NEURONAL NICOTINIC RECEPTORS MODULATE GLUTAMATERGIC TRANSMISSION ON NEONATAL RAT HYPOGLOSSAL MOTONEURONS

Thesis submitted for the degree of "Doctor Philosophiae"

S.I.S.S.A. - Neurobiology Sector

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NOTE

During my PhD years in S.I.S.S.A., I took part to the following studies:

1) Pagnotta S.E., Lape R., **Quitadamo C.**, and Nistri A. (2005) Pre- and postsynaptic modulation of glycinergic and GABAergic transmission by muscarinic receptors on rat hypoglossal motoneurons in vitro. ***Neuroscience.*** *130(3): 783-95.*

2) **Quitadamo C.**, Fabbretti E., Lamanauskas N., and Nistri A. (2005) Activation and desensitization of neuronal nicotinic receptors modulate glutamatergic transmission on neonatal rat hypoglossal motoneurons. (submitted to ***Eur. J. Neurosci***.)

All the work reported here arises solely from my own experiments and data analysis, unless otherwise stated. In particular, experiments concerning Immunostaining and Western-blot techniques were carried out by Dr. E. Fabbretti and N. Lamanauskas.
Results published in the work by Pagnotta *et al.* (2005), in which I am co-author, are not presented in this thesis.

ABSTRACT

In the neonate the muscles of the tongue, which are exclusively innervated by the XII cranial nerves originating from the brainstem nucleus hypoglossus, must contract rhythmically in coincidence with breathing, suckling and swallowing. These motor commands are generated by hypoglossal motoneurons excited by glutamatergic inputs. Since in forebrain areas the efficiency of glutamatergic transmission is modulated by neuronal nicotinic receptors (nAChRs), the role and identity of nAChRs within the nucleus hypoglossus of the neonatal rat were explored using an in vitro brainstem slice preparation. This area expressed immunoreactivity for α4, α7 and β2 subunits. Whole cell patch clamp recording from hypoglossal motoneurons showed lack of spontaneous cholinergic events mediated by nAChRs even in the presence of a cholinesterase inhibitor. However, pharmacological antagonism of α7 or β2 containing receptors depressed glutamatergic currents arising either spontaneously or by electrical stimulation of the reticular formation. Hypoglossal motoneurons expressed functional nAChRs with characteristics of α4β2 and α7 receptor subunits, and displaying fast desensitization (time constant of 200 ms) from which full recovery developed within one min. Low (0.5 μM) concentration of nicotine first facilitated glutamatergic transmission on motoneurons and later depressed it through receptor desensitization. When 0.1 μM nicotine was used, only depression of synaptic transmission occurred, in keeping with the suggestion that nAChRs can be desensitized without prior activation. These results highlight the role of tonic nAChR activity in shaping excitatory inputs to hypoglossal motoneurons and suggest that their desensitization by ambient nicotine could contribute to disorders of tongue muscle movements.

INTRODUCTION

1. CENTRAL CHOLINERGIC SYSTEM

Acetylcholine (Ach) was first identified as a neurotransmitter with the famous Otto Loewi experiments on heart muscle cells (1921) that advanced the chemical transmission hypothesis. Ach is synthesized by Choline-Acetyl-Tranferase (ChAT) in cholinergic terminals from choline and acetyl-coenzymeA (Ac-SCoA) (Fig. 1). Ac-SCoA is produced inside mytochondria; choline is supplied by an active transport system from extracellular fluid. After synthesis, Ach is packaged into releasing vescicles. The

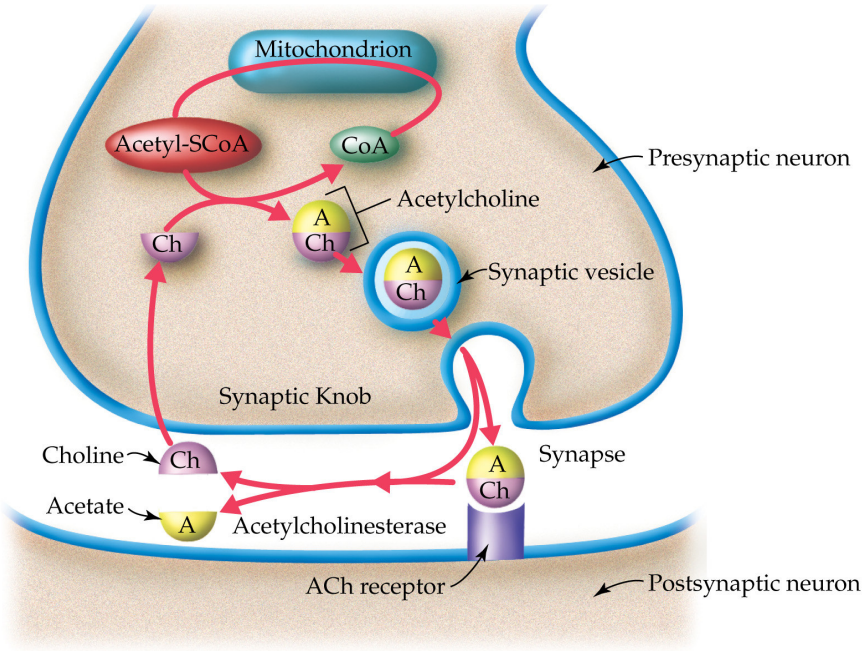


Fig. 1. Schematic representation of a cholinergic synapse.

action of Ach in the synaptic cleft is terminated by ACh-Esterase (AchE), which hydrolyzes ACh into choline and acetate. Choline is then taken up into cholinergic axon terminals becoming available as substrate for new ACh syntesis.

Besides the neuromuscular junction, Ach acts as a neurotransmitter at autonomic ganglia and at some central synapses. The central cholinergic system has not yet been completely described, but the available information suggests that it is made up of a series of closely connected subsystems (Fig. 2). The major cholinergic subsystems originate from groups of neurons located in the basal forebrain and the pons-mesencephalon. The basal forebrain contains two groups of cholinergic neurons:

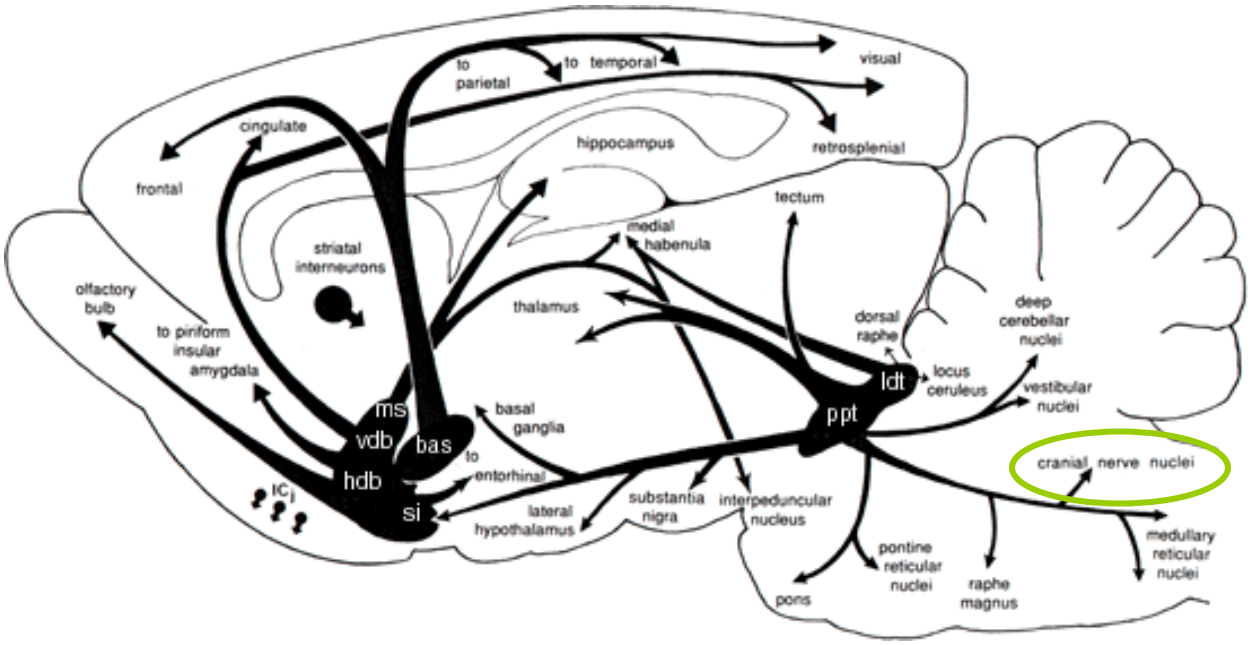


Fig. 2. Central cholinergic pathways. Cholinergic neurons in the basal forebrain and pons-mesencephalon display widespread projections (see text). (scheme adapted from Woolf, 1991).

(1) the medial septal group (medial septal nucleus and vertical diagonal band: ms and vdb) that project axons to the hippocampus and para-hippocampal gyrus and (2) the nucleus basalis group (nucleus basalis, substantia innominata and horizontal diagonal

band: bas, si, hdb) projecting to all parts of the neocortex, parts of limbic cortex and to the amygdala. The pontomesencephalon cholinergic neurons (laterodorsal tegmental and pedunculopontine tegmental nuclei: ldt and ppt) project onto hindbrain (including brainstem), thalamus, hypothalamus and basal forebrain. The striatum contains an intrinsic local circuit of cholinergic neurons (reviewed in Woolf, 1991). Each cholinergic neuron innervates a discrete area making contacts with the dendrites of many other neurons. This extensive interconnection may lead to coordinate firing of closely spaced neurons.

