes contained (in mM): KCl 130, NaCl 5, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, BAPTA 10, ATP-MG 2, sucrose 2 (pH 7.2 with KOH, 280-300 mOsm). In some experiments CsCl 130mM was used instead of KCl (using CsOH instead of KOH to adjust pH).
- LIQUID JUNCTION POTENTIALS. All potential values were corrected off-line for the liquid junction potential (Vj) between external and internal solution (Neher, 1992). Vj was measured with a 3 M KCl-agar bridge and was equal to 10mV for solutions used in current clamp experiments, while it was about 0mV for solution used in voltage clamp experiments (in this case no correction was applied).
- DRUG APPLICATION. Drugs were applied in two different ways: either bath-applied via the extracellular solution superfused at 2-5 ml/min (for a minimum of 5-10 min to reach apparent equilibrium conditions), or applied by means of pressure pulses. For the latter method, a thin-walled glass micropipette was pulled by a two-stage puller (3P-A, List Medical, Germany), to obtain a DC resistance of 6-10 MΩ (when filled with the patch electrode solution). The pipette,

visually positioned about 50 μm away from the HM soma of superficial motoneurons, was filled with the selected drug (diluted to the final concentration in the external recording solution), and connected to a Pneumatic Picopump (WPI, Sarasota, FL, USA).

In voltage clamp experiments, unless otherwise stated, 2mM kynurenic acid was routinely added to the recording solution to block ionotropic glutamatergic currents. The following drugs were used: AFDX116, atropine methylnitrate (atropine), bicuculline methiodide (bicuculline), carbamylcholine chloride (carbachol), 4-DAMP, GABA, glycine, glutamate, 4-hydroxyquinoline-2-carboxylic acid (kynurenic acid), muscarine chloride (muscarine), pirenzepine, strychnine hydrochloride (strychnine), tetrodotoxin (TTX), tropicamide.

3. RECORDING TECHNIQUES

- PATCH CLAMP RECORDING

The conventional whole-cell patch clamp technique (Hamill *et al.*, 1981) was employed. Briefly, a small heat-polished pipette, pulled from thin–walled borosilicate glass capillaries with a two-stage puller (3P-A, List Medical, Germany), to a DC resistance of 3-5 MΩ, was pressed against the cell membrane, forming an electrical seal with resistance of the order of 50 MΩ. Gentle suction, applied to the pipette interior, led to an increase in series resistance (1-10 GΩ). After the giga-seal formation an additional suction applied to the pipette interior led to the membrane rupture, and access to the cell interior.

An L/M PCA patch-clamp amplifier (List Medical, Germany) was used for voltage clamp experiments, while an Axoclamp-2B amplifier (Axon Instruments) was utilized for current clamp experiments. The first one was not suitable for current clamp experiments, since compensation of pipette resistance was not available in current clamp operation mode. Moreover, it has been shown that most amplifiers designed for voltage clamp experiments give distortions to fast occurring events like action potentials (Magistretti *et al.*, 1996). Cells were clamped at -60, -70 mV holding potential (V_h), and series resistance (5-25 MΩ) was routinely monitored, without any compensation. In current clamp mode the bridge was routinely balanced. Voltage or current pulse generation and data acquisition were performed with a PC using

pClamp 8.1 software (Axon Instr. Inc.). All recorded currents were filtered at 3 kHz and sampled at 5-10 kHz.

- EXTRACELLULAR STIMULATION

Glycinergic or GABAergic postsynaptic currents were evoked by placing a single bipolar tungsten electrode in the lateral reticular formation to stimulate afferent interneurons. Stimuli of variable intensity and duration (10-100 V, 0.02-0.2 ms) were delivered at 15 s interval. Evoked synaptic currents were then stored in a PC as individual files and averaged with pClamp 8.1 after discarding failed events.

4. DATA ANALYSIS

Cell input resistance (R_{in}) was calculated by measuring the current response to 5 or 10 mV hyperpolarizing pulses (from –60, -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). Single postsynaptic currents were detected using AxoGraph 4.6 (Axon Instruments, Foster City, CA) software, while Sigma Plot (Jandel Scientific, San Rafael, CA) and Clampfit (Axon Instruments) softwares were used for linear regression analysis of experimental data. Results were quantified as means ± SE; statistical significance was assessed with the Student's paired or unpaired *t*-test, or with the Kolmogorov-Smirnov test for probability distributions; $P < 0.05$ was considered as the acceptable level of statistical significance.

RESULTS

"Memento homo!"

(G. Taccola)

1. FUNCTIONAL ROLE OF GLYCINE AND GABA

The clear depolarizing action exerted by glycine and GABA on neonatal rat HM (Donato, 2000), raises the question about the effect of these two transmitters on cell excitability. Previous studies performed in our laboratory indicated that, on HMs, exogenous application of glycine or GABA (100 μM) prevents cells from firing in response to a current pulse injection.

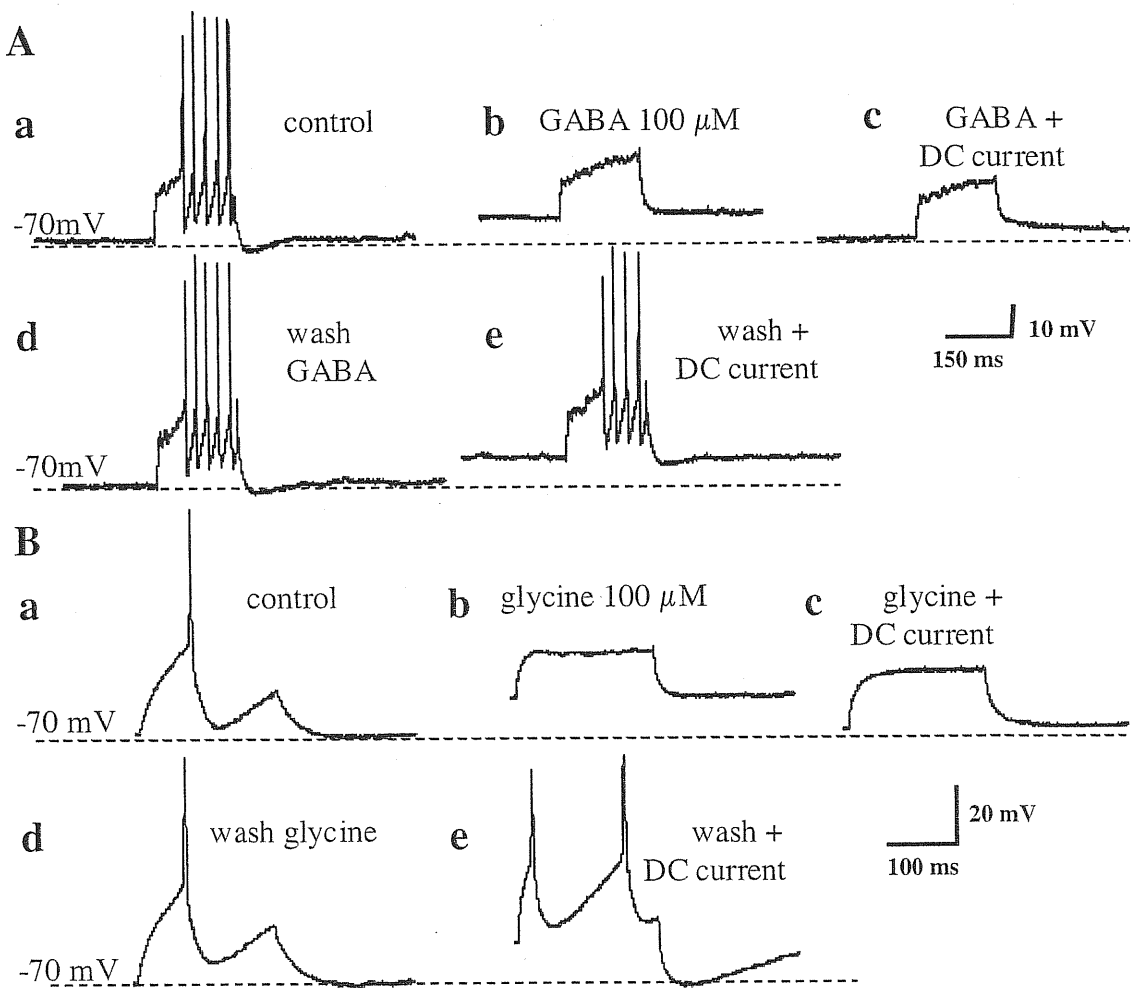

Fig.11: Exogenous application of GABA and glycine inhibits firing of hypoglossal motoneuron. (Taken from: R.Donato, 2000, Relative contribution by GABA or glycine to Cl

mediated synaptic transmission on neonatal rat hypoglossal motoneurons in vitro, *PhD thesis*, SISSA).

This inhibition was ascribed to cell membrane shunting by the large input resistance fall due to opening of Cl^- channels (Donato, 2000). However, the glycinergic or GABAergic signal used for these experiments was not the physiological one. Hence the resulting inhibition might due to activation of non-synaptic receptors normally not involved in synaptic activity. To elucidate this point better it became necessary to carry out further experiments as detailed below.

1.1 EXOGENOUS APPLICATION OF NEUROTRANSMITTERS

First, it was important to check whether the inhibition induced by bath applied GABA (or glycine) was caused by membrane depolarization with partial inactivation of voltage sensitive Na^+ channels and consequent depression of motoneuron firing. This possibility was examined by comparing the effect of bath-applied glutamate with that of GABA (or glycine) whereby both glutamate and GABA evoked the same degree of membrane depolarization.

Thus, in current clamp experiments cells were induced to fire action potentials (APs) in response to small current pulses injection (fig. 12Aa, 250 pA, 100 ms). Bath application of 50 μM glutamate evoked 6mV depolarization associated with an increase in firing probability (same current pulse as in control conditions, fig. 12Ab), without any significant change in input resistance measured from the amplitude of hyperpolarizing electrotonic potentials (not shown; on average 103±6%, $p>0.05$). On a sample of five cells the average depolarization induced by 50 μM glutamate was 5.0±1.8 mV from –70.5±0.3 mV resting membrane potential. After repolarizing the cell membrane potential to resting level, cell firing returned to control (fig. 12Ac), which was maintained after glutamate washout (fig. 12Ad).

The same protocol was repeated in the presence of strychnine (0.4 μM) and bicuculline (10 μM; fig. 12Ba-d) when increased firing was again observed during glutamate application (n=2) indicating that the excitatory action of glutamate was direct and did not involve release of endogenous GABA or glycine. These depolarizing responses to glutamate are very similar to the depolarization evoked by GABA (or glycine) (Donato, 2000) which, however, induced strong inhibition of cell firing.

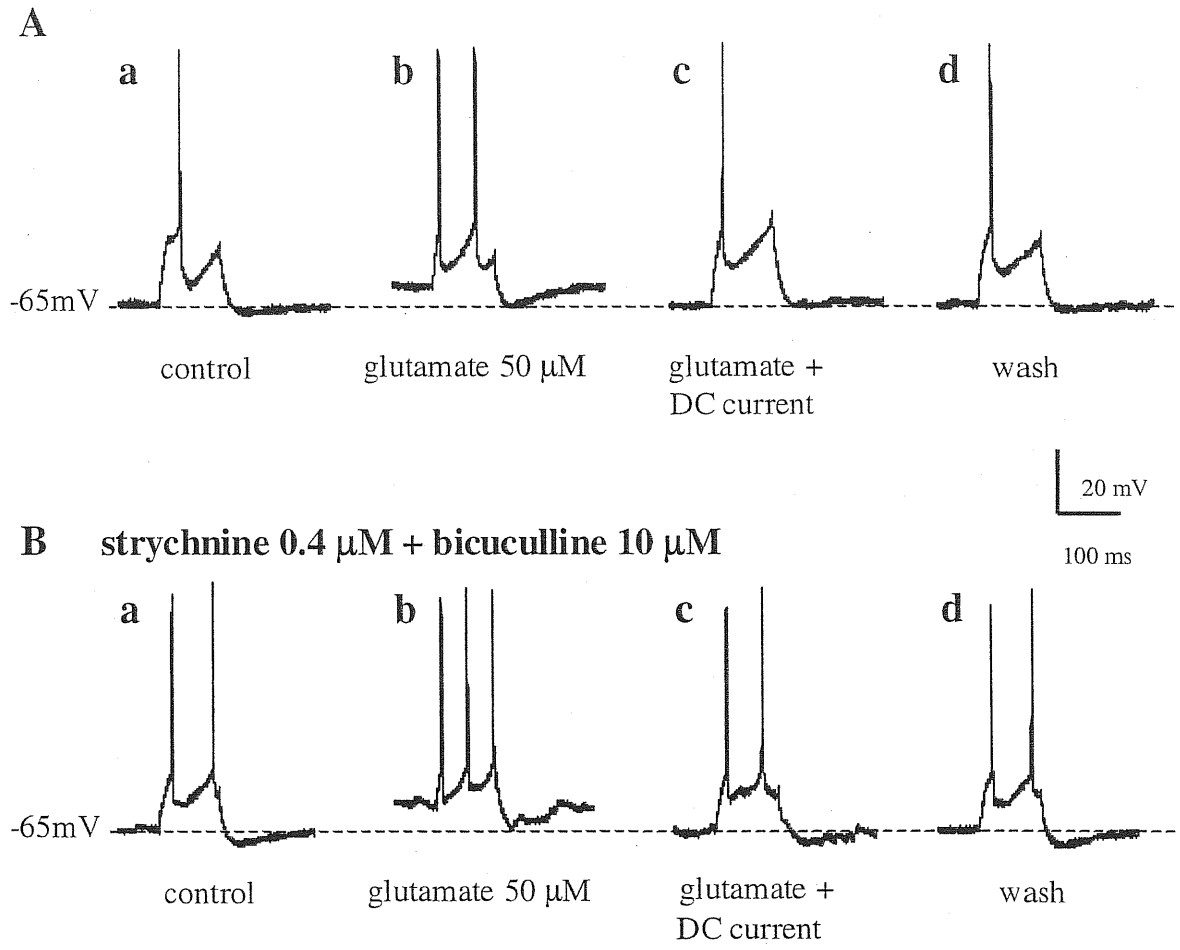

Fig.12: Exogenous application of glutamate does not inhibit motoneuron firing.
(A) a: control firing in response to small current step (0.25 nA, 100ms). b: after adding glutamate (50 μM), cell membrane potential is depolarized (6 mV), and firing in response to the same current step is doubled. c: after repolarizing cell membrane potential with DC current injection, in the continuous presence of glutamate, motoneuron recovers its original firing pattern. d: after glutamate wash out, cell injected with current step fires again as in control. (B) a-d: the same protocol as in A is repeated on a different cell in the presence of strychnine and bicuculline. Similar behaviour as in A is observed.

1.2 ENDOGENOUS RELEASE OF GABA AND GLYCINE

To explore if GABAergic or glycinergic transmission was indeed inhibitory on hypoglossal motoneurons, I evoked synaptic GABAergic or glycinergic responses (fig. 13Ab, 14Ab) by electrically stimulating the lateral reticular formation (Umemiya & Berger, 1995; Donato & Nistri, 2000, 2001) and tested how they might affect the firing pattern induced by small current pulse injection. The glycinergic or GABAergic component was isolated by adding to the extracellular medium either 10 μM bicuculline or 0.4 μM strychnine, known to be transmitter selective at these concentrations (Donato & Nistri, 2000).

As shown by the example in fig. 13, in the presence of bicuculline, the cell fired one AP in response to the intracellularly-injected current pulse (fig. 13Aa, control condition). A single pulse applied to the lateral reticular formation suppressed this firing (fig. 13Ac) as long as the synaptic response was timed to occur immediately before the action potential (see downward deflection representing stimulus artifact). Examples of glycinergic events evoked at 0.1 Hz are superimposed in fig. 13Ab (the white trace is the average from 10 events). The time course of the effect of glycinergic events on cell firing is illustrated in fig. 13B (same cell as in fig. 13A). In this test, the probability to fire one action potential was 70 % without synaptic stimulation (i.e., the cell fired 7 times out of 10). In coincidence with lateral reticular stimulation (0.1 Hz), the cell never fired in response to current pulse injection (0 % firing probability). Upon stopping synaptic stimulation, firing promptly resumed and occurred for each depolarizing current injection. A subsequent period of synaptic stimulation again suppressed firing.