Cholinergic signal is transduced by two different classes of receptors, classified according to their pharmacological sensitivity to the exogenous ligands, muscarine and nicotine. It is now common knowledge that these two classes of cholinergic receptors are members of two receptor super-families structurally and functionally unrelated: nicotinic Ach receptors (nAChRs) are ligand gated ion channels, while muscarinic ACh receptors (mAChRs) produce their effect through interaction with GTP-binding proteins, and are therefore referred to as metabotropic receptors. For a long time it was thought that central cholinergic pathways worked mainly through muscarinic synapses. The importance of central nAChRs was disclosed by the effect of tobacco smoking on behavioral and cognitive functions. Nicotine enhances attention and arousal, diminishes anxiety, produces mild analgesia, and can even improve acquisition and retention of short-term memories. Furthermore, studies on patients with cognitive or behavioral disorders such as Alzheimer's disease, Tourette syndrome, Parkinson's disease and schizophrenia demonstrate significant alterations in the expression of CNS nAChRs and in some case have shown therapeutic effects of nicotine administration (Role and Berg, 1996; Paterson and Nordberg, 2000).

2. NICOTINIC ACETYLCHOLINE RECEPTORS

2.1. MOLECULAR STRUCTURE

Best studied at the vertebrate neuromuscular end plate and in the *Torpedo* electric organ, nicotinic acetylcholine receptors (nAChRs) have been, for a long time, a model of ligand-gated ion channels (Fig. 3). This class of receptors includes gamma aminobutyric acid ($GABA_A$ and $GABA_C$), glycine and 5-hydroxytryptamine ($5\text{-}HT_3$) receptors. All these proteins are assembled in a pentameric structure across the plasma membrane, where they act both as "receptor" and as "effector": after binding of agonist to the ligand-binding sites, the inner pore is opened via a conformational change, permitting ions to flow through.

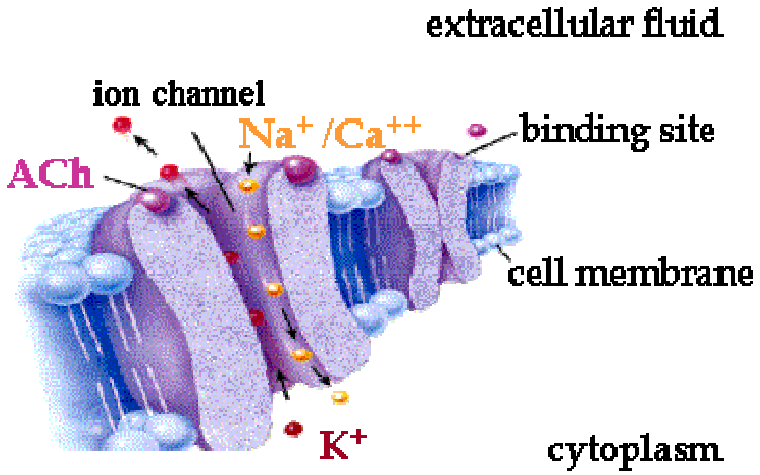


Fig. 3. The nicotinic acetylcholine receptor works as an ionic channel.

During 1970s the nAChR was purified from muscular tissue and identified as a 290kDa glyco-protein. Thereafter, it was identified as made up by five monomers of four different types named α, β, γ and δ according to their increasing molecular weight (Changeux, 1990).

Each subunit is a protein with four putative trans-membrane domains (M1, M2, M3, M4) (Fig. 4). Other features are: a long N-terminal extra-cellular part presenting glycosilation sites; a large intracellular loop comprised between the M3 and M4

segments, containing putative phosphorylation sites; a short C-terminal extracellular domain. Affinity labeling experiments have shown that: (1) the aminoacids involved in the binding of Ach are situated in the long extra-cellular N-terminal domain of two adjacent subunits; (2) the ion channel is lined with the second trans-membrane domain (M2) of all five subunits, which are crucial for the ion selectivity (Cordero-Erausquin, 2000; Corringer *et al.*, 2000).

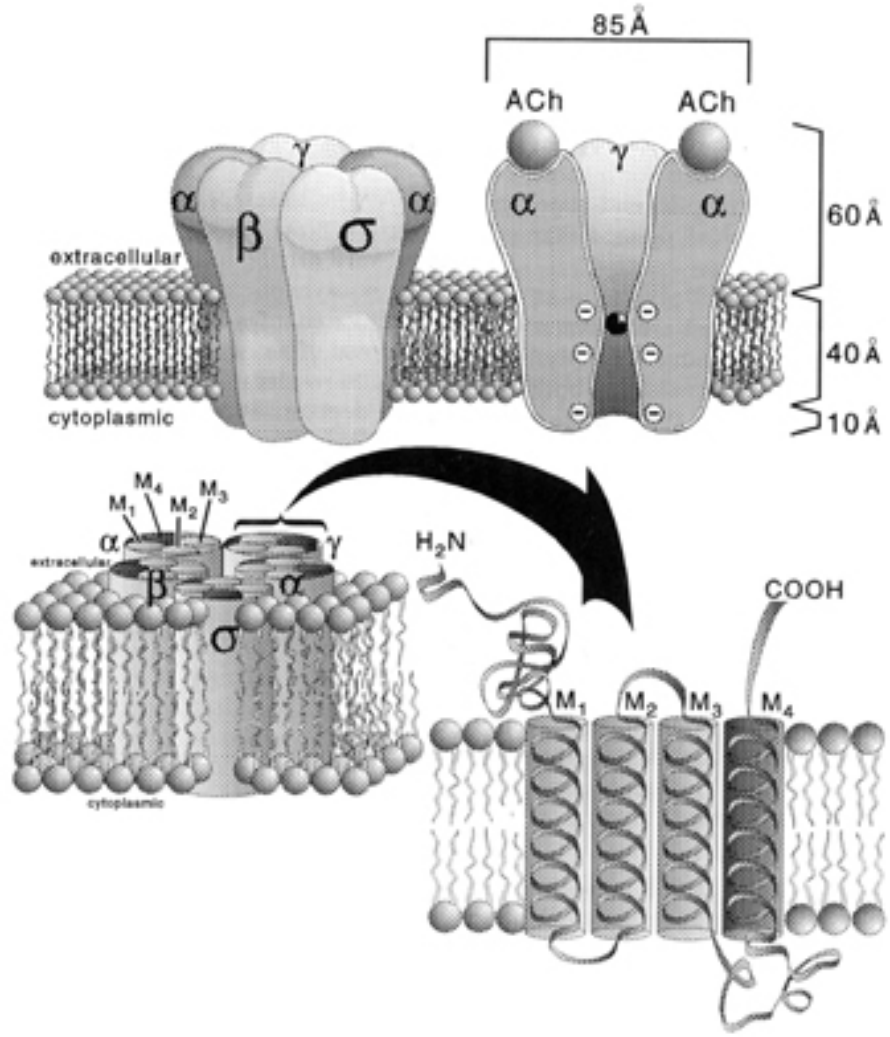


Fig. 4. Schematic model of nAChRs structure. *Top*, this receptor is a membranal heteropentameric glyco-protein. *Bottom*, *left* and *right*, the topology of the subunits: M1, M2, M3 and M4 represent four hydrophobic membrane spanning domains.

2.2. NEURONAL RECEPTOR SUB-TYPES

Following the identification of the genes encoding the neuromuscular receptor subunits, molecular cloning revealed the existence of homologous sequences in other tissues. So far, in vertebrate, twelve more genes that encode for nAChR subunits have been identified: 9 α-type (α2-α10) and 3 β-type (β2-β4) (Sargent, 1993; McGehee and Role, 1995; Itier and Bertrand, 2001). The subunits are called α or β according to their homology to the muscle α1 subunit: assignment to the α subtype group requires the presence of two adjacent cysteines, which are though to participate to the Ach binding site. The existence of such a large number of subunits available for channel formation suggests that they may combine in several ways, creating different channel subtypes with distinct biophysical and pharmacological properties. Since individual gene products can assemble with different partners depending on the available combinations, it is not possible to predict receptor composition simply on the basis of the set of genes expressed by the neuron.

The best characterized receptor subtype is the one found at the skeletal muscle and electric organ. On the other hand, on neurons two major classes of nAChRs can be described (Fig. 5). Expression of α7, α8 and α9 alone is sufficient for the formation of so called "homomeric" channels (although α8 subunit has been found only in avians, not in mammals, and the α9 subunit is only expressed by certain endocrine cells and sensory-end organs, namely cochlear hair cells). They are thought to contain five ligand binding sites, one at each interface between one α and the adjacent subunit (Elgoyhen *et al.*, 1994; Broide and Leslie, 1999; Millar, 2003). Whereas channels formed by co-expression of α and β subunits are said to be "heteromeric", α2, α3 and α4 subunits can form functional AChRs when expressed in combination with β2 or β4 subunits. The predominant brain subtypes preserve the stoichiometry of muscle receptors, with two α and three non-α subunits, and consequently two agonist binding sites. The prevalent brain subtype has been shown to possess the stoichiometry $(\alpha4)_2$ $(\beta2)_3$. It has also been reported that a co-assembly of the α7 and β2 subunits might occur, when these subunits

are co-expressed in *Xenopus* oocytes; perhaps this co-assembly may help to explain some nAChR channel diversity in the nervous system (Khiroug *et al.*, 2002).

An even more complex subgroup of heteromeric subtypes has been described: "triplex" receptors containing more than one type of α or β subunit, including the α5, α6 and β3 subunits; these subunits are unable to form channels when expressed alone or as a pair with any other single α or β subunit (Conroy and Berg, 1995; Le Novere *et al.*, 1996; Wang *et al.*, 1996; Colquhoun and Patrick, 1997; Groot-Kormenlink *et al.*, 1998; Yu and Role, 1998; Lindstrom, 2000). These subunits are also known as auxiliary subunits because they do not directly participate in the formation of the ligand binding site; they probably occupy the same relative position as β1 subunit in muscle nAChRs, and may have a role in controlling either ion permeability, desensitization or receptor location.

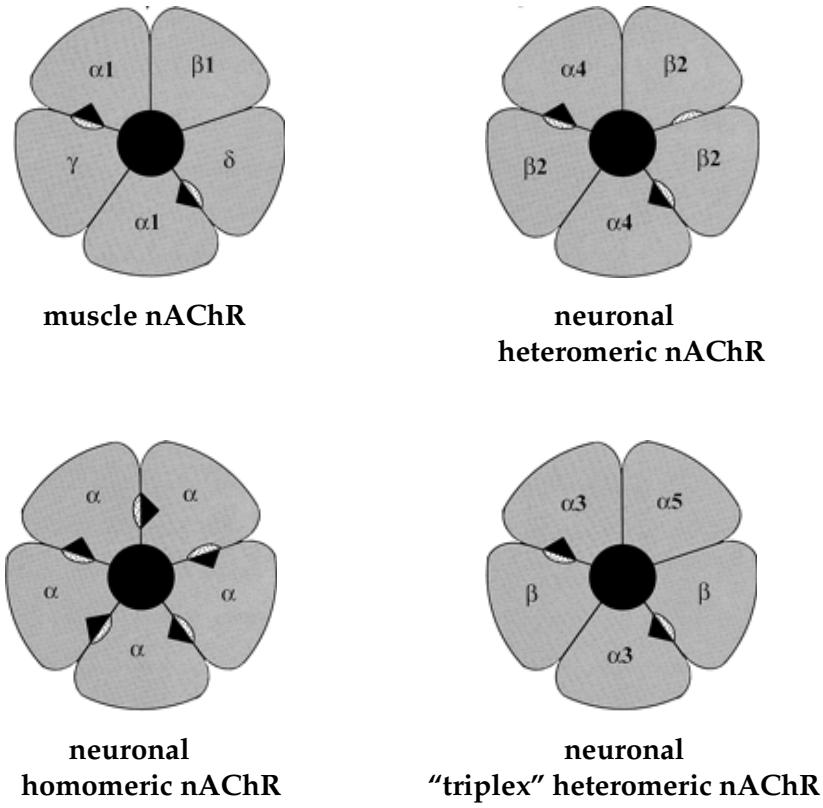


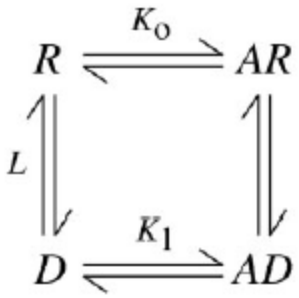
Fig. 5. Different classes of nAChR subtypes: subunit stoichiometry and arrangement (modified from Wang *et al.*, 1996).

2.3. FUNCTIONAL PROPERTIES

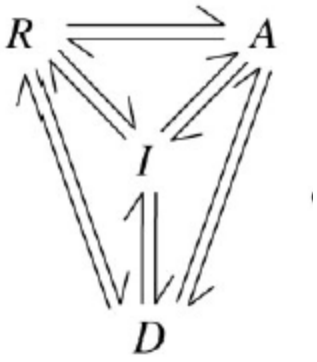
After agonist binding, the ion channel is stabilized in the open conformation for several milliseconds. Through the opened pore cations can freely flow, causing a local depolarization of the cell membrane and eventually an intracellular signal. Beside sodium and potassium, also calcium takes part in this ionic current (Vernino *et al.*, 1992; Seguela *et al.*, 1993; Fucile, 2004; Oikawa *et al.*, 2005). Calcium entry mediated by nAChRs is different from its flow through voltage-gated channels or the N-methyl-D-aspartate (NMDA) glutamatergic receptor subtype. Nicotinic receptors can mediate calcium entry at resting or hyperpolarized membrane potential, while voltage-gated calcium channels and NMDA receptors require membrane depolarization to activate. Moreover, calcium entry may have different biological relevance depending on the spatial distribution of nAChRs on the cell surface.

Beside their closed resting and the open conducting states, nicotinic receptors show another basic conformational state, namely the desensitized one. As a consequence of prolonged exposure to the agonist, the channel closes to a reversible, non-conducting state that remains unresponsive until agonist comes off the binding sites. Katz and Thesleff (1957) first described in detail desensitization of muscle nAChRs. In their report they proposed a cyclical scheme in which nAChRs may exist in two functional states: the resting or activable (R) and the desensitized (D) (Fig. 6). The R conformation has a relatively low affinity and requires high concentrations of agonist to couple binding to channel opening. In contrast, the D state has a higher affinity for agonist; therefore at low concentrations, ligand binds to pre-existing D receptors, stabilizing the desensitized conformation. The disrupted equilibrium between R and D states is restored by transition of receptor molecules from R to D, and, after sufficiently long time, all receptors will end up desensitized. Hence desensitization can occur even at quite low concentration of agonist (Katz and Thesleff, 1957; Quick and Lester, 2002).
During the past decades the idealized model for nAChRs has changed from a simple on-off cationic conductance to a sophisticated allosteric molecule (Changeux *et al.*,

1984). Changeux and co-workers suggested that desensitization represents a classical form of allosteric protein behavior, which the receptor acquires in the absence of the agonist. Moreover, they introduced an intermediate desensitized state (I), in order to reflect the non-exponential time course of desensitization onset and recovery (Feltz and Trautmann, 1982; Boyd, 1987; Edelstein and Changeux, 1998). In this picture nicotinic receptors can exist in at least four interconvertible and functionally distinct conformational states (Fig. 6). In the absence of the ligand, the receptor spontaneously changes state, but even if a transition is possible, the probability of channel opening is extremely low. The four described allosteric states are named R, A, I and D. At resting conditions, the closed (R) state is the main one, however, some molecules are always in the desensitized (D) state. Exposure to the agonist preferentially stabilizes the active (A) open state and then subsequently the intermediate (I) closed state and finally, with a slower time constant, the desensitized (D) closed state.



(Katz and Thesleff, 1957)



(Changeux *et al.*, 1984)

Fig. 6. Kinetic models for nAChR desensitization. *Left*: cyclical scheme for desensitization of nAChRs, where L is the allosteric constant reflecting the distribution of receptors in the R/D state, and K_0 and K_1 are the ligand constant affinities relative to the R and D state, respectively. *Right*: cyclical model that includes two desensitized states, of intermediate (I) or high (D) affinity. (modified from Quick and Lester, 2002).

Both nicotine and ACh can produce desensitization at concentrations that do not activate a significant fraction of their receptor population necessary to elicit a

macroscopic response (Paradiso and Steinbach, 2003). The physical nature of the multiple desensitized states is not known, but it has been proposed that one (or more) of the slowly recovering processes may reflect phosphorylation changes. Phosphorylation may also regulate movements between functional and inactive pools of receptors (Huganir *et al.*, 1986; Boyd, 1987; Fenster *et al.*, 1999).

The kinetic constants governing the rate of conformational changes, as well as the ion permeability in the open state, depend on many factors including the subunit composition. The relations between channel structure and its function can be mainly established thanks to heterologous expression studies in which host cells, usually *Xenopus* oocytes, are injected with the appropriate mRNA or cDNA. Oocytes do not normally express native nicotinic receptors, yet after appropriate injections, functionally active channels are generated, and their currents are studied by means of electrophysiological techniques.

Both α and β subunits contribute to the pharmacological and functional profiles. In particular, there are clear distinctions among the neuronal nAChR complexes, whether generated by the expression of a single α-type subunit (homomeric) or by co-expression of α and β subunits (heteromeric) (McGehee and Role, 1995; Role and Berg, 1996). α7-homomeric receptors display greater calcium permeability and can be gated either by ACh or by choline; this receptor subtype often shows fast onset of desensitization. While the exact number of receptors that falls into the heteromeric class remains to be elucidated, it is thought that receptors containing β2 subunits desensitize faster than those containing β4 subunits, independently of the α subunit expressed. Conversely, receptors containing α3 subunits desensitize faster than those containing α4 (Fenster *et al.*, 1997; Quick and Lester, 2002; Wang and Sun, 2005). All these classes of receptors may be involved in different aspects of ACh function in the CNS. For instance, acting via homomeric receptors, ACh can induce short-lived or phasic membrane depolarizations, while acting via heteromeric receptors, ACh can generate sustained or tonic depolarization (Tribollet *et al.*, 2004).