Analogous results were obtained in the presence of strychnine, as shown in fig. 14. In fact, in response to current injection, this cell fired two action potentials (fig. 14Aa) in control conditions and only one when GABAergic synapses were activated at the same time of the intracellular current injection (fig. 14Bc). A larger delay in spike latency was also observed in fig. 14Ac with respect to fig. 14Aa (46.0 ± 4.0 ms vs. 37.2 ± 3.5 ms; n=25 events; $p<0.05$). Again the reproducibility of the effect of GABAergic postsynaptic potentials on cell firing is illustrated in fig. 14B (same cell as in fig. 14A). Because there was no apparent difference in the effectiveness of glycinergic or GABAergic or mixed synaptic events in inhibiting spike activity, data were pooled together for analysis. Thus, inhibition was assessed as either a decrease in firing probability and/or an increase in latency of firing. In 5 out of 6 cells, there was a significant increase (20.5±4.2%, $p<0.01$) in latency of the first action potential while in the remaining cell firing was fully suppressed. In four cells the latency change was also associated with a decrease in firing probability (from 95±2% to 10±3%). In a subset of 5 motoneurons lateral reticular stimulation could evoke mixed GABAergic-glycinergic responses which were, however, ineffective in inhibiting or facilitating firing.

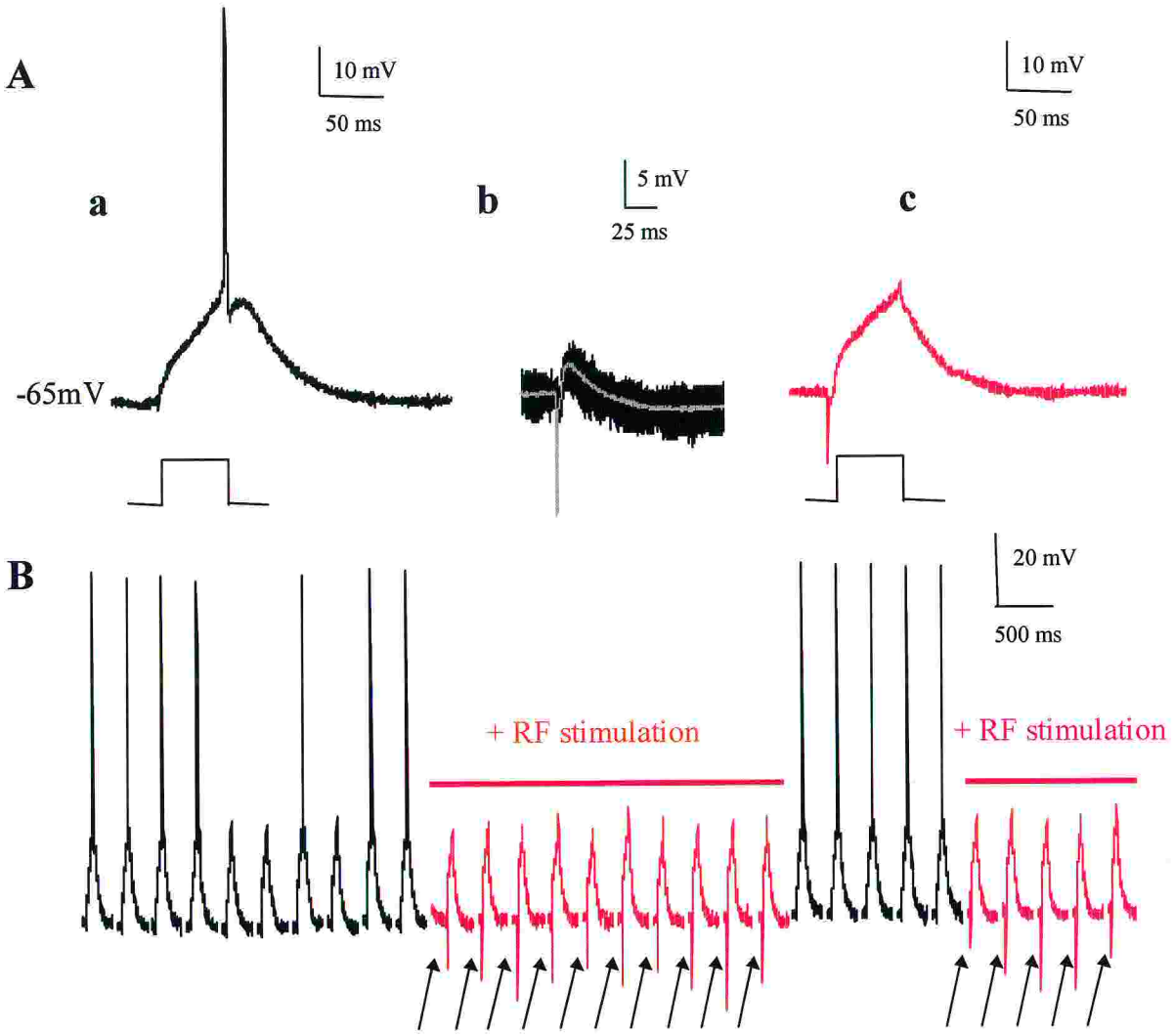

Fig.13: Endogenous release of glycine reduces motoneuronal excitability.
Experiments performed in the presence of kynurenic acid and bicuculline to isolate glycinergic transmission. (A) a: cell response to a current pulse injection (0.17 nA, 50 ms; schematized below the trace). b: cell responses to reticular formation stimulation (100 V, 0.06 ms). Ten superimposed traces (black records) with their average (gray trace) are shown. c: concurrent stimulation of reticular formation and current pulse injection lead to inhibition of firing. (B) Responses to a train of injected current pulses are displayed (same cell as in A). Initially the cell fires 7/10 times (left) while, in the case of concurrent reticular formation stimulation (middle), the cell never fires. The motoneuron completely recovers its ability to fire once control conditions are restored (right) while a subsequent test with combined synaptic stimulation again suppresses firing (arrows indicate the stimulation artefact).

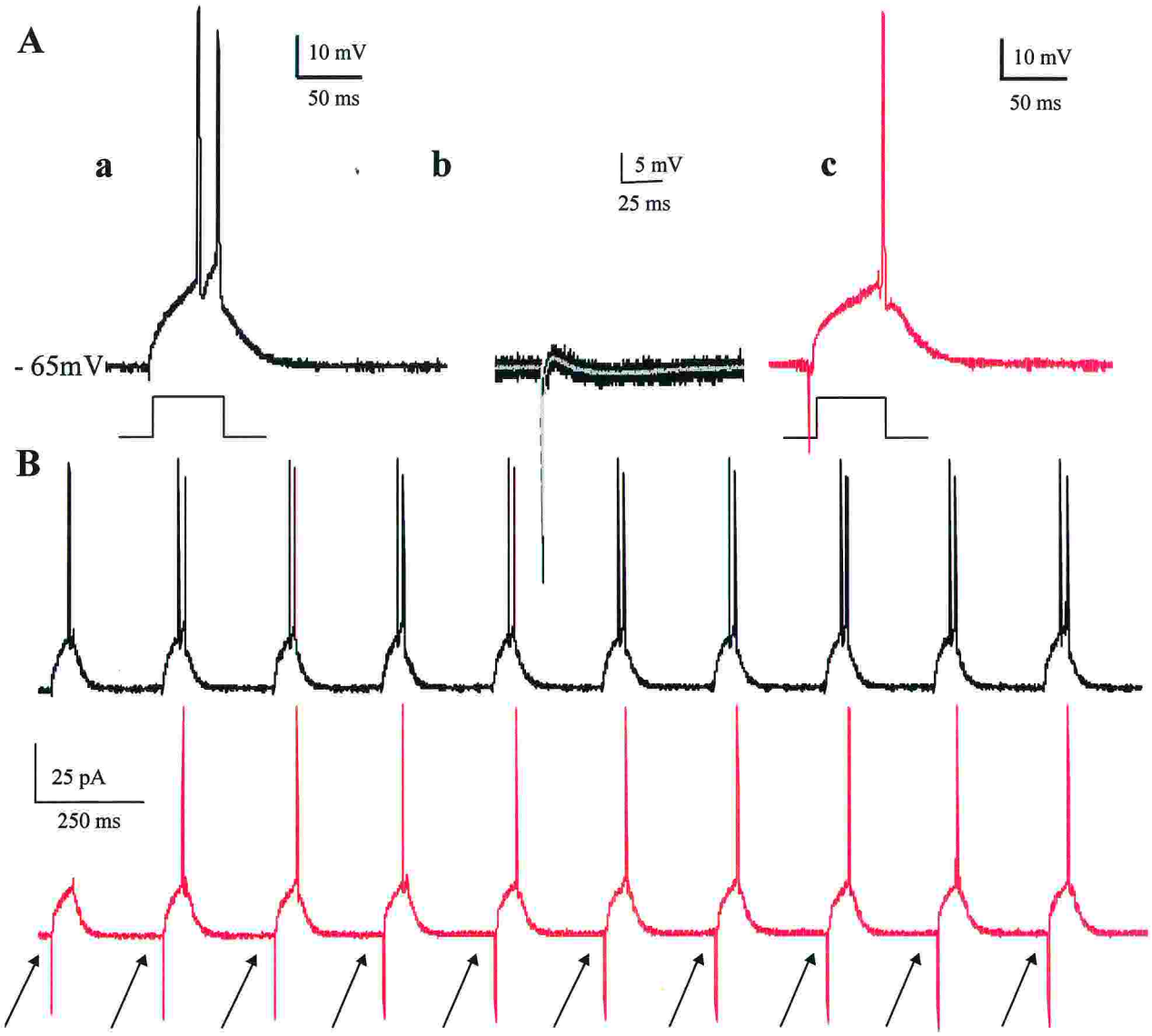

Fig.14: Endogenous release of GABA reduces motoneuronal excitability.
(A) Experiments performed in the presence of kynurenic acid and strychnine to isolate GABAergic transmission. a: cell response to a current pulse injection (0.20 nA, 50 ms; schematized below the trace). b: cell responses to reticular formation stimulation (100 V, 0.2 ms). Ten superimposed traces (black records) with their average (gray trace) are shown. c: again, firing is reduced with respect to control when the current pulse injection is superimposed to the synaptic response. Note the increase in latency of the AP in c with respect to a. (B) Responses to a train of injected current pulses are displayed (same cell as in A). In control condition the cell fires two AP for each current injection (upper traces) while, in the case of concurrent reticular formation stimulation (lower traces), the cell fires only one (or no) AP in response to the same current injection (arrows indicate the stimulus artefact).

2. GLYCINE AND GABA RECEPTOR MODULATION

Once the functional inhibitory role of glycine and GABA on HMs was confirmed, I started studying the modulation exerted on these two transmitter systems by the metabotropic mAChRs. First of all, I tested the effect of muscarine, a selective agonist of mAChRs, on hypoglossal motoneuron basic characteristics. Then, I studied the muscarinic receptor-mediated modulation of synaptically released glycine or GABA, by trying to identify the mAChR subtypes involved in this process.

2.1 MUSCARINE EFFECT ON BASIC PROPERTIES OF HMs

In current clamp mode 10 μM muscarine depolarized the HM membrane potential by 5.8 ± 0.8 mV from –66.1±0.8 mV resting value (n=8, see example in fig. 15A). This depolarization was coupled with a significant increase in cell input resistance, as summarized in fig. 15C (R_{in} became 117 ± 4% of the control, $p<0.05$, 6 cells out of 8). Moreover, in cells in which spontaneous postsynaptic potentials (sPSPs) were clearly detectable, muscarine application induced an increase in both their frequency and amplitude, 243 ± 65% and 128 ± 14%, respectively (n=4, $p<0.05$, fig. 15B-C). Muscarine effects persisted, at least partially, even after manual repolarization of membrane potential to resting value with DC current injection (potential amplitude and frequency became 131.5 ± 24.6 % and 174.2 ± 35.2 % of control value, respectively), indicating that the increase in synaptic activity was not due simply to the muscarine-induced depolarization.

Muscarinic receptor activation affected also HM excitability. In the example in fig. 15D, in response to small current pulse injections to the soma, the cell fired two APs (fig. 15Da). In presence of muscarine (10 μM), the same current pulse evoked four APs and this effect persisted, at least partially, even when the cell membrane potential was repolarized to the resting value (fig. 15Db-c). After muscarine washout the cell recovered its original firing pattern (fig. 15Dc). On an average of four cells, muscarine reduced the latency of the first spike to 53.9 ± 2.5 % of the control value.

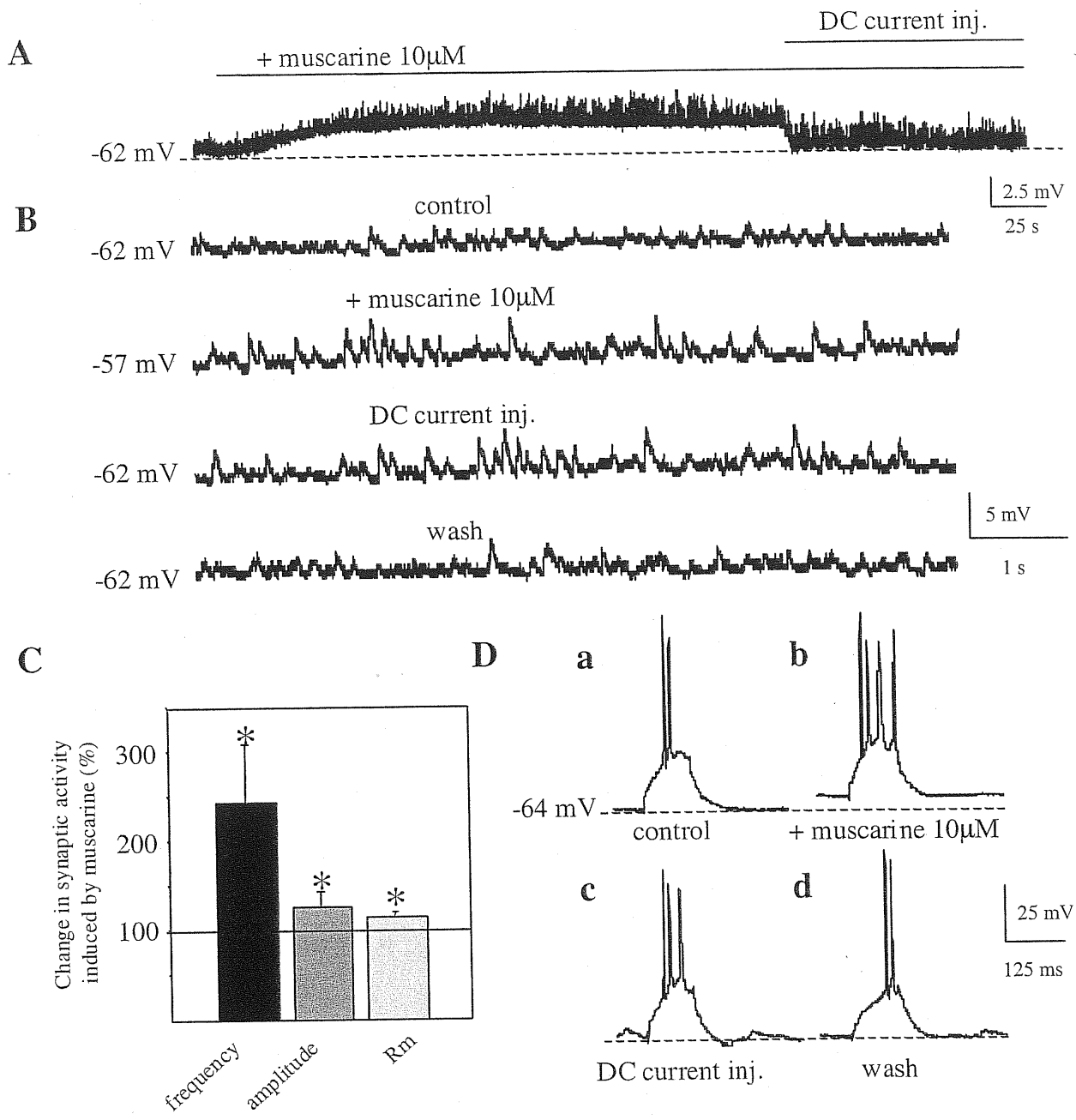

Fig. 15: Muscarine effect on hypoglossal motoneuron basic properties.
(A) Current clamp recording from a motoneuron. Bath-applied muscarine (10 µM) induces a small depolarization (4.4±0.1 mV) and an increase in both amplitude and frequency of sPSPs (163±11% and 155%, respectively). (B) The same trace as in A is showed on a faster time base. (C) Muscarine-induced fractional changes in frequency and amplitude of sPSPs and in cell input resistance, for a pool of 8 and 6 motoneurons, respectively. (D) In response to a small current pulses injection (100 pA x 100 ms) HM fires two action potentials. In the presence of 10 µM muscarine the same current pulse evokes four action potentials, indicating an increase in cell excitability. This increase persists when the cell is repolarized to resting value with DC current injection.