At a cholinergic synapse, approximately 1-3 mM ACh is rapidly released into the cleft, immediately activating nicotinic receptors. In few milliseconds ACh is hydrolyzed by the esterase and/or diffuses away. The half-time of ACh is very short and therefore desensitization is usually not thought to be important in physiological conditions. But at very active synapses when repeated pre-synaptic inputs occur, nAChRs are more susceptible to desensitization. In these conditions the onset of desensitization may prevent excessive stimulation because all nAChRs are Ca^{++} permeable (McGehee and Role, 1995), and therefore potentially excitotoxic to neurons. Moreover, several workers have suggested that the selective desensitization of one receptor subtype, while other subtypes remain active, may play a significant role in changing the plasticity of particular brain networks. The physiological role of desensitization may become especially important when considering nicotine plasma levels in smokers (Ochoa *et al.*, 1990; Dani and Heinemann, 1996), or the effect of drugs that inhibit ACh-Esterase (used, for instance, to treat Alzheimer's disease).

In addition, a variety of pharmacological agents regulating the functional states of nAChRs has been described (Lena and Changeux, 1993), like extracellular calcium which increases the ACh evoked current at α7 homomeric receptors, and a calcium recognition site was identified near the ACh binding site. Similarly, heteromeric receptors show sensitivity to zinc: in particular β4-containing receptors are potentiated by low concentrations of zinc, while β2 receptors are inhibited (Vernino *et al.*, 1992; Hsiao *et al.*, 2001).

The wide diversity of nAChR subtypes, although problematic for investigators, allows sensitive tuning of the cholinergic transmission.

2.4. PHARMACOLOGY

Different subunit combinations determine topographical variations at the ligand binding site, which in turn accounts for the pharmacological differences among nicotinic receptor populations. Ach is the major endogenous ligand for all nicotinic receptors subtypes, while its metabolite choline selectively gates the α7 subtype (Alkondon *et al.*, 1997). The exogenous alkaloid nicotine, present in *Nicotiana tabacum*, mimics the effect of ACh activating all nAChR subtypes, and even epibatidine, the most potent natural agonist at nAChRs, obtained from skin extracts of an Amazonian frog, is not subtype specific (Badio and Daly, 1994).

Pharmacological criteria have been used to classify nAChR subtypes, first of all the different sensitivity of nAChRs to the snake toxin α-bungarotoxin (α-BTX) (Clarke *et al.*, 1985). It was shown that homomeric receptors α7, α8 and α9 are blocked by α-BTX, whereas heteromeric receptors are insensitive to this toxin (McGehee and Role, 1995). Another natural compound acting on homomeric nAChR subtype is the alkaloid methyllycaconitine (MLA). The great advantage of using MLA is that its effect is completely reversible. By the way, its action is selective, rather than specific because MLA binds to α7 receptors with a Ki near to 1 nanomolar, while muscle and α4β2 nAChRs require much higher concentrations for inhibition (Alkondon *et al.*, 1992; Palma *et al.*, 1996). On the other hand, the alkaloid di-hydro-β-erythroidine (DHβE), isolated from *Erythrin*a seeds, displays heteromeric subtype specific antagonism at sub-micromolar concentrations (Harvey *et al.*, 1996; Chavez-Noriega *et al.*, 1997). Also the venoms extracted from *Conus* snails are an interesting source for pharmacologically active drugs, among which the α-conotoxin MII, at nanomolar concentrations, showed a potent and selective antagonism for the α3β2 nAChR subtype (Cartier *et al.*, 1996; Harvey *et al.*, 1997; Kaiser *et al.*, 1998). Competitive antagonists displace the agonist from its binding sites, whereas open channel blockers, such as mecamylamine, bind within the pore, and there are multiple sites for other non-competitive inhibitors and modulators such as physyostigmine and galanthamine (Pereira *et al.*, 1994; Schrattenholz *et al.*, 1996). In addition, nAChRs activity is subjected to regulation by

several other factors, including peptide transmitters, various protein kinases, and Ca^{++} (Hopfield *et al.,* 1988; Mulle *et al.*, 1992; Davis *et al.*, 2001).

A general problem in addressing nAChR function in the brain remains the lack of specific agonists and antagonists for each of the receptor subtypes.

2.5. RECEPTORS DISTRIBUTION AND LOCATION

Much of the current knowledge regarding neuronal nAChRs has been obtained from *in vitro* binding studies (Marks *et al.*, 1986). In situ hybridization and immunohistochemical experiments have furthered our understanding of native nAChRs composition (Wada *et al.*, 1989). These studies indicate a wide and non-uniform distribution of various nAChR subunits. Although one class of nicotinic receptor often predominates within a region, usually more than one subtype is detectable.

Nevertheless, the most diffuse receptor subtype in vertebrate nervous system seems to be the $\alpha4\beta2$ subtype, which can also contain $\alpha5$ subunit, and the $\alpha3\beta4$ subtype, with or without $\alpha5$. These receptors, corresponding to high-affinity binding sites for nicotine, have been detected in the thalamus, hippocampus, *substantia nigra*, striatum, cerebral cortex and cerebellum. The $\alpha6$ containig receptors, very often in conjunction with the $\beta3$ subunit, are present in the optic pathway, the *locus coeruleus* and dopaminergic neurons of the mesostriatal pathways where they control dopamine release (Picciotto *et al.*, 1998; Perry *et al.*, 2002). The $\alpha7$ containig receptors, which account for α-BgTX binding (Orr-Urtreger *et al.*, 1997), are also rather diffuse, particularly in the hippocampus, hypothalamus, cortex and brainstem, whereas $\alpha9/\alpha10$ containing receptors are present extra-neuronally in limited area such as the olfactory epithelium and the cochlea (Gotti *et al.*, 1997).

nAChRs are strongly expressed early in embryonic life, suggesting their role during development (Zoli *et al.*, 1995; Agulhon *et al.*, 1999a; 1999b). Varying the level of intracellular Ca^{++} can have strong consequences on cell metabolism, from altering gene expression to triggering apoptotic cell death (Ghosh *et al.*, 1995; Broide *et al.*, 1999).

In the periphery, neuronal nAChRs have been detected not only in ganglionic tissue, but also in developing and denervated muscle, on keratinocytes and on peripheral blood cells, notably polymorphonuclear cells and lymphocytes (Gotti and Clementi, 2004).

Anatomical and functional evidence suggests that neuronal nAChRs are preferentially located on pre-synaptic boutons where they contribute to the regulation of secretion of other neurotransmitters via sustained Ca^{++} influx (Wonnacott, 1997). In many cases the α7 subtype, which is the most permeable to calcium among the nAChRs class, mediates increased synaptic release, but in some cases other subtypes are involved. Indeed, the magnitude and time course of pre-synaptic facilitation elicited by nAChR activity differs from synapse to synapse, and these differences may be the result of receptor composition (Grady *et al.*, 1992; Guo *et al.,* 1998; Alkondon *et al.*, 1999). Activation of pre-synaptic nAChRs enhances the release of many neurotransmitters in diverse regions of the brain. In particular, pre-synaptic nAChRs have been implicated in the release of ACh, norepinephrine, dopamine, glutamate and GABA (McGehee *et al.*, 1995; Maggi *et al*., 2001). It is common knowledge that nicotine addiction is mediated by the influence of nAChRs on dopamine release in the mesolymbic pathway (Wonnacott, 1997; Fisher *et al.,* 1998; Li *et al.,* 1998; Radcliffe *et al.,* 1999). Certainly, the presence of cholinergic projections within terminal fields displaying nAChRs is suggestive of endogenous cholinergic activation via pre-synaptic nAChRs.

In addition to controlling and modulating the release of various neurotransmitters, pre-synaptic nAChRs may play an important role in regulating neuronal growth, path-finding and differentiation.

Nicotinic receptors can modulate neurotransmitter release also at peri-terminal locations. Due to the high impedance of pre-terminal fibers, the gating of relatively few axonal nAChRs may elicit significant changes in synaptic excitability. nAChRs activation may initiate Na^{+}-mediated axonal membrane depolarization, which in turn activates Ca^{++} entry through voltage gated channels. This action is blocked by

tetrodotoxin which prevents voltage-gated sodium channel opening and therefore stops depolarization advancing (Lena *et al.*, 1993). Moreover, strategically located nAChRs might enable activation of only selective portions of the axonal arborization.

There is also evidence that high-affinity, nicotine binding nAChRs are located on postsynaptic membranes (Clarke, 1993). Many neurons can generate fast inward currents in response to exogenously applied Ach, but relatively few direct nicotinic synapses have been documented in the mammalian brain. These receptors are regarded to mediate fast excitatory synaptic transmission on GABAergic interneurons in the hippocampus, and on both glutamatergic pyramidal cells and GABAegic interneurons in the developing visual cortex (Roerig *et al.*, 1997; Alkondon *et al.*, 1998). In many cases nicotinic transmission is a minor component of the excitatory input, which is overwhelmingly glutamatergic (Collingridge and Lester, 1989).

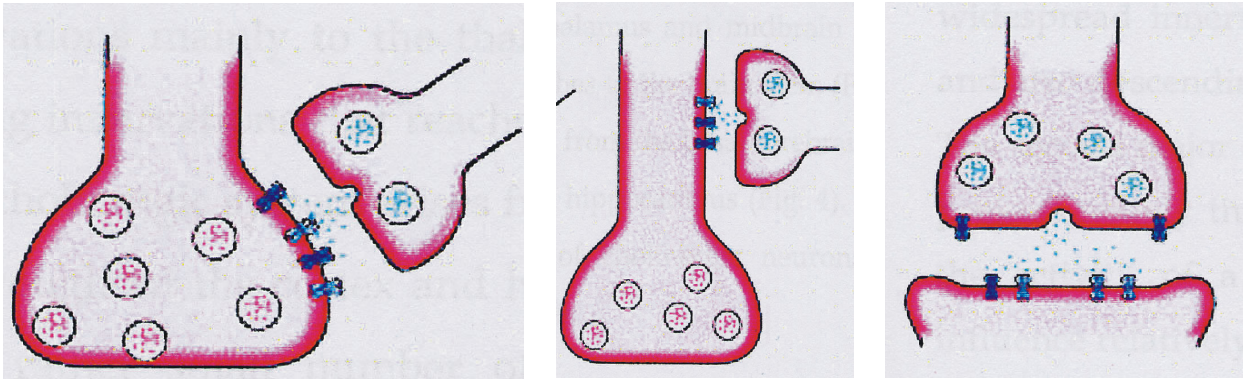


Fig. 7. Schematic representation of nAChR locations: (*left*) pre-synaptic, (*center*) peri-synaptic, (*right*) post-synaptic.

The intrinsic difficulty in studying central cholinergic synapses arises from anatomical considerations (Wolf, 1991): cholinergic projections are diffuse and nAChR containing targets are scattered throughout the brain. The density of synapses is rather low and therefore they are difficult to detect in a brain slice preparation, which is a commonly used experimental model.

It is thought that the physiological role of cholinergic systems may be regulating the gain and fidelity of synaptic processes by increasing the signal-to-noise ratio. Properly timed nAChR activity could facilitate transmitter release and boost the efficiency of synaptic transmission, ensuring that important events emerge out via enhanced synaptic probability of transmitter release (Maggi *et al.*, 2003; 2004). It is this fidelity that might be lost by the impairment in nicotinic function in the hippocampus during Alzheimer's disease.

In addition to controlling pre or post-synaptic element activation, nAChRs may regulate several cell functions, activating various downstream events simply increasing intracellular Ca^{++} levels. Receptor location itself, together with the cellular machinery accessible from that site, is highly determinant for calcium-dependent processes (Berg and Conroy, 2002).

3. A MODEL SYSTEM FOR STUDYING NICOTINIC RECEPTOR FUNCTION: THE HYPOGLOSSAL MOTONEURONS

3.1. MOTOR NEURONS

For a long time after the studies by Sherrington and Eccles, motoneurons have been considered to be paradigmatic to understand how neurons work. Many of the basic properties of neurons, and even the fundamental statement that chemical neurotransmission is the principal form of neuronal communication, were first identified in motoneurons. A huge amount of information relative to neuronal properties has been collected, but one difficulty in properly understanding all this is due to the absence of data concerning how neurons process incoming synaptic signal into actual function. In this regard motoneurons display special advantage, since their information coding is translated into action potential discharges, which, in turn, produce contraction of skeletal muscular fibers.

Although excitatory synaptic transmission to motoneurons is mainly glutamatergic, other neurotransmitters may contribute to excitatory input, among which ACh (Connaughton *et al.*,1986). In mammals, nAChRs not only play a crucial role in muscle contraction, but also modulate motoneurons excitability via Renshaw cells activation (Eccles *et al.*, 1954; Dourado and Sargent, 2002). Both spinal and cranial motoneurons are contacted by choline acetyltransferase (ChAT) containing axon terminals. Moreover, it has been shown that either class of motoneurons expresses functional nAChRs (Zaninetti *el al.,* 1999; Ferreira *el al.*, 2001; Ogier *el al.,* 2004). *In-situ* hybridization experiments performed by Wada and colleagues (1989) have revealed the presence of mRNA strands relative to many nAChR subunits in several brainstem nuclei including trigeminal (V), facial (VII), vagal (X) and hypoglossal (XII) nuclei.

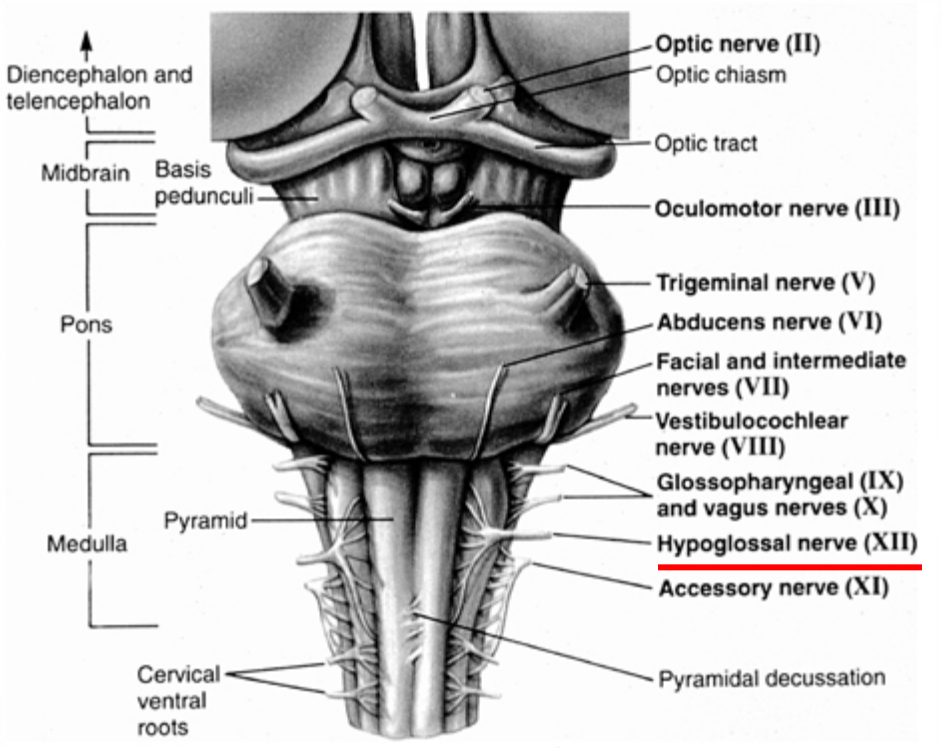


Fig. 8. Brainstem, ventral view.

3.2. NUCLEUS HYPOGLOSSUS

A suitable model for studying nAChR function in the CNS is the hypoglossal nucleus. It is a dense motor-nucleus, easily identifiable within the brainstem. It is located bilaterally to the medullary midline just beneath the floor of the forth ventricle; its rostro-caudal extent roughly covers the same length as the olive.

At least 90% of neurons within the hypoglossal nucleus are motoneurons (Viana *et al.*, 1990) although there is anatomical evidence for a small population of interneurons (Boone and Aldes, 1984; Takasu and Hashimoto, 1988). However, interneurons are smaller (10-20 μm) than hypoglossal motoneurons (HMs) themselves (20-40 μm). Moreover, interneurons show few dendritic processes and are confined to the ventrolateral or dorsolateral borders of the nucleus. On the other hand, HMs are large multipolar neurons spreading dendrites extensively within the hypoglossal nucleus and also into the neighboring reticular formation. Axons from HMs travel ventrally through the medulla and then emerge to form the hypoglossal nerve (XII cranial nerve, c.n.), which innervates the tongue (Lowe, 1980).

Lingual musculature comprises intrinsic and extrinsic muscles. The former have no bone anchoring and are located within the body of the tongue. They include vertical, transversal, superior, and inferior longitudinal muscles: their activity shapes the tongue. The extrinsic muscles have clear bony attachment, and include the genioglossus, geniohyoid, styloglossus, hyoglossus and palatoglossus, whose activity is fundamental in tongue protrusion, retraction, depression and elevation. The hypoglossal motor nucleus is myotopically organized. In the rat, HMs innervating tongue protruder muscles are located in the ventrolateral part of the XII nucleus, whereas HMs sending axons to retractor muscles are located more dorsally (Lewis *et al.*, 1971; Krammer *et al.*, 1979; Dobbins and Feldman, 1995).

Various peripheral sites (trigeminal, vagal and hypoglossal nerves), brain stem centers, and higher centers have been shown to exert an influence on XII motoneuron activity (Borke *et al.*, 1983; Takada *et al.*, 1984; Li *et al.*, 1993; Dobbins and Feldman, 1995; Ugolini, 1995; Sahara *et al.*, 1996; Hostelge *et al.*, 1997; Jean, 2001; Zhang *et al.*,