2.2 *MUSCARINIC MODULATION OF SPONTANEOUS INHIBITORY TRANSMISSION*

My previous results showed that mAChRs activation could increase frequency and amplitude of synaptic transmission on HMs. In those experiments, however, no distinction between excitatory and inhibitory transmission was made. While the effect of mAChR activity on glutamatergic transmission has been reported (Bellingham & Berger, 1996), to further investigate the modulatory effect of muscarine on glycinergic or GABAergic currents, I performed a series of voltage clamp experiments, in which kynurenic acid was routinely added to the external solution (to block glutamatergic ionotropic transmission), and bicuculline or strychnine was again used to isolate glycinergic or GABAergic transmission, respectively.

2.2.1 SPONTANEOUS POSTSYNAPTIC CURRENTS

First of all, in the continuous presence of kynurenic acid, bath-application of 10 μM muscarine to HMs clamped at V_H=–60 mV, reversibly induced a slowly developing inward current (-42 ± 10 pA), without any significant change in input resistance (103.4 ± 4.0 % of control value, $p > 0.05$, n=21).

In the continuous presence of 10 μM bicuculline, glycinergic transmission was recorded as postsynaptic currents, inwardly directed due to the symmetrical chloride transmembrane concentration. An example of such recordings is depicted in fig. 16A (upper trace), showing fast inward-directed glycinergic inhibitory postsynaptic currents (IPSCs), with a mean amplitude of -79.0 ± 1.8 pA and a frequency of about 7.5 Hz in control conditions. On this cell, bath application of 10 μM muscarine induced a marked reduction in sIPSC amplitude and frequency that became 38.7 ± 1.0 pA and 3.7 Hz, respectively (fig. 16A, lower trace).

GABAergic transmission showed the same kind of response to muscarine (fig. 16B). In this example, muscarine application reduced the amplitude and frequency of the slower, inwardly-directed GABAergic sIPSCs to 31.5 ± 2.4 pA and 0.99 ± 0.12 Hz, respectively (control values were 55.4 ± 1.8 pA and 1.22 ± 0.21 Hz). On a pool of six motoneurons the muscarine-induced reduction in amplitude and frequency of glycinergic sIPSCs was 59.2 ± 15.9 % and 63.9 ± 12.2 %, respectively (fig.17B, $p<0.05$). On a pool of five motoneurons the muscarine-induced reduction in amplitude and

frequency of GABAergic sIPSCs was 60.5 ± 9.8 % and 71.0 ± 21.6 %, respectively (fig.17B, $p<0.05$). In either case (glycinergic or GABAergic events), muscarine did not change the kinetics of the IPSCs (fig. 17Aa-b, right traces).

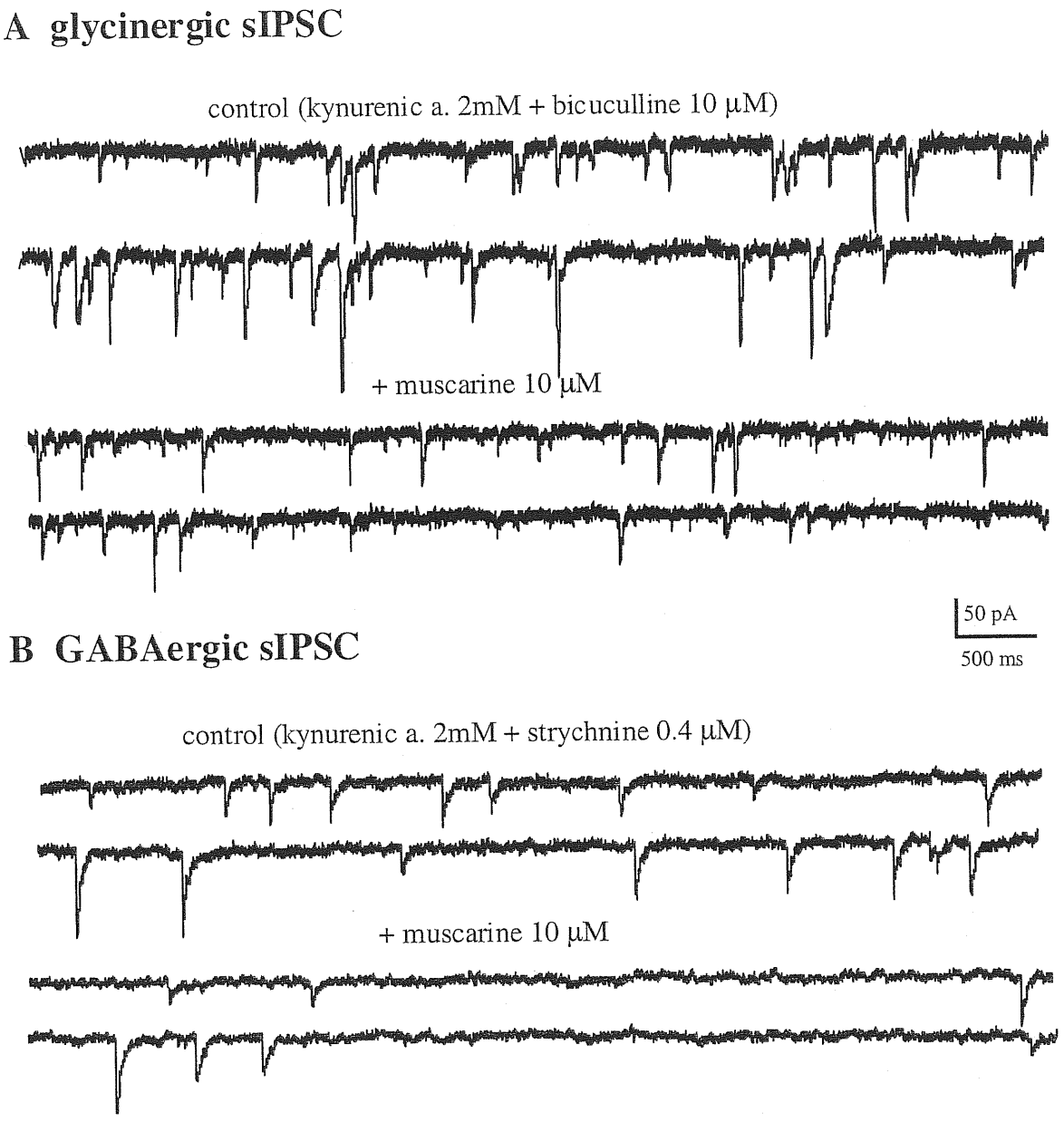

Fig.16 : Muscarine effect on spontaneous glycinergic or GABAergic currents.
(A) Spontaneous glycinergic currents, recorded in presence of kynurenic acid and bicuculline, before and during bath-application of 10 µM muscarine (upper and lower traces respectively). (B) Spontaneous GABAergic currents, recorded in presence of kynurenic acid and strychnine, before and during bath-application of 10 µM muscarine (upper and lower traces respectively, different cell from A). Calibration bars are the same for both panel A and B.

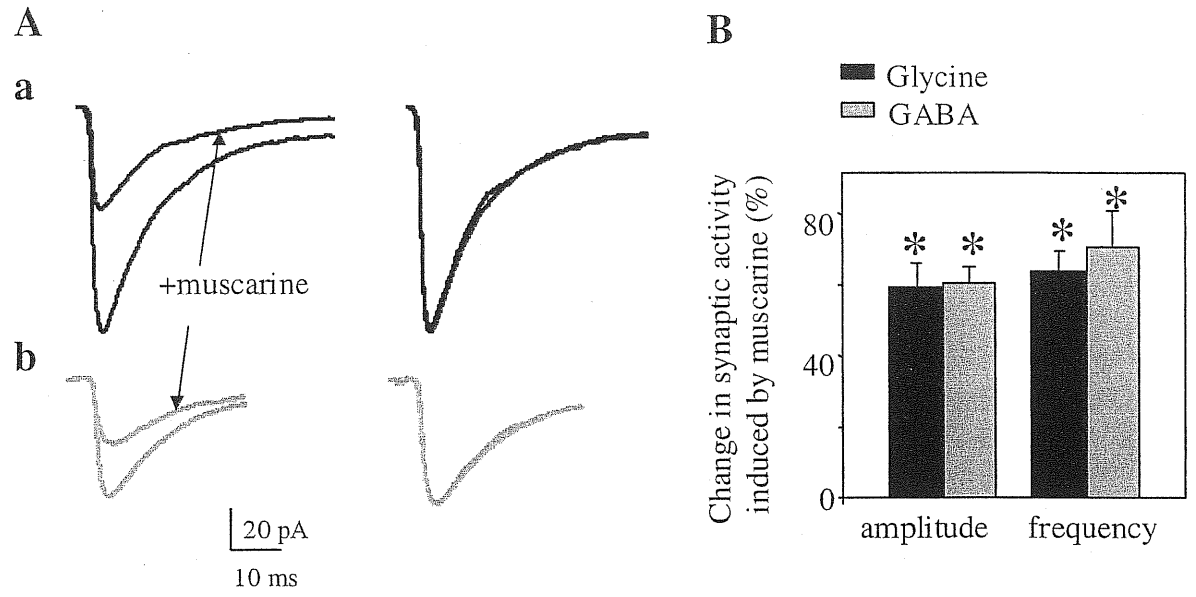

Fig.17: (A) Glycinergic (a) and GABAergic (b) average currents (n>150 events) in control conditions and during muscarine application (left traces, same cells as in fig. 16A and 16B). On the right, the same traces are normalized and superimposed. (B) Histogram summarizing muscarine effect on amplitude and frequency of spontaneous currents. Application of 10 μM muscarine significantly reduces the amplitude of glycinergic and GABAergic currents to 59.2 ± 15.9 % and 60.5 ± 9.8 %, respectively ($n \geq 5$ for each transmitter, $p<0.05$). The frequency of events is also significantly decreased to 63.9 ± 12.2 % (glycine) and 71.0 ± 21.6 % (GABA) of control values ($n \geq 5$ for each transmitter, $p<0.05$).

2.2.2 MINIATURE EVENTS

Spontaneous transmission is composed of network independent and network dependent events. To assess if muscarine-induced depression of chloride-mediated synaptic transmission took place at the level of synaptic terminals or at network level, I performed experiments with 1 μM tetrodotoxin (TTX), which allowed recording of miniature inhibitory postsynaptic currents (mIPSCs). Examples of such recording are reported in fig. 18 for both glycinergic and GABAergic transmission.

Fast glycinergic mIPSCs were only slightly affected by muscarinic receptor activation. In the motoneuron shown in fig. 18A, the current amplitude shifted from -38.9 ± 2.3 pA in control condition (upper traces), to -32.8 ± 2.8 pA during muscarine application (lower traces). The corresponding histogram and cumulative probability distribution for current amplitude are depicted in fig.19A, showing significant muscarine-induced depression ($p<0.05$). On average, muscarine reduced glycinergic miniature events amplitude to 87.5 ± 9.4 % of the control value ($p<0.05$, $n=7$, fig. 19C). For the same

pool of motoneurons no average variation in the frequency of events was observed (p= 0.521, n=7, fig. 19C).

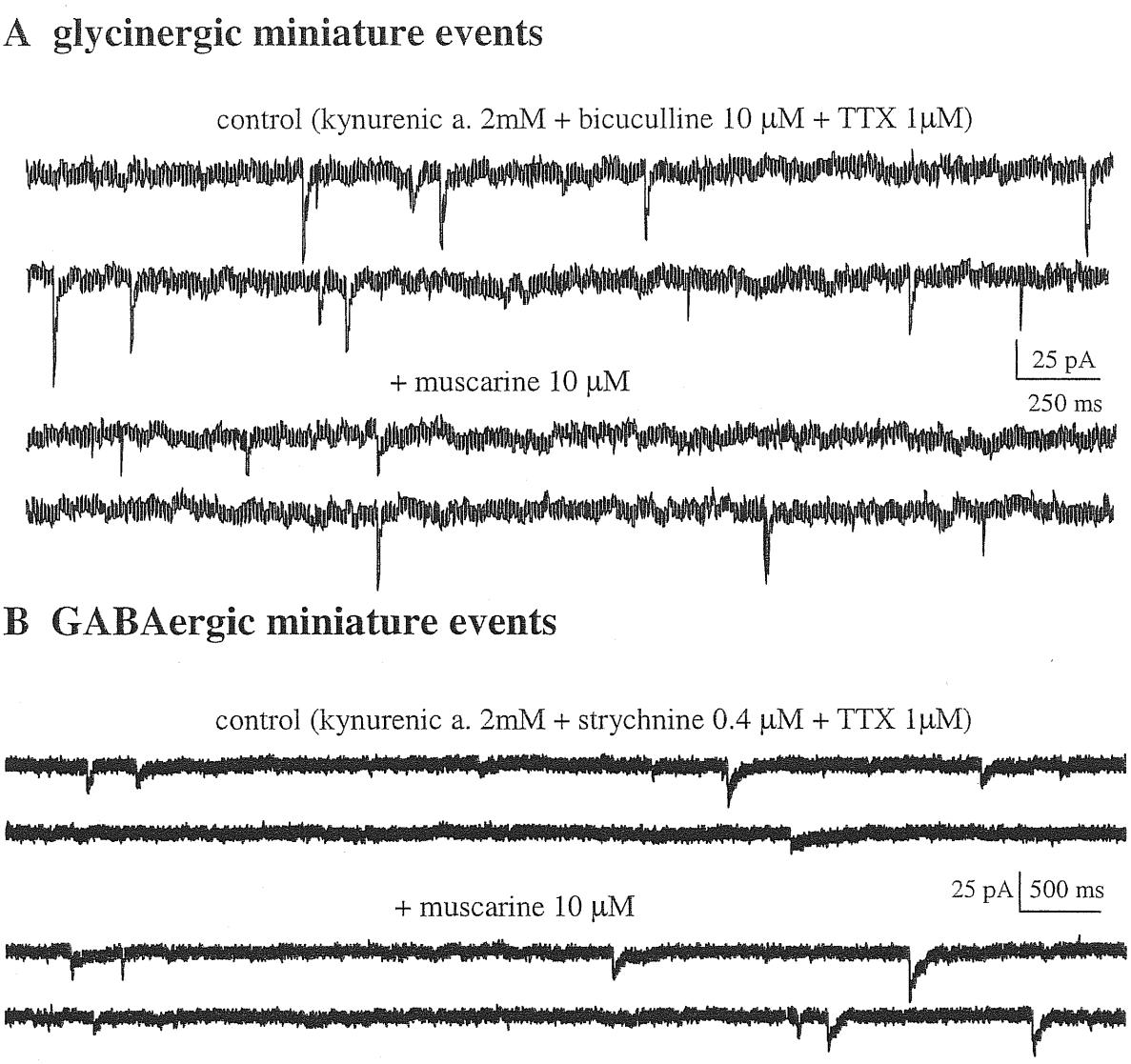

Fig.18: Muscarine effect on glycinergic or GABAergic miniature events.
(A) Glycinergic miniature events, recorded in the continuous presence of kynurenic acid and bicuculline. Current recorded from one motoneuron in control condition and during muscarine bath-application. (B) GABAergic miniature events, recorded in the continuous presence of kynurenic acid and strychnine. Current recorded from one motoneuron (different from A) in control condition and during muscarine application.

On the other hand, when I looked at the GABAergic transmission, I found that mIPSCs were left unchanged by muscarine application, both in amplitude and in frequency, as shown (for mIPSCs amplitude) in the histogram and cumulative probability distributions in fig. 19B (same cell as fig. 18B). The average changes in

amplitude and frequency of GABAergic mIPSCs were 106.1 ± 9.3 % and 113.9 ± 29.0% of control values, respectively (n=3, p>0.05, fig. 19C).