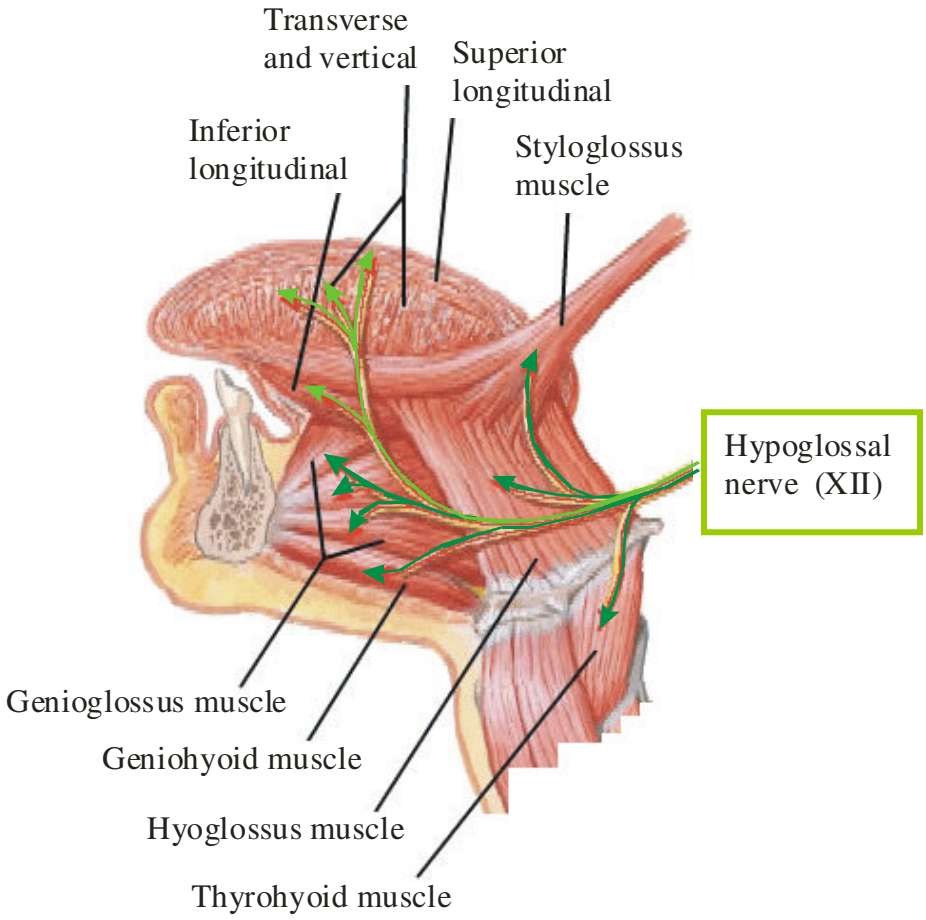


Fig. 9. Sagittal section of the head showing tongue's musculature and its relative innervation. (freely adapted from Frank H.Netter's Anatomical atlas).

2001); anyway, no direct cortical connections to orofacial motor nuclei, including the XII, have been demonstrated in rats, confirming that voluntary motor commands to motoneurons pass through various relay stations (Travers and Norgren, 1983).

It is the lateral reticular formation (LRF) that provides the primary source of inputs to the XII nucleus. In particular, hypoglossal premotor neurons are ventrolateral and dorsolateral in the medullary reticular formation (Borke *et al.*, 1983; Travers and Norgren, 1983). The origin of LRF projections was localized to the nucleus reticularis parvocellularis (RPc) (Zhang and Luo, 2003). And in turn, RPc receives afferents from the trigeminal (V) and solitary nuclei (Lowe, 1980; Borke *et al.*, 1983; Dobbins and Feldman, 1995; Luo *et al.*, 2001). RPc is regarded as the brainstem center that coordinates

motor sequences of mastication, swallowing, respiration and vocalization. Some of hypoglossal premotor neurons are GABAergic, glycinergic, or glutamatergic (Collingridge and Lester, 1989; Singer *et al.,* 1996; Li *et al.,* 1997; O'Brien *et al.,* 1997; O'Brien and Berger, 1999). The integration of all these synaptic inputs, in concert with the intrinsic membrane properties of motoneurons themselves, determines the output signals (Berger, 2000).

The cholinergic neurons in the laterodorsal tegmental and pedunculopontine tegmental nuclei (LTD/PPT) are thought to provide the primary source of cholinergic projections to the hypoglossal nuclei (Connaughton *et al.,* 1986). Immuno-cytochemical experiments performed using Ab against choline acetyl-transferase (ChAT) and the vescicular ACh trasporter (VAChT) has revealed the presence of staining fibers forming large puncta/spots around motoneuron cell bodies and dendrites in the hypoglossal nucleus, suggesting that this nucleus is the target of cholinergic innervation (Gilmor *et al.*, 1996; Arvidsson *et al.*, 1997; Schäfer *et al.*, 1998; Ferreira *et al.*, 2001). An intriguing possibility is that these motoneurons, by virtue of recurrent axon collaterals, could influence the activity of neighbouring cells via post-synaptic nAChRs. Such a direct synaptic coupling between motoneurons, already established in some motor systems, could facilitate motor unit recruitment, and enhance the synchrony of synergistic motoneuron firing (Perrins and Roberts, 1995).

Electrophysiological studies reported that all HMs, regardless of their location, respond to stimulation by nicotinic agonists (Zaninetti *et al.,* 1999; Chamberlin *et al.,* 2002; Shao and Feldman, 2005). Very recent studies demonstrate positive immunoreaction in HMs for various nAChR subunits, suggesting a role for nicotinic receptors in the regulation of hypoglossal nucleus activity (Ferreira *et al.*, 2001; Dehkordi *et al.,* 2005). The density and distribution of nAChRs, assessed through binding experiments, is higher in the whole brainstem during embryonic and postnatal development in comparison with adult animals. Similar data are found in cortical and hippocampal tissue as well, suggesting a role for nAChRs in the maturation of all these brain structures (Tribollet *et*

al., 2004). It is feasible that increased nAChR expression may be related, and possibly contribute, to the establishment of functional neuronal connectivity.

As the XII nerve innervates the tongue, HMs are engaged in several motor functions, including suckling, swallowing, and mastication (Jean, 2001). Moreover, because of tongue's critical position in the upper airways, the hypoglossal nucleus is also active during respiration (Strohl *et al.*, 1980). Rhythmic HMs activity, strictly correlated with the inspiratory burst generated in the pre-Bötzinger complex of the lower brain stem, has been monitored in different experimental systems, both in "in vivo" (Richmonds and Hudgel, 1996; Pierrefiche *et al.*, 1997) and "in vitro" conditions (Suzue, 1984; Murakoshi *et al.*, 1985; Smith *et al.*, 1991; Funk *et al.*, 1993; Ballanyi *et al.*, 1999). It is noteworthy that nicotine not only increases respiratory rhythm, but also the activity of HMs, in particular of those projecting to the genioglossus muscle (the major extrinsic tongue protruder muscle), whose function is fundamental for the maintenance of upper airway patency (Remmers *et al.*, 1978; Shao and Feldman, 2001; Robinson *et al.*, 2002; Shao and Feldman, 2002). Indeed, many investigators suggest a clinical use of nicotinic agonists in the treatment of obstructive-sleep-apnea, a disease that involves sleep related loss of genioglossus muscle tone (Strohl *et al.*, 1980; Gothe *et al.*, 1985; Bellingham and Funk, 2000).

4. AIMS OF THE PRESENT STUDY

The purpose of this study was to investigate whether nAChRs expressed in the nucleus hypoglossus are functional, to explore their pharmacological characteristics and their role in modulating glutamatergic transmission at early postnatal age. Furthermore, we investigated how nicotine can desensitize nAChRs and consequently affect glutamatergic transmission on HMs. Although nAChRs can control the activity of

inhibitory neurons, a large number of studies have indicated that the main action of nAChRs is to modulate glutamate mediated transmission. Hence, in the present report, we deliberately restricted the experimental variables of our in vitro model by systematically blocking GABA and glycine mediated inhibition. This enabled us to study glutamatergic events in isolation and to ascertain their efficiency in the presence of nicotinic agonists or antagonists in concert with changes in postsynaptic nACh activity.

In view of the adverse effect of prenatal nicotine exposure on nervous system development (Nordberg *et al.*, 1991; DiFranza and Lew, 1995; Slotkin *et al.*, 1999; Robinson *et al.*, 2002), it is still an open question the mechanism by which maternal smoking affects the neonate's ability to generate motor commands to the tongue muscles responsible for breathing and milk suckling. The basic mechanisms that might underlie this important issue were investigated by testing the effect of small concentrations of nicotine, similar to those found in the plasma of smokers, on glutamatergic transmission in neonatal rat HMs.

METHODS

1. TISSUE PREPARATION

Experiments were carried out on brainstem acute slices obtained from neonatal Wistar rats (from 0 to 5 days old; P0-5) according to the method previously described by Viana *et al.,* (1994). Animals were decapitated after being anaesthetized with intraperitoneal injection of urethane (2 g/kg body wt). This procedure is in accordance with the regulations of the Italian Animal Welfare Act following the European Community directives and approved by the local authority veterinary service.

Thereafter, the brainstem was quickly removed and submerged in ice-cold saline (for composition see below). Cooling of the preparation was particularly important, because it minimized damage from anoxia and improved the texture of the tissue for slicing. A vibrating tissue slicer (Vibracut, FTB, Weinheim, Germany) was used to cut 200 µm thick slices. Mechanical stability of the tissue during slicing was essential. For this purpose a tissue block containing the lower medulla was fixed (using insect pins) onto an agar block (4 % in 0.9 % NaCl) shaped to contain the tissue in the correct orientation (caudal-end up). The block was then glued to the stage of the vibro-slicer and immersed in ice-cold physiological saline, saturated with 95% O_2 -5% CO_2.

Slices were first transferred to an incubation chamber containing gassed Krebs solution, kept there for 1 hour at 32°C and then allowed to slowly reach room temperature. After this recovering period, single brainstem slices, were placed in a small recording chamber, held in place by a fine nylon net glued to a horse-shaped platinum wire and continuously perfused (2-3 ml/min) with the recording Krebs solution (see below). Hypoglossal motoneurons (HMs) were identified within the nXII (Fig. 10) with a Zeiss Axioscope microscope connected to an infrared video camera (Fig. 11). Under x40

magnification single HMs were clearly visible and showed a large soma (20-40 μm diameter).