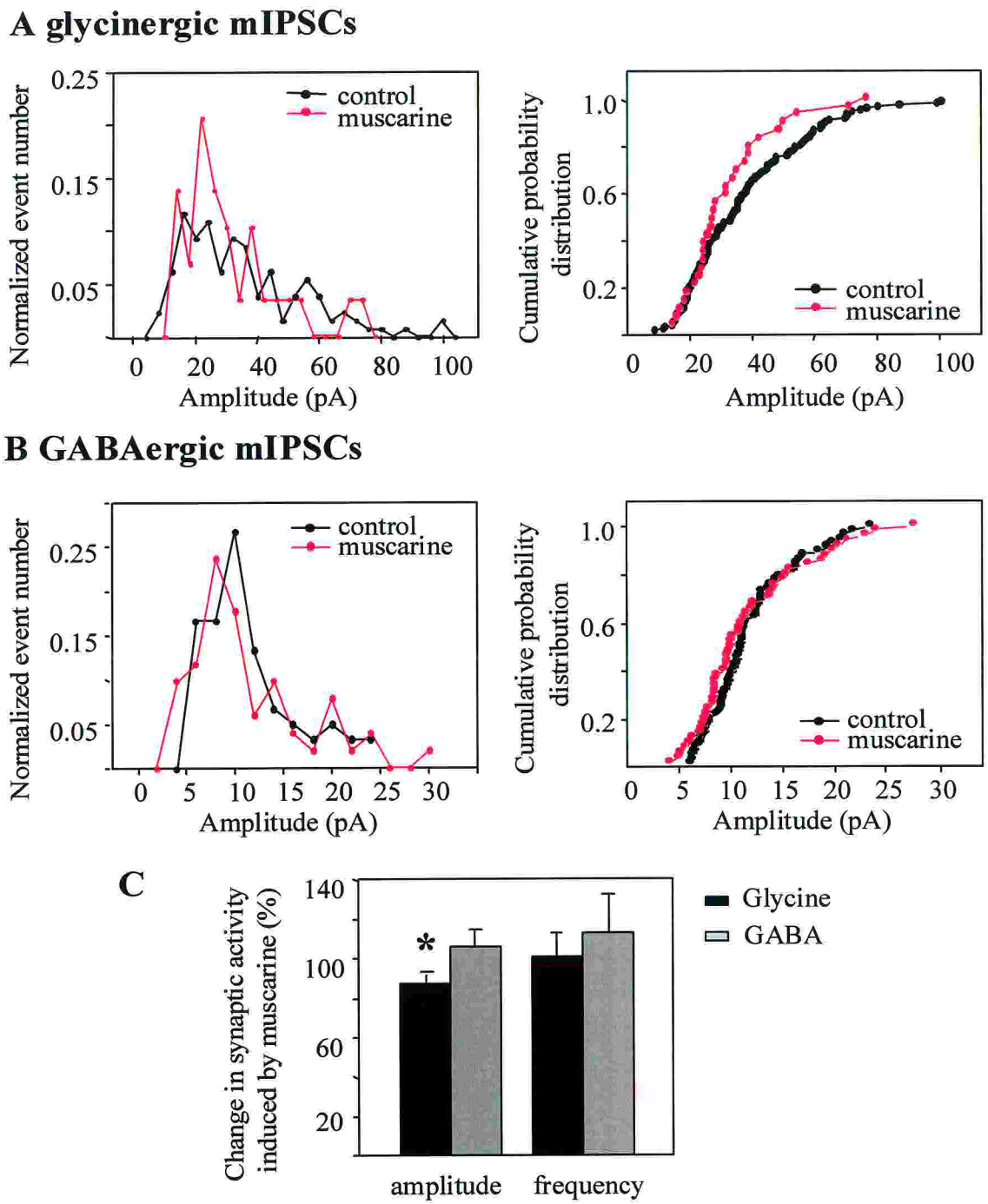

Fig.19 : (A) Histogram and cumulative probability distribution for amplitude of glycinergic mIPSC from the same motoneuron shown in fig. 18A. A significant reduction was observed during the application of 10 µM muscarine. (B) Histogram and cumulative probability distribution for amplitude of GABAergic mIPSC from the same motoneuron shown in fig. 18B. No variation was observed during the application of 10 µM muscarine (C) Muscarine significantly reduces the amplitude of glycinergic miniature events to 87.5 ± 9.4 % of the control value (n=7, p<0.05), while it leaves unchanged their frequency (100.9 ± 29.3 % of control value, n=7, p>0.05). GABAergic miniature events are not affected by muscarine application (amplitude and frequency are 106.1 ± 9.3 % and 113.9 ±29.0 of control, n=3, p>0.05).

2.2.3 EFFECT OF SELECTIVE mAChR ANTAGONISTS ON SPONTANEOUS TRANSMISSION

The weak effect exerted by muscarine on miniature events suggested that its major contribution to the depression of sIPSCs originated from network-located mAChRs. Since these receptors may be tonically activated by endogenous acetylcholine, I investigated if their pharmacological block could produce any effect on spontaneous inhibitory transmission. As discussed in details in the introduction, mAChRs are subdivided into five subtypes, M1-M5. Pirenzepine (2 μM), AFDX116 (500 nM), DAMP (1 μM) and tropicamide (1 μM) were used as antagonists towards M1, M2, M3 and M4 receptor subtypes, respectively. No selective antagonist against the M5 receptor subtype was commercially available.

Fig. 20 summarizes the effect of each subtype selective antagonist on glycinergic sIPSCs.

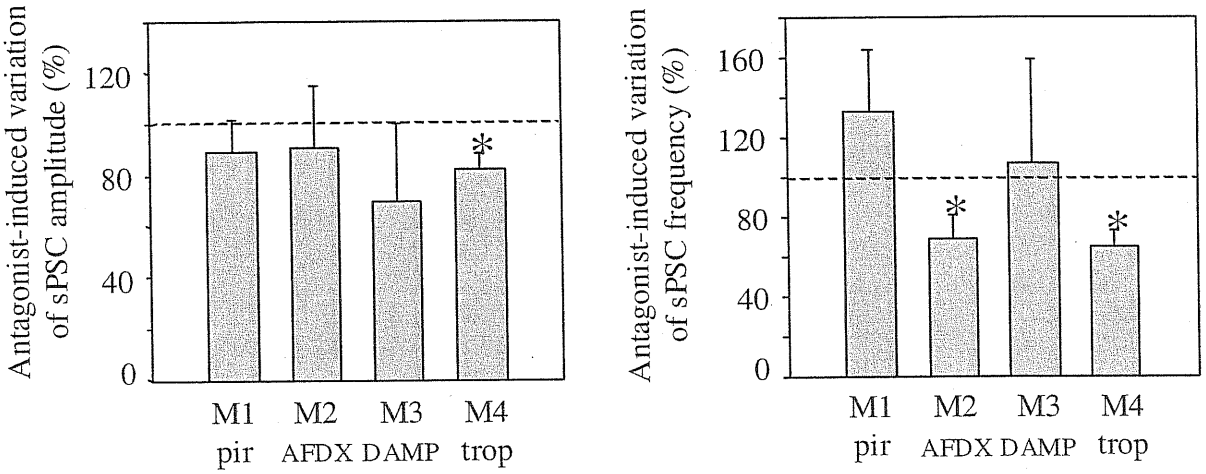

Fig.20: Effect of mAChR selective antagonists on glycinergic spontaneous transmission. Only tropicamide (antagonist for M_4 receptor subtype) and AFDX-116 (antagonist for M_2 receptor subtype) modulate sIPSCs.

On a sample of four motoneurons, bath application of 1μM tropicamide (M_4 selective) significantly reduced the amplitude and frequency of sIPSC (83.24 ± 6.10 % and 65.52 ± 7.63 % of control values, respectively, $p<0.05$, fis. 20).

On a different sample of cells, bath application of 500 nM AFDX-116 (M_2 selective) reduced solely the frequency of sIPSCs (68.73 ± 12.61 % of control value, n=4, $p<0.05$, fig.20). Antagonists for M_1 and M_3 receptor subtypes were ineffective in producing any significant variation in spontaneous transmission ($p>0.05$, $n \geq 4$ for each

antagonist, fig. 20). The situation was quite different as far as GABAergic spontaneous transmission was concerned (fig. 21).

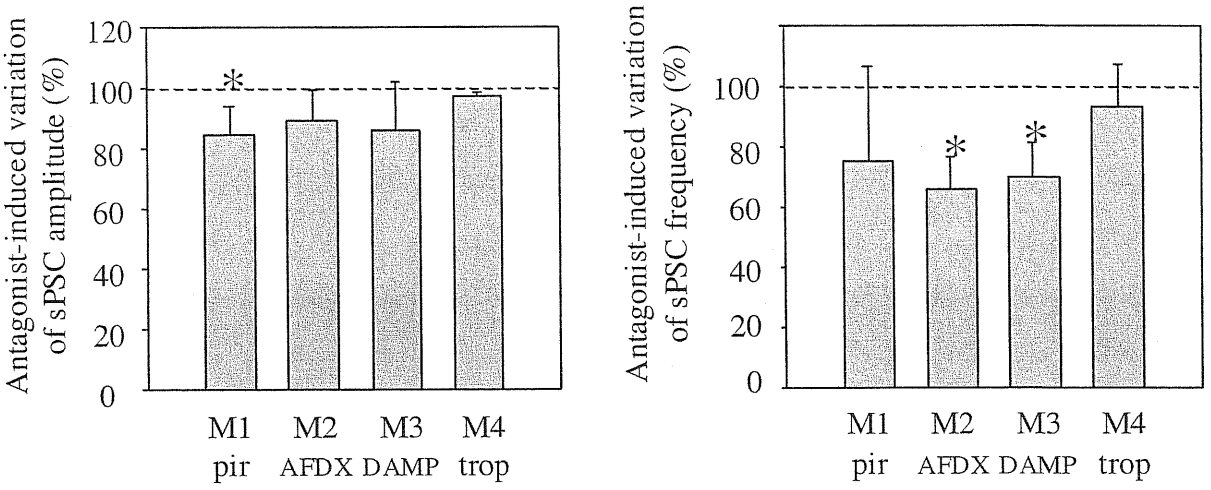

Fig.21: Effect of selective mAChR antagonists on GABAergic spontaneous transmission. Pirenzepine (antagonist for M_1 receptor subtype) modulates the amplitude of sIPSCs, while AFDX-116 (antagonist for M_2 receptor subtype) and DAMP (antagonist for M_3 receptor subtype) reduce the frequency of sIPSCs.

In this case tropicamide was completely ineffective on spontaneous transmission, while application of 2 μM pirenzepine (M_1 selective), reduced the amplitude of sIPSCs to 84.62 ± 9.41 % of control value (n=4, $p<0.05$), leaving unchanged their frequency. AFDX-116 (500nM) and DAMP (1 μM) could also modulate GABAergic transmission, by reducing the frequency of sIPSC to 65.91 ± 14.56 % and 69.76 ± 11.49 % of control value, respectively (n=4 for both antagonists, $p< 0.05$).

2.2.4 EFFECT OF SUBTYPE SELECTIVE ANTAGONISTS ON MUSCARINE-MEDIATED CHANGES IN GLYCINERGIC OR GABAERGIC TRANSMISSION

sIPSCs were recorded while bath-applying 10 μM muscarine in the continuous presence of subtype selective antagonists. This protocol was repeated for each receptor subtype, and for both glycinergic and GABAergic transmission.

In the presence of 2 μM pirenzepine, muscarine still reduced the amplitude and the frequency of spontaneous glycinergic currents. In the presence of 1 μM DAMP, sIPSC amplitude (but not frequency) was also reduced by muscarine. Conversely, in the presence of 500 nM AFDX-116 or 1 μM tropicamide, frequency of sIPSCs (but not amplitude) was significantly reduced. Percent reductions for glycinergic transmission are summarized in table 2 and fig. 22A.

A different pharmacological pattern was observed in the case of spontaneous GABAergic transmission, as summarized in fig. 22B and in table 2.

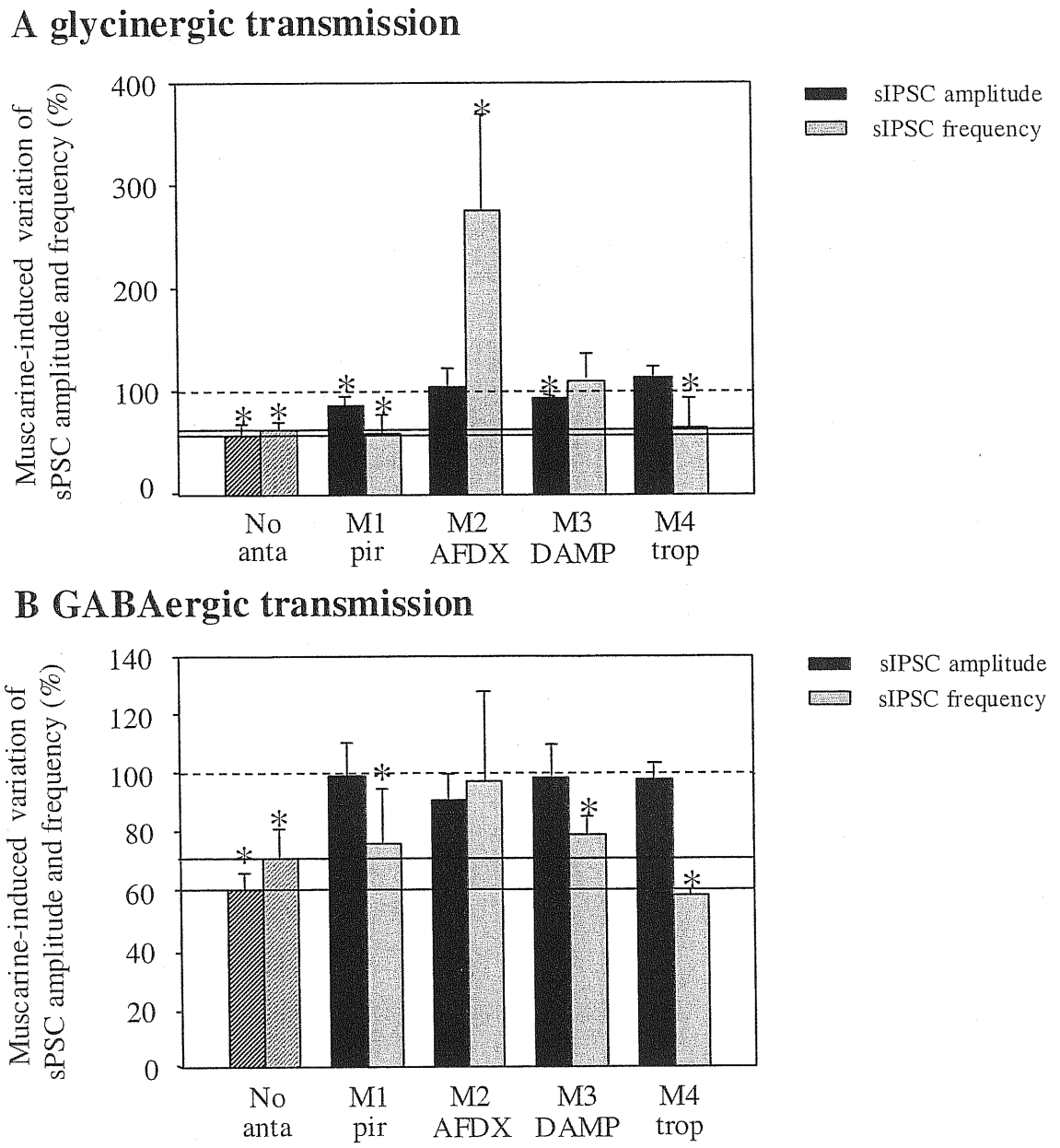

Fig.22: Muscarine-induced percent variation of amplitude and frequency of glycinergic and GABAergic spontaneous currents, in the presence of different mAChR antagonist (for values see table 2). Note that antagonists per se decreased sIPSC amplitude and/or frequency (as shown in fig. 20 and fig. 21); thus all control data were obtained in the presence the respective antagonist. Asterisks indicate a significant ($p<0.05$) variation with respect to control values measured in the presence of antagonist alone.

Selective antagonist	A_{gly} var (%)	F_{gly} var (%)	A_{GABA} var (%)	F_{GABA} var (%)
No antagonist	59.2 ± 7.1 *	63.9 ± 5.4 *	60.5 ± 4.4 *	71.0 ± 9.7 *
M_1, pirenzepine	85.6 ± 9.2 *	58.6 ± 18.2 *	99.4 ± 10.3	76.4 ± 18.2 *
M_2, AFDX-116	102.8 ± 16.5	275.9 ± 90.8 *	91.5 ± 7.8	97.7 ± 29.8
M_3, DAMP	93.2 ± 0.5 *	109.8 ± 23.5	99.1 ± 9.8	79.5 ± 5.1 *
M_4, tropicamide	113.2 ± 7.9	63.5 ± 27.9 *	98.0 ± 4.4	58.9 ± 1.8 *

Table2: Muscarine-induced percent variation in amplitude and frequency of glycinergic or GABAergic sIPSCs, in the presence of different antagonists for mAChR subtypes. Note that antagonists per se decreased sIPSC amplitude and/or frequency (as shown in fig. 20 and fig. 21); thus all control data were obtained in the presence the respective antagonist. Values are expressed as mean ± SE. (A = amplitude, F = frequency, asterisks indicate a significant variation respect to control values measured in the presence of antagonist alone, n≥ 4 for each antagonist and each transmitter.)