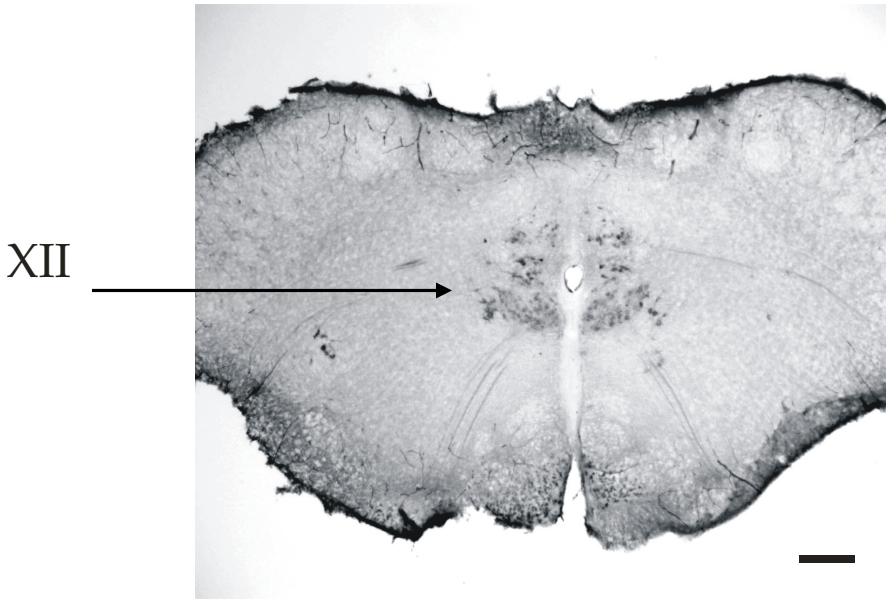


Fig. 10. Neonatal rat brain-stem transverse slice; immunostaining of the hypoglossal nucleus (XII) with anti-ChAT (choline acetyl-transferase) antibodies. (scale bar: 200 μm, courtesy of E. Fabbretti)

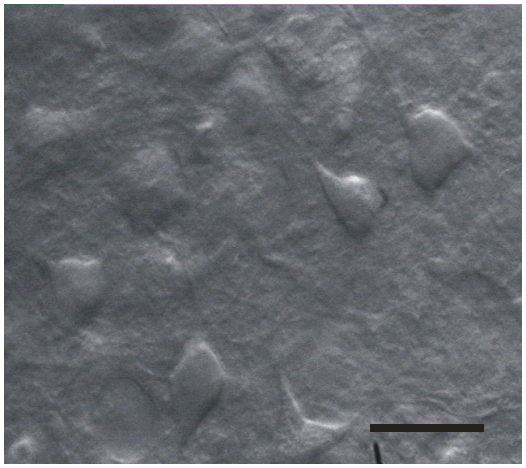


Fig. 11. Hypoglossal motoneurons from P2 rat brain stem slice (scale bar: 50 μm, courtesy of S.E. Pagnotta)

2. SOLUTIONS AND DRUGS

2.1. SLICE PREPARATION AND MAINTENANCE

The solution used for slice cutting and maintenance contained (in mM): NaCl 130, KCl 3, NaH_2PO_4 1.5, $CaCl_2$ 1, $MgCl_2$ 5, $NaHCO_3$ 25, glucose 10 (pH 7.4 adjusted with NaOH; 290-310 mOsm).

2.2. VOLTAGE CLAMP RECORDINGS

The extracellular solution used to perfuse slices during recording contained (in mM): NaCl 130, KCl 3, NaH_2PO_4 1.5, $CaCl_2$ 1.5, $MgCl_2$ 1, $NaHCO_3$ 25, glucose 15 (pH 7.4 with NaOH, 300-320 mOsm). Patch pipettes were filled with intracellular solution containing (in mM): CsCl 130, NaCl 5, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, BAPTA 10, ATP-Mg 2, sucrose 2 (pH 7.2 with CsOH; 280-300 mOsm).

2.3. DRUG APPLICATION

Drugs were applied in two different ways: either bath-applied via the extracellular solution superfused at 2-3 ml/min (for a minimum of 5-10 min to reach apparent equilibrium conditions), or via fast, focal pressure pulses. For the latter method, a thin-walled glass micropipette was pulled in the same way as a patch pipette using a two-stage puller (3P-A, List Medical, Germany) in order to obtain a DC resistance of 5-6 MΩ. The pipette was filled with nicotine (diluted to the final concentration of 2 mM in the external recording solution), and positioned approximately 20-50 μm away from the soma of the recorded cell, under microscopic control. The puffer pipette was connected to a Pneumatic Picopump (WPI, Sarasota, FL, USA); pulses duration ranged from 10 ms to 60 s (4-8 p.s.i. pressure). As long as applications were spaced at intervals of 1 min, observed responses were closely reproducible (see also Khiroug *et al.*, 1998; Pagnotta *et al.*, 2005).

All experiments were performed in the continuous presence of bicuculline (10 μM) and strychnine (0.4 μM) in the bathing solution; these drugs were used as pharmacological

tools to block GABA and glycine-mediated transmissions (Donato and Nistri, 2000; Marchetti *et al.*, 2002). In this way glutamatergic transmission could be studied in isolation.

The following drugs were used: 6-cyano-7nitroquinoxaline-2,3-dione (CNQX), D-amino-phosphonovaleriate (APV) and methyllycaconitine citrate (MLA) purchased from Tocris; bicuculline methiodide (bicuculline), dihydro-β-erytroidine hydrobromide (DHβE), ethyl[*m*-hydroxyphenil]-dimethylammonium chloride (edrophonium), ([-]-1-methyl-2-[3-pyridyl]pyrrolidine) hydrogen tartrate salt (nicotine) and strychnine hydrochloride (strychnine) from Sigma; tetrodotoxin (TTX) from Latoxan.

3. ELECTROPHYSIOLOGICAL TECHNIQUES

3.1. PATCH-CLAMP RECORDING

Recordings were performed at room temperature. The conventional whole-cell patch clamp technique (Hamill *et al.*, 1981) was employed. Briefly, a small heat-polished glass pipette, pulled from thin–walled borosilicate glass capillaries (Hingelberg, Germany) with a two-stage puller (3P-A, List Medical, Germany) to a DC resistance of 3-4.5 MΩ (opening diameter between 0.5 and 1 μm), was pressed against the cell membrane. Gentle suction, applied to the pipette interior, led to the formation of an electrical seal with resistance in the order of 2-10 GΩ (giga-seal). After the giga-seal formation, additional suction applied to the pipette interior led to the membrane rupture, and direct low resistance access to the cell interior.

An L/M-EPC-7 patch-clamp Amplifier (List Medical, Germany) was used for voltage clamp experiments. Cells were clamped at -60, -65 mV holding potential (V_h), and series resistance (5-25 MΩ) was routinely monitored, without any compensation. Voltage pulse generation and data acquisition were performed with a PC using pClamp 8.1-9.2 softwares (Axon Instruments Inc.). All recorded currents were filtered at 3 kHz and sampled at 5-10 kHz.

3.2. ELECTRICAL STIMULATION

Glutamatergic postsynaptic currents were evoked by placing a single bipolar tungsten electrode in the dorso-lateral reticular formation (Fig. 12) to stimulate afferent interneurons to the hypoglossal nucleus (Borke *et al.*, 1983; Cunningham and Sawchenko, 2000). Stimuli of variable intensity and duration (10-100 V, 0.02-0.2 ms) were delivered at 10 s intervals, and were selected in each experiment in order to elicit submaximal responses. Electrically evoked synaptic currents were recorded and stored in a PC as individual files and then averaged with pClamp 8.1-9.2 after discarding failed events.

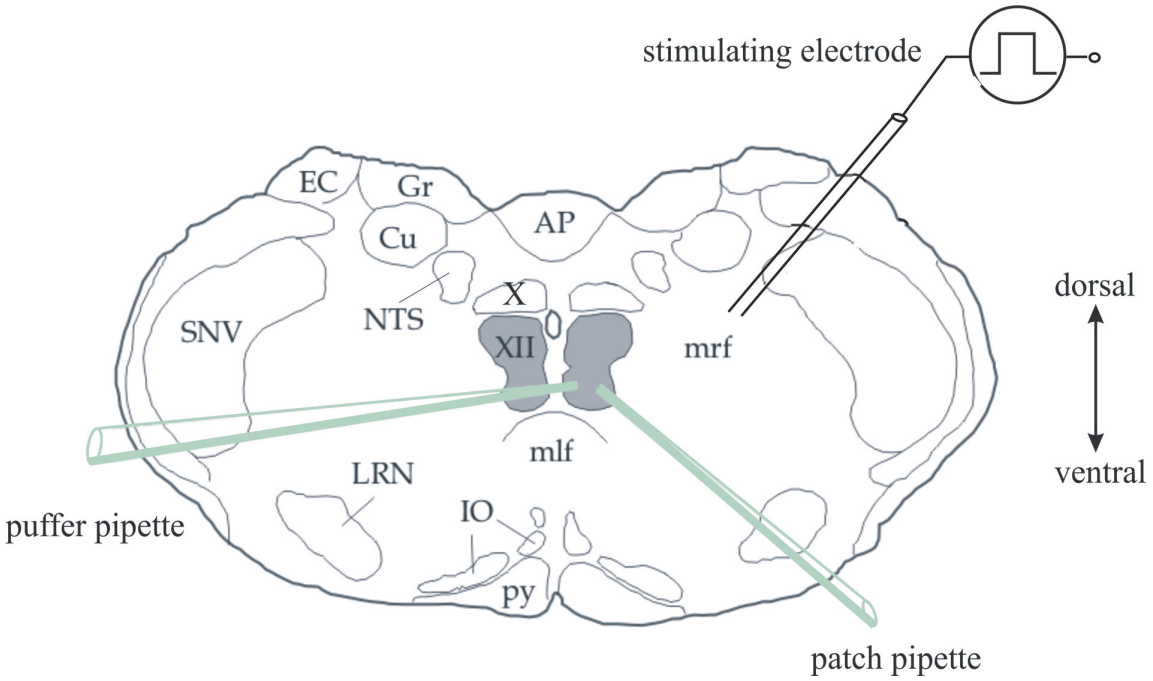


Fig. 12. Schematic representation of a brain stem slice. Puffer pipette approaching HMs on the left, patch pipette on the righ. The stimulating electrode is placed in the dorso-lateral medullary reticular formation (mrf). X, vagal nucleus; XII, hypoglossal nucleus; AP, area postrema; Cu, cuneate nucleus; EC, external cuneate nucleus; Gr, gracile nucleus; IO, inferior olive; LRN, lateralreticular nucleus; mlf, medial longitudinal fasciculus; NTS, nucleus of the tract solitarius; py, pyramidal tract; SNV, spinal nucleus of the trigeminal nucleus.