In conclusion, the present data indicate that spontaneous inhibitory transmission is modulated by a heterogeneous group of mAChRs, predominantly located within a sparse network of brainstem structures and partly activated by endogenous ACh. This realization suggested the usefulness of studying the role of mAChRs in inhibitory transmission evoked by discrete electrical pulses. This approach should limit the network contribution to muscarine actions especially when mono (or oligo) synaptic connections are investigated, thus making the interpretation of the effects of mChRs activation simpler.

2.3 MUSCARINIC MODULATION OF EVOKED INHIBITORY TRANSMISSION

IPSCs were evoked as previously described (Donato & Nistri, 2000;Donato & Nistri, 2001) by placing a bipolar stimulating electrode in the lateral reticular formation (lRF) adjacent to the hypoglossal nucleus. The frequency of stimulation was kept low enough to avoid short term change in synaptic responses (0.067 Hz, that is 1 stimulus every 15 seconds). The stimulus intensity was adjusted to obtain either a minimal stimulation (with approximately equal number of failures and successes) or submaximal stimulation. In response to such stimuli, inwardly directed currents were recorded from HMs as shown in fig. 23Aa-Ba. The weak stimulus used to evoke IPSCs and their relatively short, constant latency (4.7 ± 0.6 ms, n=10) suggested them to be monosynaptic responses. Again, glycinergic and GABAergic components were pharmacologically isolated.

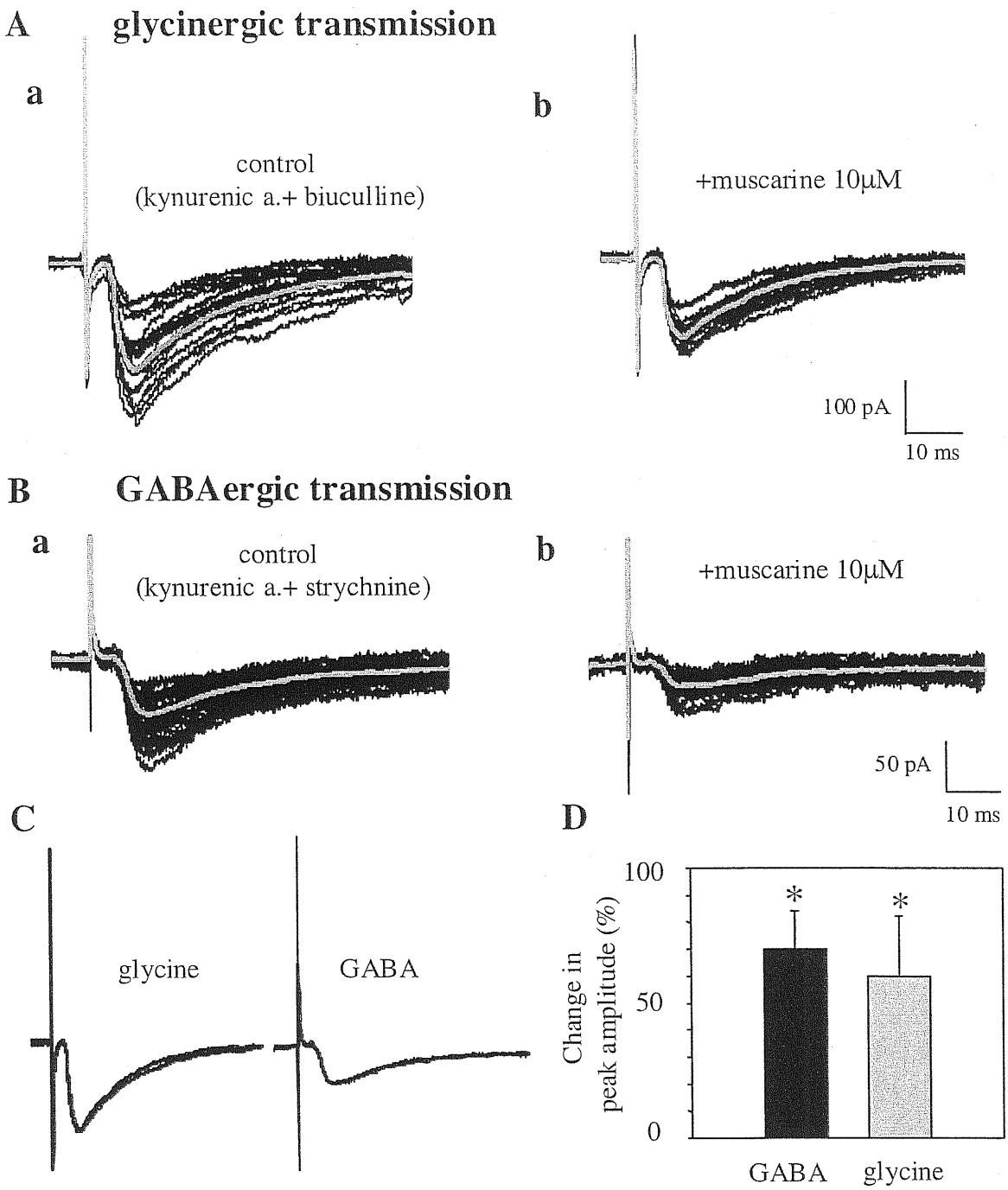

Fig.23: Muscarine effect on glycinergic and GABAergic evoked transmission.
(A) Currents recorded in the presence of kynurenic acid and bicuculline to isolate glycinergic transmission, are evoked by lRF stimulation. Fifteen superimposed current traces (black ones) with their average (gray trace) are shown in control condition (a) and during bath-application of muscarine (10 μM) (b). Eventual failures are not depicted. (B) The same protocol is applied on a different motoneuron, in the continuous presence of kynurenic acid and strychnine to isolate evoked GABAergic transmission: superimposed currents in control condition (a) and during muscarine application (b) are shown. Again eventual failures are not shown. (C) Averaged and normalized glycinergic or GABAergic currents, in control condition and during muscarine application, are superimposed. Muscarine application does not change the current kinetics. (D) Histogram of current amplitude fractional change, induced by muscarine application for a sample of 5 cells (for each transmitter). Both glycinergic and GABAergic transmissions are significantly depressed by muscarine (59.2 ± 16.3 % and 71.0 ± 20.8 % of the control value respectively, $p<0.05$).

In fig. 23A-B examples of muscarine-induced effects on glycinergic and GABAergic evoked transmission are shown. Fifteen evoked currents are superimposed both in control condition and during drug application. Like in the case of spontaneous transmission, muscarine had a depressant effect, leading, for a pool of motoneurons, to an amplitude reduction for glycinergic and GABAergic currents of 59.2 ± 16.3 % and 71.0 ± 20.8 %, respectively (n=5, $p<0.05$, fig. 23D). The percent number of failures (respect to the total number of stimuli) during muscarine application was 28.7 ± 14.2 % or 42.8 ± 10.6 % for glycinergic or GABAergic eIPSCs, respectively (in control condition the percent number of failures was zero or 12.1 ± 9.4%). After normalizing and superimposing the average responses in control condition and during muscarine application, no variation in current kinetics was observed (fig. 23C).

2.3.1 EFFECT OF SUBTYPE SELECTIVE ANTAGONISTS ON MUSCARINE-MEDIATED CHANGES IN GLYCINERGIC OR GABAERGIC EVOKED TRANSMISSION

Applying the same protocol used with spontaneous transmission, I tested the effect of muscarine on evoked glycinergic or GABAergic IPSCs in the continuous presence of mAChR selective antagonists.

Experiments on glycinergic transmission are shown in fig. 24, where examples of evoked currents, recorded in presence of different antagonists and during muscarine application, are depicted (fig. 24a-d). Depending on the antagonist applied, muscarine produced different effects. In particular, AFDX116 completely blocked muscarine-induced depression of eIPSCs (fig. 24b and 24e), while in presence of pirenzepine, DAMP or tropicamide, muscarine still retained its depressant action (fig. 24a, 24c, 24d, 24e). On average, in the presence of pirenzepine (2 μM), AFDX116 (500 nM), DAMP (1 μM) or tropicamide (1 μM), muscarine application reduced evoked glycinergic currents to 71.4 ± 4.6 %, 110.6 ± 13.6 %, 75.4 ± 15.7 % or 82.7 ± 7.4 % of control value, respectively (fig. 24e, $n \geq 4$ for each antagonist, $p<0.05$).

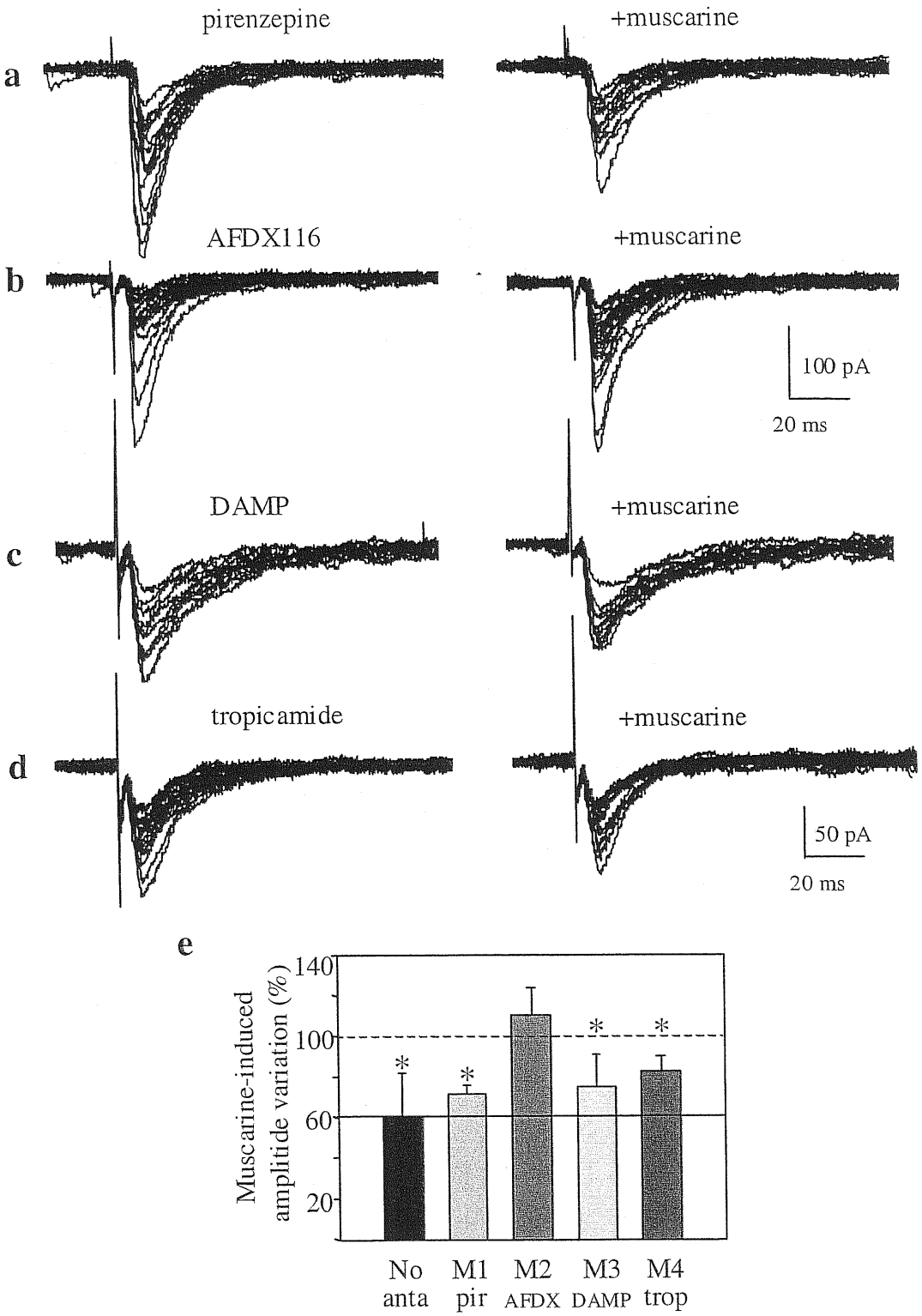

Fig. 24: Muscarinic effect on glycinergic eIPSCs in the presence of muscarinic receptor antagonists. Evoked glycinergic transmission is pharmacologically isolated as previously dscribed. Currents are recorded in the presence of 2 µM pirenzepine, 0.5 µM AFDX116, 1 µM DAMP or 1 µM tropicamide, (superimposed traces from different cells depicted in panel a, b, c and d, respectively), before and during muscarine (10 µM) bath-application. (e) Results from a pool of motoneurons are summarized in this histogram. Muscarine effect on glycinergic transmission is prevented only by 0.5 µM AFDX116 ($n \geq 4$ for each antagonist). Asterisks indicate a significant ($p<0.05$) variation with respect to control values measured in the presence of antagonist alone, or between control and muscarine.

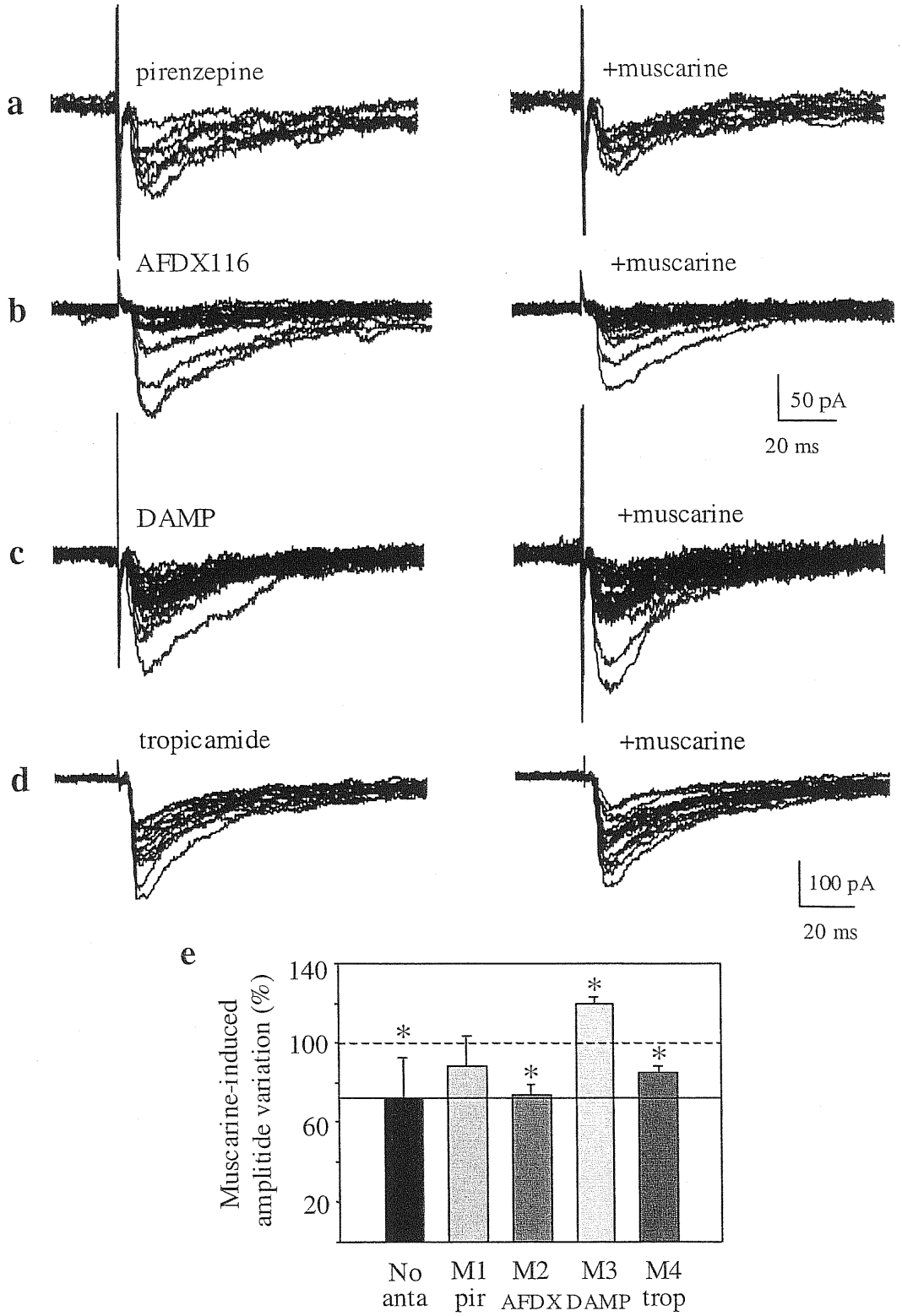

Fig.25: Muscarinic effect on GABAergic transmission in the presence of muscarinic receptor antagonists. Evoked GABAergic transmission is pharmacologically isolated as previously described. Currents are recorded in the presence of 2 µM pirenzepine, 0.5 µM AFDX116, 1 µM DAMP or 1 µM tropicamide, (superimposed traces from different cells depicted in panel a, b, c and d, respectively), before and during muscarine (10µM) bath-application. (e) Results from a pool of motoneurons are summarized in this histogram. Muscarine effect on GABAergic transmission is prevented by pirenzepine (2 µM) or DAMP (1 µM) application (n ≥4 for each antagonist). Asterisks indicate a significant ($p<0.05$) variation with respect to control values measured in the presence of antagonist alone, or between control and muscarine.

The situation was quite different for GABAergic evoked transmission, since the muscarine-induced depressant effect was prevented by pre-application of selective antagonists for M_1 and M_3 receptor subtypes. In particular, when the M_1 receptor subtype was pharmacologically blocked with pirenzepine, muscarine application was completely ineffective in modulating GABAergic eIPSCs (fig.25a-e). On the other hand, when the M_3 receptor subtype was pharmacologically blocked with DAMP, muscarine inverted its effect, increasing the amplitude of eIPSCs (fig. 25c-e). On average, in the presence of pirenzepine (1 μM), AFDX116 (500 nM), DAMP (1 μM) or tropicamide (1 μM), muscarine-induced change in eIPSCs was 88.3 ± 14.0%, 71.9 ± 5.1, 119.8 ± 3.5 or 85.0 ± 3.3 % of control value, respectively (fig. 25e, n ≥ 4 for each antagonist). Data on evoked glycinergic and GABAergic are compared in fig. 26.

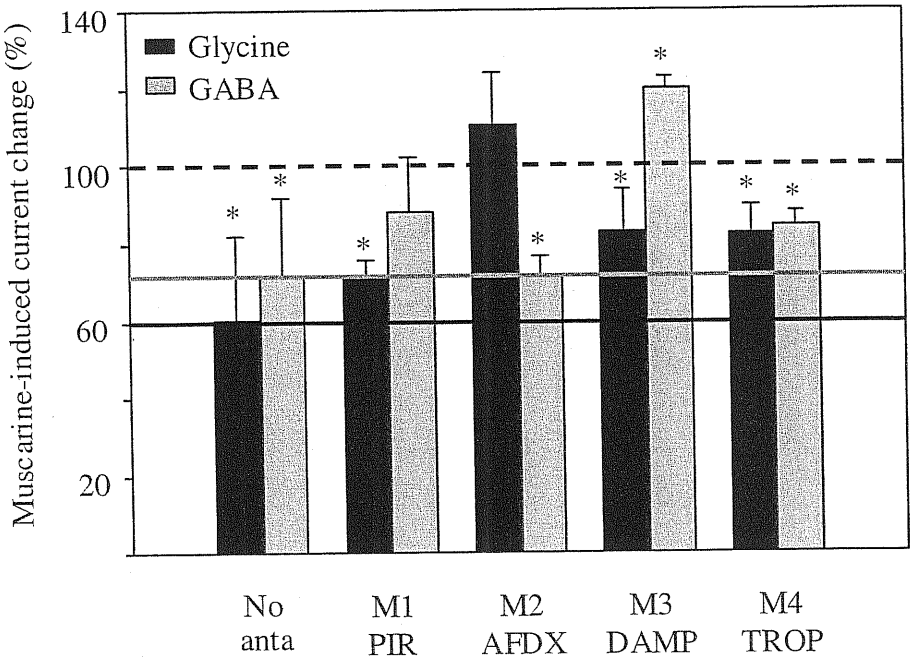

Fig.26: Subtype selective antagonists block of muscarine effects: comparison between glycinergic and GABAergic transmission. (Same data as in fig. 24-25)

2.4 GLYCINERGIC AND GABAERGIC POSTSYNAPTIC RECEPTORS

Muscarinic modulation of glycinergic and GABAergic transmission could be due (other than to presynaptic modulation) to postsynaptic alterations in glycine or GABA-gated ion channels. To investigate this possibility, I evoked pure postsynaptic glycinergic or GABAergic currents with pressure pulse application of the two transmitters directly on the soma of motoneurons, using a fine tipped puffer pipette (see methods).

2.4.1 BASIC PROPERTIES OF PRESSURE-PULSE EVOKED GLYCINERGIC OR GABAERGIC CURRENTS

Pressure pulse application of glycine was always performed in presence of bicuculline (10 μM), to prevent glycine-induced depolarization of GABAergic synaptic terminals contacting the HM from which I was recording. In fact, such depolarization could facilitate the release of GABA from presynaptic terminals, eliciting a postsynaptic GABAergic current contaminating the glycinergic one. Alternatively, if glycine was taken up by presynaptic terminals, it might have caused GABA release via heterochange mechanism. For analogous reasons, GABAergic currents were always recorded in presence of strychnine (0.4 μM).

Glycine (100 μM) pulses, of different time duration, elicited currents like those shown in fig. 27A (lower traces). These currents were insensitive to TTX (1 μM) and were fully suppressed by 0.4 μM strychnine application (n=3), indicating that they were genuine postsynaptic, glycine-mediated, currents (fig. 28B lower trace). Analogously, 100 μM GABA application via pressure pulses, evoked currents, insensitive to TTX, that were completely and reversibly blocked by 10 μM bicuculline (n=2, fig. 27A-B upper traces). While applying glycine or GABA pressure pulses of increasing time duration, I recorded responses of increasing amplitude, obtaining the dose-response curve shown in fig. 28 (Di Angelantonio & Nistri, 2001). Current amplitude (normalized with respect to the response evoked by 50 ms glycine or GABA pulse) versus glycine or GABA pulse duration is depicted.

In general, glycinergic and GABAergic currents had quite different characteristics. On average, in response to the same pressure pulse (same time duration and amount of transmitter applied), glycinergic currents were faster and smaller than GABAergic ones (fig. 27A, notice the different calibration bars).

	Amplitude (pA)	T_{rise} (ms)	τ_{decay} (ms)
Glycine	219.7 ± 37.4	44.6 ± 3.2	175.5 ± 44.6
GABA	1127.5 ± 166.0	244.3 ± 23.9	733.1 ± 47.0

Tab.3: Mean characteristics of currents elicited with 50 ms pulses of glycine or GABA (100 μM)

Mean amplitude, rise time and decay time, characterizing currents elicited with a 50 ms pulse of 100 μM glycine or GABA, are reported in table 3 (n=4).

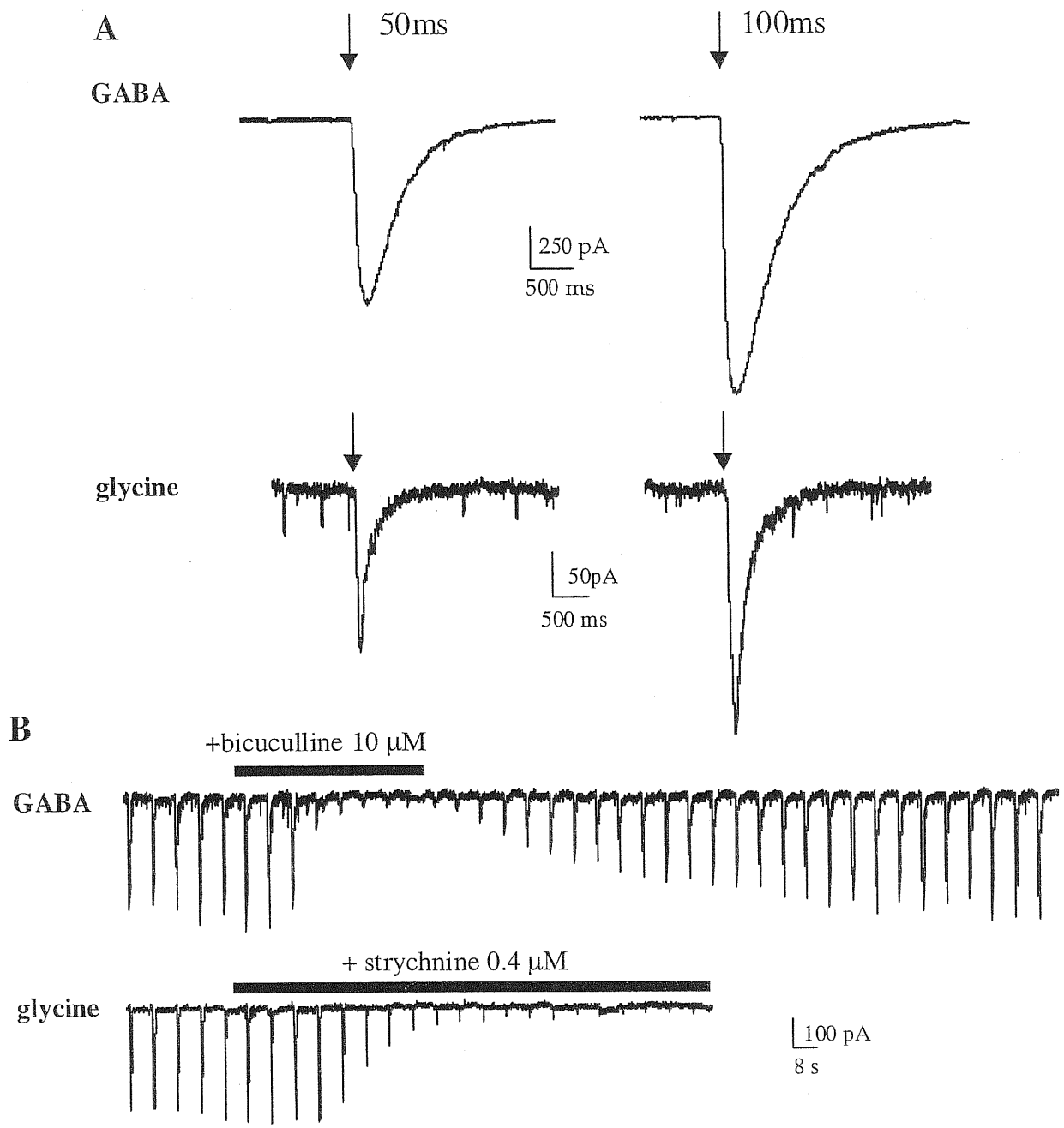

Fig.27: Glycinergic an GABAergic postsynaptic currents.
Examples of glycinergic and GABAergic currents evoked with pressure pulse application of 100 μM glycine or GABA. (A) Currents recorded in response to GABA (upper traces) or glycine (lower traces) pulses of different time duration (currents on the left are elicited in response to 50 ms pressure pulse, while currents on the right are elicited in response to 100 ms pressure pulse of GABA or glycine; arrows indicate the time of application). (B) Upper traces: 10 μM bicuculline fully and reversibly suppresses currents elicited by 10 ms GABA pulses. Lower traces: 0.4 μM strychnine completely blocks currents elicited by 50 ms glycine pulses. The time interval between two stimuli is 30 s for both GABAergic and glycinergic currents.

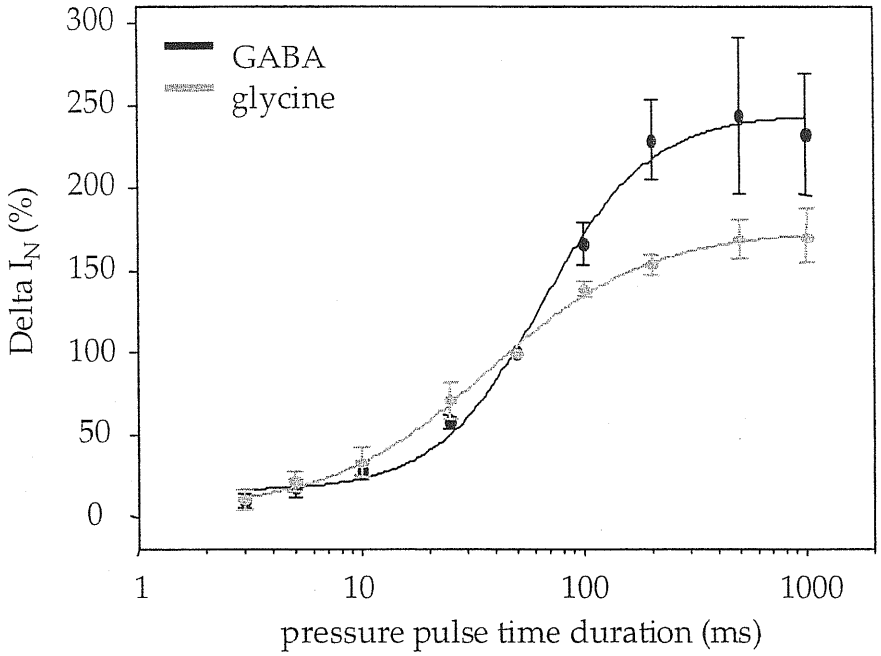

Fig.28: Glycine and GABA dose-response curve. Both glycinergic and GABAergic current amplitudes are normalized to the value recorded with 50 ms transmitter application.

2.4.2 MUSCARINE MODULATION OF POSTSYNAPTIC RECEPTORS

Currents evoked by 50 ms glycine pulses were recorded in control condition and during muscarine (10 μM) application. The example in fig. 29A shows the time course of such an application, with muscarine reducing the current amplitude to -111.5 ± 4.3 pA (control value was -161.0 ± 6.9 pA). On average, muscarine-induced reduction of pressure pulse evoked glycinergic currents was 65.9 ± 5.5 % of control (n=4, $p<0.05$). This effect was prevented by pre-application of 500 nM AFDX (n=2).

In fig. 29B muscarine is applied to a different motoneuron, from which I recorded currents elicited by 30 ms GABA pulses. Current amplitude was reduced to -480.2 ± 7.3 pA (control value was -576.8 ± 7.2 pA). On average, muscarine-induced reduction of pressure pulse evoked GABAergic currents was 85.9 ± 2.5 % of control (n=3, $p<0.05$), and the effect was prevented by pre-application of 1 μM DAMP (n=3).

The effect of muscarine (10 μM) was tested also on glycinergic and GABAergic currents of increasing amplitude (again elicited with pulses of increasing duration). In fig. 30, glycine and GABA average dose-response curves (panel Aa and Ba, respectively), obtained from a pool of five motoneurons, are depicted both in control condition and during 10 μM muscarine application. Current amplitudes are normalized with respect to the response evoked by 50 ms glycine or GABA pulse in

control solution. Muscarine reduced glycinergic and GABAergic currents without changing their kinetics, as shown by normalized and superimposed average responses (to 50 ms pulses) depicted in fig. 30Ab-Bb.

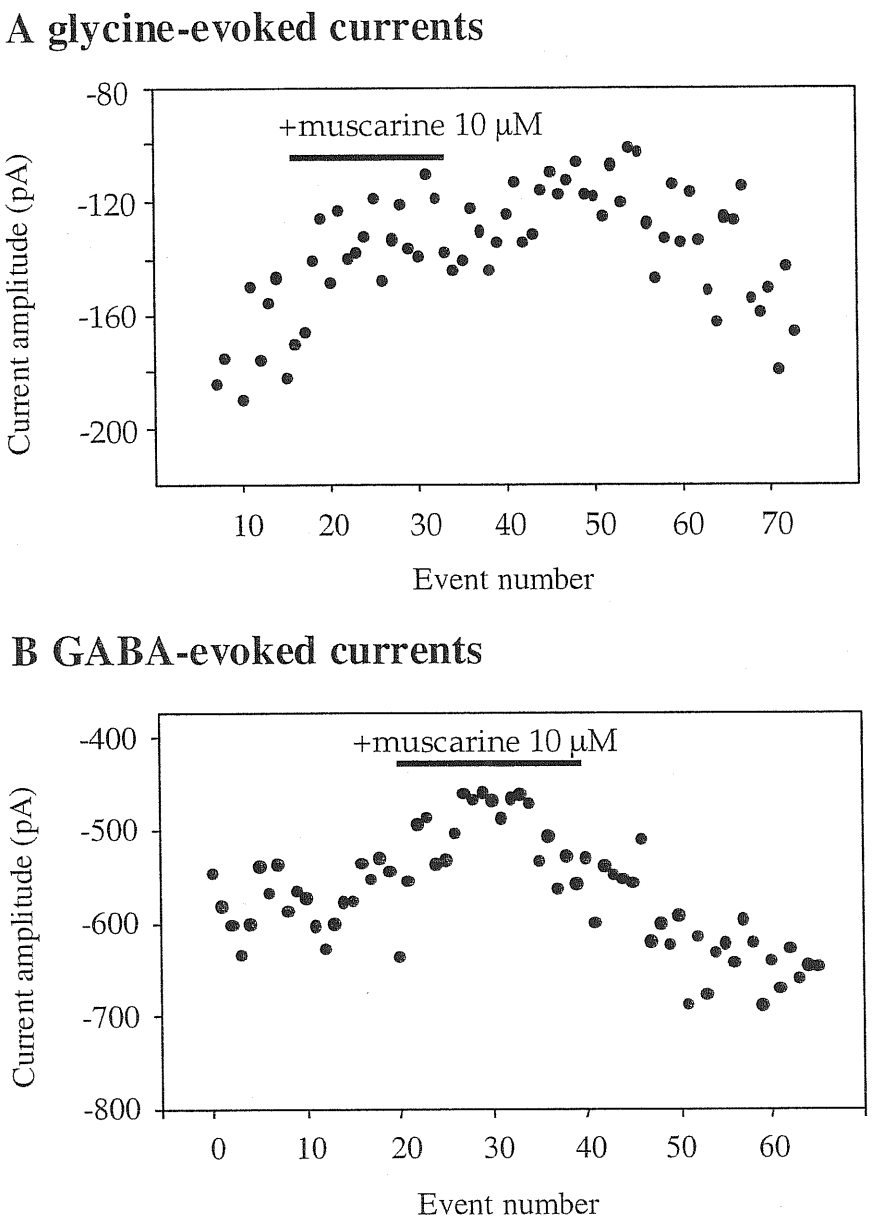

Fig.29: Time course of muscarine action on pressure pulse evoked currents.
(A) Glycinergic currents evoked by 50 ms pulses. (B) GABAergic currents evoked by 30 ms pulses (different cell from A). The time interval between pulses is 30 s for both transmitters.

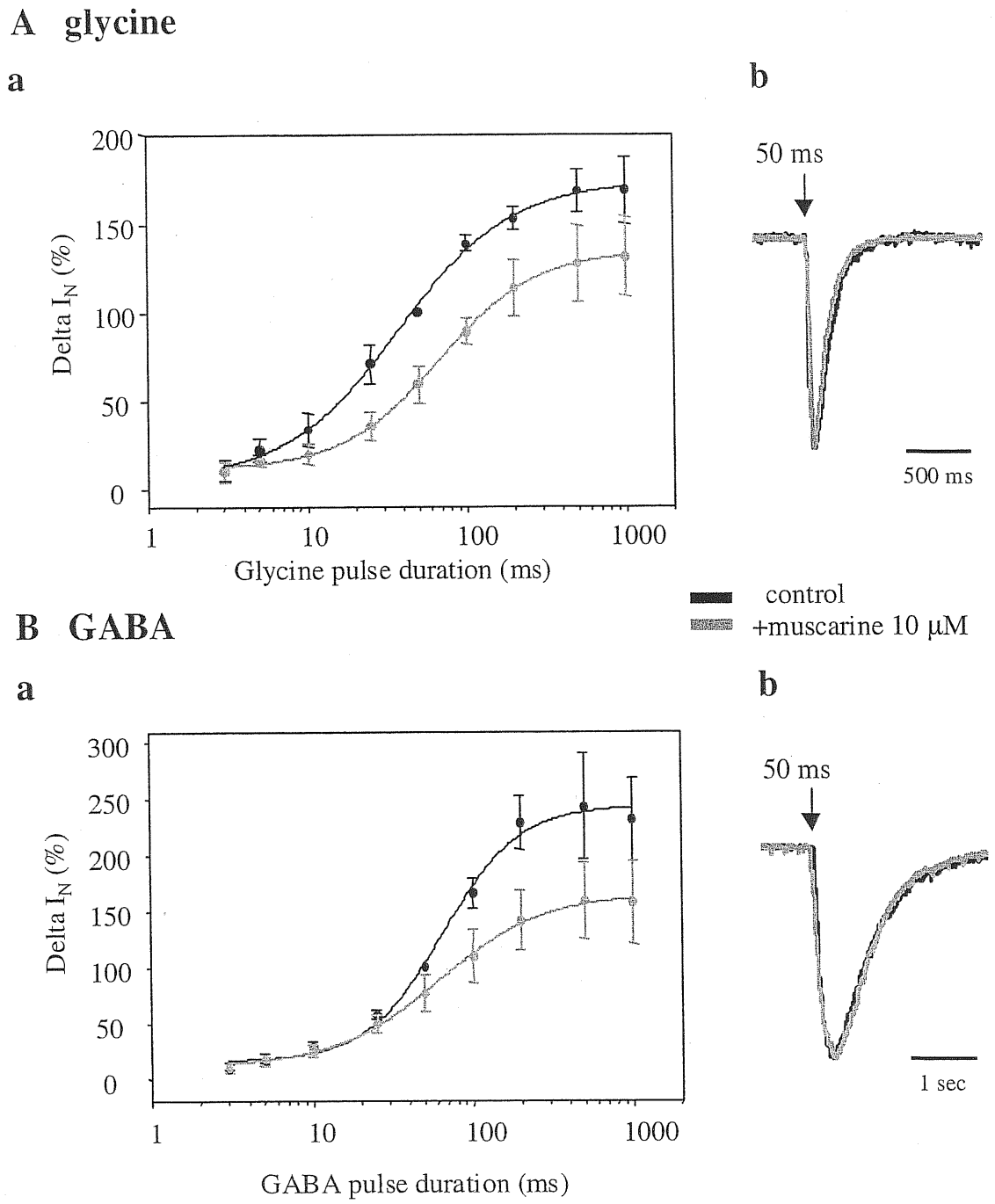

Fig.30: Effect of 10 µM muscarine on glycinergic and GABAergic dose-response curves.
(A) a: plot of glycine current amplitude versus increasing duration of glycine pressure pulses, in control solution and in presence of 10 µM muscarine (bath-applied to the HM). Current is normalized with respect to the response evoked by 50 ms in control solution. b: Normalized average currents recorded in control solution and in presence of muscarine, for one HM, are superimposed. Currents are elicited by 50 ms pressure pulses. (B) a: plot of GABA current amplitude versus increasing duration of GABA pressure pulses, in control solution and in presence of 10 µM muscarine (bath- applied to the HM). Again current is normalized with respect to the response evoked by 50 ms in control solution. b: Normalized average currents recorded in control solution and in presence of muscarine, for one HM, are superimposed. Currents are elicited by 50 ms pressure pulses. Note the different time scale between glycine and GABA current in panel Ab and Bb.

DISCUSSION

"Io stimo più il trovare un vero, benché di cosa leggiera, che l' disputar lungamente delle massime questioni senza conseguir verità nissuna "

(G. Galilei, Opere, IV)

1. GENERAL REMARKS ON THE EXPERIMENTAL MODEL

In the present work I used neonatal rat brainstem slices as a model system for the study of glycinergic and GABAergic transmission.

As described in detail in the introduction, glycine and GABA are differentially distributed in the CNS, the former being mainly located in the spinal cord and the latter in the forebrain. However, in some regions, such as the brain stem cranial nuclei and in particular the nucleus of the hypoglossus, both neurotransmitters are present and functional (Umemiya & Berger, 1995; Singer *et al.*, 1998; Donato & Nistri, 2000, 2001). The co-existence and extensive overlap between these two transmitter systems, make the hypoglossal nucleus a suitable model for studying glycine and GABA relative contribution to synaptic microphysiology, their interplay as transmitters and differential role at a functional level.

An important point is the choice of the acute brain slice preparation. Brain slices have greatly facilitated the investigation of the electrical properties of neurons and the analysis of synaptic transmission between them in the central nervous system, mainly because in this preparation neurons are readily identified and accessible, and remain viable for hours with a degree of network connections. Obviously the use of brain slices has also some limitations, when compared, for example, with dissociated neurons or entire tissue. First of all, using brain slices neurons, space clamp problems may arise. However, the study by Viana *et al.* (1994) has suggested that HMs are compact neurons, with an electrotonic length of 0.99. A similar result (0.5-0.85 λ) has been reported for spinal motoneurons (Clements & Redman, 1989; Thurbon *et al.*, 1998). The use of thin slices (in which some part of the dendritic tree might have been cut off) and visual identification of cells (with the possibility of choosing smaller cells)

may help to reduce space clamp errors. A second disadvantage in using slices is the difficulty in identifying the fibres projecting onto the neuron under study. For the hypoglossal nucleus, for example, it is known that the main inputs originate from the reticular formation (RF), which is apparently a diffusely organized area forming the central core of the brainstem. This particular feature of the RF prevented us from using focal stimulation of single glycinergic or GABAergic fibres. Nevertheless, stimulation of projecting interneurons was still possible with bipolar electrodes, while glycine or GABA-mediated transmission was dissected out with pharmacological tools.

A final comment concerns the animal age. Clinical interest in studying hypoglossal nucleus from neonatal animals arises from recent studies, demonstrating its involvement in the Sudden Infant Death Syndrome (SIDS) (Konrat *et al.*, 1992; O'Kusky & Norman, 1995; Lamont *et al.*, 1995). From a more general point of view, however, it should be stressed that neonatal rat glycine or GABA receptors often show different subunit composition and electrophysiological properties with respect to those present in the adult animal (see introduction for details). For this reason the first part of the present study investigated their functional role.

2. FUNCTIONAL ROLE OF GLYCINERGIC AND GABAERGIC TRANSMISSION

Although GABA and glycine are inhibitory transmitters in the adult brain, their role during early neuronal development is still not completely clear. As discussed in detail in the introduction, in neonatal animals glycine or GABA action varies from the spinal cord, where it is mainly inhibitory, to the higher regions of the neuraxis, where it is mainly excitatory. In structures such as the brainstem, however, it is still uncertain if these transmitters actually inhibit motoneuron firing. Thus, the first part of the present study attempted to elucidate this issue concerning the hypoglossal nucleus.

Recent studies performed in our laboratory (Donato, 2000) gave the first direct demonstration of the inhibitory action exerted by glycine and GABA on HMs, showing that exogenous application of these two transmitters prevents cells from firing in response to a current pulse injection. In those experiments the reversal potential for Cl^-, calculated with the Nernst equation, was –37 mV, the same value

observed by Singer *et al.* (1998) with perforated patch recording. It is noteworthy that responses evoked by GABA or glycine were membrane depolarizations with comparable amplitude and with substantial reduction in input resistance. During application of GABA or glycine, motoneuron firing was suppressed, an effect readily reversed on washout. By returning the membrane potential to the control value, it became apparent that GABA or glycine still inhibited firing presumably because the cell membrane was shunted by the large input resistance fall due to opening of Cl^- channels. Starting from these observations, I further investigated the functional effect of glycine or GABA released on HMs.

There are two main objections to the previous findings. First of all, bath-applied transmitters might have operated via activation of extrasynaptic receptors not normally accessible to synaptically released GABA or glycine, and second, inhibition could be due to membrane depolarization which partially inactivated voltage sensitive Na^+ channels and consequently depressed motoneuron firing. In particular, this last effect could not be completely excluded even if firing inhibition induced by exogenous application of glycine (GABA) persisted when the cell membrane was manually repolarized to resting value. In fact, due to space clamp problems, the dendritic arborization membrane potential could have been less sensitive to DC current injection.

To confute these objections, I first observed that, in contrast to glycine or GABA, the exogenous application of the putative excitatory transmitter glutamate, evoked membrane depolarization, without significant change in input resistance (perhaps because of the remote location, coactivation of AMPA/NMDA receptors and/or lower conductance of glutamate channels) and actually facilitated spike firing. Thus, bath-application of exogenous transmitters, despite the widespread distribution of their receptors, could reveal major functional differences between glutamate and GABA (or glycine).

The major demonstration of GABA or glycine inhibition of hypoglossal motoneurons was obtained by testing how evoked synaptic GABAergic or glycinergic responses affected the firing pattern induced by small current pulse injection. As previously mentioned, on hypoglossal motoneurons it is difficult to identify the source of glycine or GABA releasing fibres, which originate sparsely from the adjacent reticular

formation. Notwithstanding the diffuse location of such fibres, it is, however, possible to elicit GABAergic and glycinergic synaptic responses by discrete stimulation (Umemiya & Berger, 1995; Singer *et al.*, 1998; O'Brien & Berger, 1999; Donato & Nistri, 2000, 2001). When mixed GABAergic and glycinergic PSPs (or pharmacologically isolated GABA or glycine synaptic responses) were evoked immediately prior to action potentials, there was a decrease in firing probability and an increase in firing latency despite the associated membrane depolarization which might have been expected to facilitate firing. This result clearly indicated the inhibitory nature of GABA or glycine mediated signals. Similar results have been previously obtained in rat superior cervical ganglion neurons and in rat DRG neurons, where GABA-induced chloride-mediated depolarization was associated with a decrease in excitability, presumably due to the membrane shunt (Feltz & Rasminsky, 1974; Adams & Brown, 1975).

The timecourse of these PSPs was too rapid to allow injection of current pulses sufficiently long to fully charge the cell membrane capacitance and to monitor changes in input resistance. This problem prevented direct measurement of input resistance alterations. However, in view of the large and reproducible decrease in input resistance induced by application of GABA or glycine, it seems likely that spike firing inhibition was brought about by the membrane shunt induced by synaptically released GABA and glycine. A number of hypoglossal motoneurons exhibited GABAergic and glycinergic PSPs which were ineffective to block spike firing. This is not entirely surprising in the light of the sparse distribution of GABAergic and glycinergic boutons (Li *et al.*, 1997) and the extensive severance of GABAergic and glycinergic fibres during the slice preparation. It seems feasible that in those instances there was an insufficient number of active inhibitory synapses close to the motoneuron soma to suppress (or retard) the activation of the somatic sodium conductance responsible for generating the action potential (Lape & Nistri, 2001). Indeed, in such cases, GABAergic and glycinergic PSPs were presumably generated too distant from the cell soma so that the associated resistance fall was electrotonically filtered out along the dendritic arbor.

2.1 FACTORS RESPONSIBLE FOR GLYCINE (OR GABA) MEDIATED INHIBITION OR EXCITATION OF NEONATAL NEURONS

It might be of interest to examine why opposite functional effects of GABA are reported, for example, on hippocampal (and hypothalamic) neurons and motoneurons at the same developmental age and with similar resting potential value. The reversal potential for GABA is about –50 mV for hippocampal pyramidal cells (Ben Ari *et al.*, 1989), and about –40 mV for hypothalamic neurons (Chen *et al.*, 1996), both cell types apparently excited by GABA. These values are, however, similar to the Cl^- reversal potential of hypoglossal (Singer *et al.*, 1998) or spinal (Wu *et al.*, 1992) motoneurons and consistently positive with respect to the spike threshold. These data therefore suggest that the driving force for GABA or glycine mediated responses is comparable for different neurons and not responsible for the fact that these amino acids can be excitatory on some cells and not on others.

Another possibility is that, despite membrane depolarization, GABA inhibits certain neurons by shunting their membrane resistance (via a large increase in Cl^- permeability) to make them less responsive to excitatory signals. The single channel conductance of neonatal $GABA_A$ receptors is not dissimilar between distinct neuronal populations (mouse spinal neurons in culture: 15-28 pS, Bormann *et al.*, 1987; Macdonald *et al.*, 1989; neonatal hippocampus: 24-35 pS, Hokosawa *et al.*, 1994). Although the $GABA_A$ single channel conductance of neonatal hypoglossal motoneurons is unknown, the one for glycine (which inhibits, like GABA, via Cl^- permeability increase) is 30 pS (Singer *et al.*, 1998), that is near the data reported for GABA on other neurons.

Despite relatively similar values of reversal potential and of single channel conductance, membrane conductance during transmitter activity also depends on channel number and/or opening probability (see Nistri & Gutman, 2001) which, if differentially expressed by certain neurons, could then generate opposite functional effects of GABA (or glycine). A further factor contributing to the functional diversity of GABA (or glycine) action might be the varying distribution of their receptors with respect to the source of excitatory synaptic potentials or the site of spike generation. Even in the neonatal hippocampus a recent report describes the effect of GABA (via $GABA_A$ receptors) as inhibitory (Lamsa *et al.*, 2000) in contrast with previous studies

(Ben-Ari *et al.*, 1989). Perhaps the variable location of $GABA_A$ receptors might indeed be responsible for its contrasting effects even on the same hippocampal cell type. Alternatively, at least for the hippocampus, GABA-mediated excitation might require activation of a distinct membrane receptor class ($GABA_C$ receptor, Martina *et al.*, 1995; Bormann, 2000).

3. GLYCINERGIC AND GABAERGIC RECEPTOR MODULATION

3.1 FUNCTIONAL ROLE OF MUSCARINIC MODULATION

Metabotropic receptors, and in particular mAChRs, are well known to modulate motoneuronal excitability in several areas of the brain stem and spinal cord (see for a review: Rekling *et al.*, 2000).

More in general, acetylcholine receptors are widely diffused in all the brainstem (Caulfield, 1993; Mallios *et al.*, 1995; Zaninetti *et al.*, 1999), and their presence in several nuclei, on both pre and postsyanptic neurons, has raised an increasing interest in their functional role. However, while many studies are focussed on the role of nicotinic acetylcholine receptors, especially concerning respiratory nuclei (Ferguson *et al.*, 2000; Shao & Feldman, 2001; Shao & Feldman, 2002), fewer investigations on their metabotropic counterpart are available.

From a functional point of view, as mentioned in the introduction, ACh is involved in central respiratory control including central chemosensitivity (see introduction for references), and defects in the ventral medullary muscarinic system may play a role in disorders of respiration such as SIDS (Kinney et al. 1995). Clinically, muscarinic antagonists are particularly effective against bronchoconstriction with a central and peripheral site of action (Brown & Taylor, 1996).

Cholinergic pathways have been also associated with perception and expression of normal and excessive levels of motion stimuli, and broad-spectrum antagonists of mAChRs, such as scopolamine, are often used to prevent motion sickness (Brown & Taylor, 1996). Even if their action is mainly exerted either on the cortex or on the vestibular apparatus sickness (Brown & Taylor, 1996), the complexity of the cholinergic system and the interaction of scopolamine with this system, left open the possibility that pharmacological doses of drugs specific to the cholinergic system might exert significant modulatory influences at alternative sites as well. Among

them, respiratory and emesis related nuclei, mainly located in the brainstem, hold a predominant role, and the hypoglossus nucleus is certainly part of them.

3.2 MUSCARINE EFFECT ON BASIC PROPERTIES OF HM

The first effect observed in response to muscarine application to HMs, was a small depolarization coupled with an increase in synaptic activity and facilitation of firing. In those experiments, however, no discrimination between excitatory or inhibitory inputs was made, revealing an overall increase in motoneuron excitability induced by muscarinic receptor activation. In HMs, mAChR effects on spike firing has been previously reported (Viana *et al.*, 1993; Lape & Nistri, 2000).

3.3 MUSCARINE MODULATION OF SPONTANEOUS AND EVOKED CURRENTS

In the present study we found that muscarinic receptor activation strongly reduced amplitude and frequency of spontaneous glycinergic and GABAergic transmission (with no major differences between the two transmitters). The situation was quite different when looking at miniature events. In fact, in this case, GABAergic currents were unaffected by muscarine, while glycinergic ones were slightly reduced in amplitude. Muscarine did not change the kinetic parameters of both spontaneous and miniature currents. Classically, a variation in the frequency of events is ascribed to a presynaptic site of modulation, while a variation in amplitude predominantly to a postsynaptic one. Thus, at first glance, the different actions exerted by muscarine on mIPSCs and sIPSCs seem to indicate that a major contribution to the spontaneous current depression arises from network-located receptors, without any involvement of the presynaptic terminals in the modulation process. To elucidate better this point we then studied evoked transmission, and we observed again that muscarine application lead to a reduction in both evoked glycinergic and GABAergic current amplidude, with no alteration in their kinetics.

What mechanisms could be responsible for such a depression of inhibitory neurotransmission? One might consider three actions:

1) Muscarinic receptor activation could inhibit the release of transmitter from the presynaptic terminal via calcium dependent or independent mechanisms.

2) Muscarinic receptor activation could depress the excitability of presynaptic interneurons located within the slice, thus reducing the amount of released transmitter.

3) Muscarine could modulate glycinergic or GABAergic currents via postsynaptically located receptors on HMs.

Let's examine in more detail these three possibilities in the light of the present data.

1) The general dependence of evoked transmitter release on Ca^{2+} influx through voltage-dependent Ca^{2+} channels makes a mAChR-induced decrease in calcium influx an obvious mechanism for decreasing neurotransmitter release (Caulfield, 1993; Brown *et al.*, 1997). Moreover, calcium insensitive miniature events are a common feature of several CNS areas (Lupa, 1987; Thesleff, 1988; Scanziani *et al.*, 1995), thus explaining the unchanged glycinergic and GABAergic mIPSC frequency that we observed after muscarine application. However, if in response to mAChR activation, such a calcium dependent mechanism actually occurred, it should have been associated with changes in the kinetics (especially the rise time) of the monosynaptic evoked current. This phenomenon, however, was never observed. Modulation of evoked inhibitory transmission via muscarinic receptors located on presynaptic terminals might also be caused via calcium independent mechanisms. Direct muscarinic modulation of the molecular apparatus involved in exocytosis of neurotransmitter vescicles (by altering, for example, the level of phosphorylation of one of the proteins involved) has been previously observed (Scanziani *et al.*, 1995; Capogna *et al.*, 1995). However, in such cases, a corresponding alteration in frequency of miniature events should be also observed. Again this phenomenon was absent.

2) Once the hypothesis of a presynaptic terminal site of modulation is ruled out, one might postulate a muscarine-induced reduction in interneuron excitability, that could account for the observed depression of evoked and spontaneous inhibitory transmission. The precise location of mAChRs responsible for such an effect is, however, still debatable. As shown in fig. 31, different possibilities can be considered. Muscarinic receptors could be located on the soma of the first order interneurons impinging on HMs (fig. 31, interneuron **a**, receptor **1**). There, they could produce somatic inhibition of the glycinergic (GABAergic) interneuron via several mechanisms, like those found on hippocampal CA1 pyramidal cells on which they

activate an inwardly rectifying K^+ conductance (Brown *et al.*, 1997;Seeger & Alzheimer, 2001). This would expect to depress transmitter release on HMs.

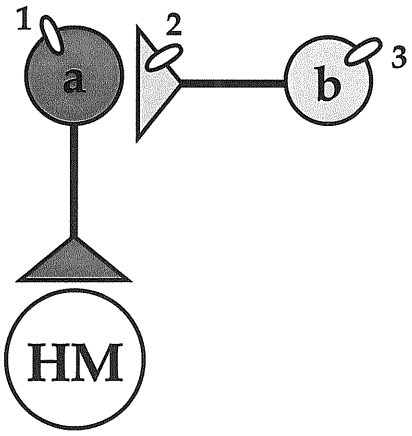

Fig.31: Schematic representation of mAChR possible locations.

Another possible location for mAChRs, again depicted in fig. 31 (interneuron **b**, receptor **2**), is on the synaptic terminal of a second order inhibitory interneuron. In this case an excitatory action of muscarine on interneuron **b** could lead to the inhibition of interneuron **a**. Several examples of presynaptic excitatory actions of muscarine can be found in literature (Caulfield, 1993; Brown *et al.*, 1997). A similar excitatory effect could be exerted, in principle, also on the soma of a second order interneuron (fig. 31, interneuron **b**, receptor **3**), even if it becomes necessary to assume substantial preservation of interneuronal connections in 200 μm thin slices. Nevetheless, network-based mAChRs appear to be responsible for at least part of the observed depression of inhibitory transmission.

3) The last possible explanation for muscarine-induced depression of evoked inhibitory transmission, is its postsynaptic origin, directly on the HM membrane. Even if this possibility, by itself, cannot account for the reduction in spontaneous event frequency, it can reinforce the inhibitory action exerted on evoked transmission at network level. Moreover, a postsynaptic action of muscarine is important to explain the observed reduction of miniature glycinergic current amplitude, as it will be discussed in the next paragraph.

3.4 POSTSYNAPTIC MODULATION: COMPARISON BETWEEN PRESSURE-PULSE EVOKED AND MINIATURE EVENTS

The possibility of a postsynaptic localization (and action) of muscarinic receptors was investigated by means of pressure-pulse evoked glycinergic or GABAergic currents.

First of all, we observed that glycinergic and GABAergic currents evoked in this way have kinetics resembling those of spontaneous and evoked synaptic events (Donato & Nistri, 2000). In particular, glycinergic events remained faster than GABAergic ones, with shorter rise and decay times. A major difference was observed in the amplitude of currents elicited by glycine or GABA applied for the same time, as GABAergic currents were about fivefold larger than glycinergic one.

Bath-applied muscarine depressed the maximal amplitude of both glycinergic and GABAergic dose-response curves, indicating the presence of postsynaptic mAChRs interacting with chloride-permeable channels. However, a differential effect of muscarine was observed on responses induced by glycine or GABA. In particular, small glycinergic currents, evoked by pulses shorter than 20 ms, were consistently depressed by muscarine application, while small GABAergic responses were not. This behavior resembles that of miniature events: also in that case only glycinergic events were affected by muscarine application.

This observation raises an obvious question: is it realistic to compare the properties of miniature GABAergic currents with those of short pulse evoked currents?

Although miniature events are axiomatically presumed to be caused by release of a large concentration of transmitter to saturate postsynaptic receptors (Mozrzymas *et al.*, 1999), this is not necessarily a universal condition. In fact, experiments on both excitatory and inhibitory transmission in several brain regions, indicate that often the amount of transmitter released by a single vesicle (eliciting a miniature current) is far from being saturating for the postsynaptic receptor (Clements *et al.*, 1992; Liu & Tsien, 1995; Clements, 1996). If we consider this possibility applicable to GABAergic miniature events recorded from hypoglossal motoneurons, the comparison between mIPSCs and short pulse evoked currents becomes straightforward, and the postsynaptic action of muscarine accounted for. However, clear resolution of this issue will require future work based on quantal analysis of synaptic transmission.

3.5 PHARMACOLOGICAL DISCRIMINATION BETWEEN DIFFERENT MUSCARINIC RECEPTOR SUBTYPES

Different receptor subtypes might have mediated the muscarinic action on glycinergic and GABAergic transmission. We explored this possibility by using apparently selective antagonists against four of the five different mAChR subtypes. These

antagonists were pirenzepine (M_1), AFDX-116 (M_2), DAMP (M_3) and tropicamide (M_4). Some general comments on the selectivity of these compounds are, however, warranted. Unfortunately, muscarinic receptor research has been hindered by lack of antagonists with high affinity for one receptor subtype and very high selectivity. This implies that a particular receptor subtype should be defined with a range of selective antagonists (Caulfield, 1993). However, such a kind of pharmacological discrimination can become really problematic in tissues expressing more than one receptor subtype. In those cases, in fact, experiments to assess specificity and optimum doses of different antagonists are difficult. Furthermore, developmental maturation and/or tissue-dependent changes in receptor subtype expression may occur.

Bearing in mind these limitations, I first observed that spontaneous inhibitory transmission was differentially affected by the application of selective antagonists against muscarinic receptor subtypes. These data, even if of difficult interpretation, clearly indicated the presence of tonic acetylcholine, endogenously present in the brain stem slices, and affetting the network-based activity impinging upon HMs.

Then, I tested the ability of different antagonists to block muscarinic effects on inhibitory transmission. The muscarine-mediated action on evoked GABAergic transmission was blocked by pirenzepine and DAMP, apparently indicating that it was mediated by M_1 and M_3 receptors. However, because DAMP can act on M_1 and M_3 receptors (Caulfield, 1993), any identification of a response as mediated by M_3 receptors requires demonstration of minimal sensitivity to a typical M_1 blocker like pirenzepine. Hence, the present results, based on DAMP and pirenzepine antagonism, can be interpreted as due to M_1 plus M_3 receptors or to M_1 alone. The latter possibility is made more likely by my preliminary immunohistochemical experiments, performed on brain stem slices containing the hypoglossal nucleus, that did not show any positive staining with a fluorescent antibody against M_3 receptors (data not shown).

The muscarine-mediated action on evoked glycinergic IPSCs was blocked by AFDX application, apparently indicating that it was mediated by M_2 receptors. Even if AFDX is not considered to be strongly selective for M_2 receptors (Caulfield, 1993), the

absence of any action exerted on GABAergic evoked transmission, seems to confirm its selectivity, at least in our experimental conditions.

As far as spontaneous transmission is concerned, the pharmacological pattern of antagonist block was more complex, presumably reflecting the heterogeneity of network inputs responsible for spontaneous events. Muscarine effects on spontaneous GABAergic current amplitude were apparently prevented by each one of the four antagonists. However, muscarine-induced reduction in sIPSC frequency was prevented only by AFDX pre-application. These results seem to indicate that M_2 receptors are mainly presynaptically localized.

Muscarine effects on spontaneous glycinergic currents were blocked by AFDX or tropicamide, in terms of amplitude reduction, and by DAMP, in terms of frequency reduction. AFDX application strongly reverted the depressant effect exerted by muscarine on sIPSC frequency. These data again show the predominant role of putative, presynaptically-located M_2 receptors.

Finally, when I studied postsynaptic glycinergic or GABAergic currents, the muscarine action was blocked by AFDX or DAMP, respectively.

Even if transduction mechanisms, activated by muscarinic receptor stimulation, were not investigated in the present work, some speculations can be done on this point[6]. As previously discussed, depression of inhibitory transmission could be due to the activation of mAChRs located either on the soma of first order interneurons (where they should cause inhibition) or on second order interneurons (where they should produce facilitation of transmitter release).

In general, postsynaptic muscarinic inhibition can be induced by G-protein-activated inward rectifier potassium channels, whose activation hyperpolarizes the cell membrane. This action seems to be mediated mostly by M_2 receptor subtype. Membrane hyperpolarization can be also due to K_{Ca} opening in response to M_1 or M_3 receptor stimulation. This last effect is mediated by PLC stimulation of Ca^{2+} release from IP_3-sensitive intracellular stores.

On the other hand, muscarinic facilitation of transmitter release can be related an increase in the intracellular calcium induced either by PLC stimulation of Ca^{2+} release from intracellular stores, or by a direct coupling to Ca^{2+} channels in the cell

[6] See for a review (Caulfield, 1993; Brown *et al.*, 1997)

membrane. Also several potassium conductances are known to be inhibited by muscarinic receptor activation, thus producing cell membrane depolarization. Among them K_M channels are preferentially inhibited by M_1 and M_3 receptors, via a PLC-mediated transduction pathway. Also SK (K_{Ca}) channels, K_{IR} and some fast-gating M-like potassium channels can be blocked by mAChRs activation, via less clearly identified intracellular pathways.

4. CONCLUSIONS

With the present work I obtained two main findings. First of all, I demonstrated that glycinergic and GABAergic transmission exert an inhibitory action on hypoglossal motoneurons, during early postnatal development. Thereafter I showed the role of metabotropic cholinergic system on inhibitory transmission. Muscarinic receptor activation strongly depressed glycinergic and GABAergic transmission, via different receptor subtypes located both on the postsyanptic membrane of motoneurons and at network level. Even if the overall action of muscarine on HMs seem to be an increase in cell excitability, muscarinic receptors are known to depress also glutamatergic evoked transmission in hypoglossal nucleus (Bellingham & Berger, 1996). This apparent discrepancy points to the relative weight of synaptic excitatory and inhibitory inputs for motoneuron firing.

Another important conclusion arises comparing muscarinic actions on glycinergic and GABAergic transmissions. It is known that, in some regions such as for example the spinal cord (Jonas *et al.*, 1998) and the abducens nucleus (Russier *et al.*, 2002), GABA and glycine can be co-released from the same presynaptic vesicle. Analogous observations were made also in the hypoglossal nucleus (O'Brien & Berger, 1999), even if in this regions data were controversial (Donato & Nistri, 2000). The present study helps in clarifying this point, showing that glycinergic and GABAergic transmissions are modulated by different muscarinic receptor subtypes, thus indicating their release from different interneurons. So, in HMs, glycinergic and GABAergic co-release from the same synaptic terminal, if present, represents a limited phenomenon only.

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