4. DATA ANALYSIS

Continuous recordings of spontaneous activity were stored on magnetic tape and transferred to a PC after digitalization with an A/D converter (Digidata 1200).

Cell input resistance (R_{in}) was calculated by measuring the current response to 5 or 10 mV hyperpolarizing steps (from -60,-65 mV, holding potential), or from the slope of the linear part of the *I-V* relation obtained by applying a slowly rising voltage signal (ramp test: from –80 to +20 mV, 41.7 mV/s).

Single postsynaptic currents were detected using AxoGraph 4.6 (Axon Instruments, Foster City, CA) and Clampfit 9.2 (Axon Instruments) softwares, while SigmaPlot 2001 (Jandel Scientific, San Rafael, CA) software was used for linear regression analysis of experimental data.

Results were quantified as means ± S.E.M., with "n" indicating the number of cells in which the experiment has been repeated. Statistical significance was assessed with the Student's paired *t*-test, applied only to raw data, or with the Tukey-test and ANOVA for non-parametric values; according to convention, two groups of data were considered statistically different if $P<0.05$.

RESULTS

The database of the present study comprises 178 HMs, with 281±30 MΩ mean input resistance.

1. INVESTIGATING THE PRESENCE OF TONIC CHOLINERGIC ACTIVITY IN THE BRAINSTEM SLICE PREPARATION

Previous studies have indicated that the nucleus hypoglossus expresses neuronal nAChRs (Zaninetti *et al.*, 1999; Chamberlin *et al.*, 2002; Dehkordi *et al.*, 2005) and it is contacted by cholinergic fibers (Gilmor *et al.*, 1996; Arvidsson *et al.*, 1997; Schäfer *et al.*, 1998; Ferreira *et al.*, 2001). In this study I first explored whether it was possible to observe direct cholinergic network activity on HMs, using a simple brainstem slice preparation. I started recording spontaneous synaptic currents (sPSCs) from HMs. Bicuculline and strychnine were routinely added to the recording solution, in order to block synaptic inhibitory processes (Donato and Nistri, 2000); the remaining activity was considered to be mediated by glutamate (Sharifullina *et al.*, 2004, 2005) (Fig. 13A, top trace). Then the ionotropic glutamate receptor antagonists, CNQX (10 μM) and APV (30 μM), were bath-applied to reveal any non-glutamatergic excitatory currents (Fig. 13A, middle traces): however, no events could be detected, confirming that spontaneous events were glutamatergic. This observation suggests the lack of spontaneously active cholinergic synapses on HMs (n=6), at least in this preparation.

I also wondered if blocking ACh hydrolysis, with the cholinesterase inhibitor edrophonium (20 μM), could boost ACh availability and unmask cholinergic events (Kouznetsova and Nistri, 2000). However, as shown in Fig. 13A (bottom traces), even in this case there was lack of synaptic activity when CNQX and APV were present (n=3 cells). There was also no change in baseline current. Because lack of spontaneous

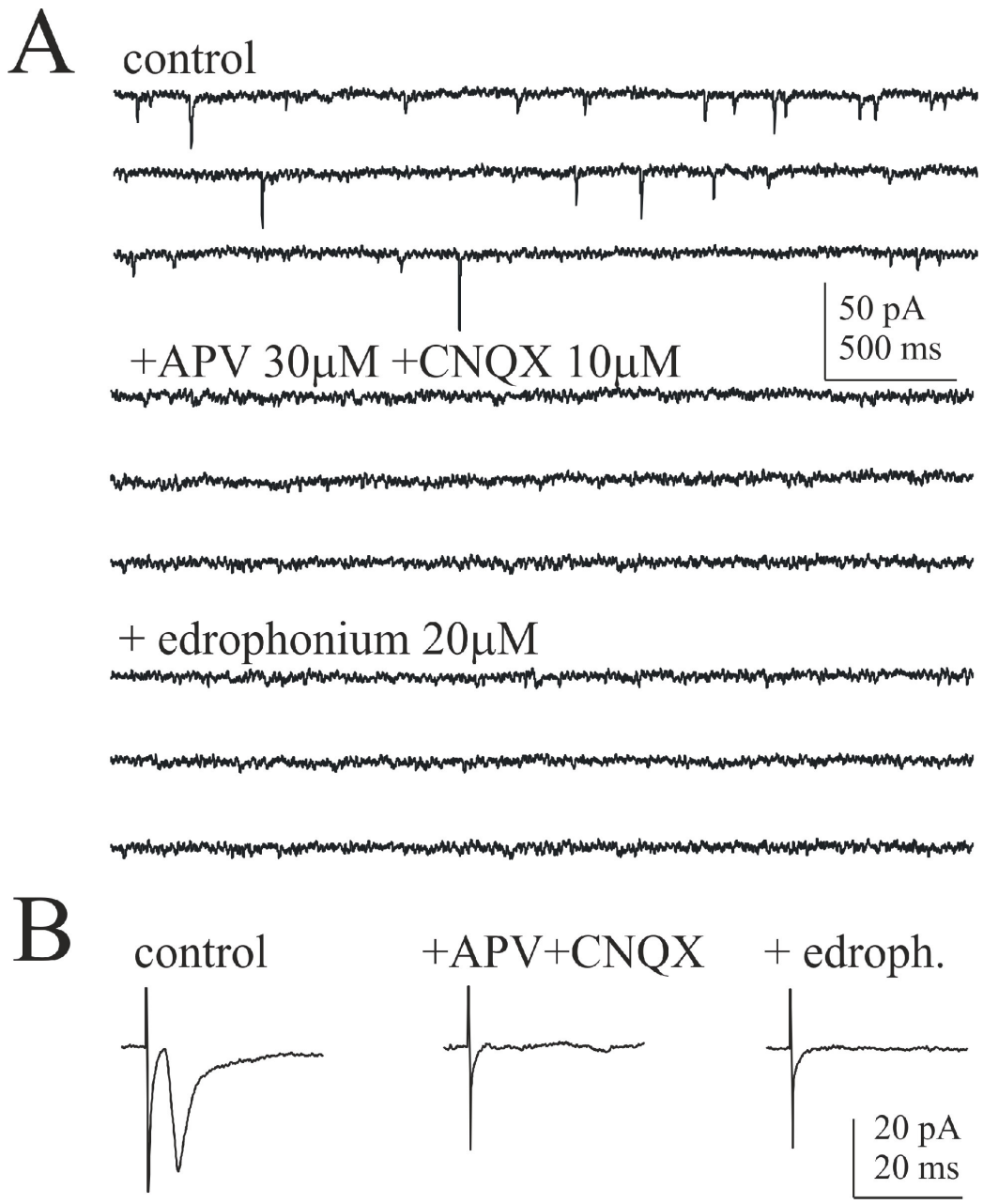


Fig.13. Lack of endogenous cholinergic activity on HMs. (A) Upper traces: consecutive recording from a voltage clamped motoneuron (in the presence of 10 µM bicuculline and 0.4 µM strychnine. Middle traces: no residual activity is detectable after the application of APV (30 µM) and CNQX (10 µM)(n=6). Bottom traces: addition to the bathing solution of the AChE inhibitor edrophonium (20 µM) is ineffective to disclose any endogenous and spontaneous release of ACh (n=3). Reproduced traces are representative of the same cell. (B) ePSCs elicited by sub-maximal stimulation of the dorso-lateral mrf (see methods): average traces of 20 pulses (10 Hz train) recorded in control solution, or in the presence of glutamatergic transmission blockers, alone and together with edrophonium (20 µM). Same cell as in (A).

cholinergic events might have suggested low release probability, it was attempted to elicit cholinergic currents by stimulating the reticular formation (mrf) with 10 Hz trains. Under these conditions and using a protocol which produces monosynaptic glutamatergic currents (Essin *et al.*, 2002; Sharifullina *et al.*, 2004), confirmed by the relatively short and constant lantency (4.8 ±0.3 ms, n=13) of the currents, I could record robust electrically evoked postsynaptic currents (ePSCs; Fig. 13B, left). These responses were fully suppressed in the presence of CNQX plus APV (Fig. 13B, middle; n=3), confirming that them to be primarily glutamatergic. No other evoked activity could be recorded, in the presence of glutamatergic ionotropic receptor blockers, even after applying edrophonium (Fig. 13B, right; n=3).

However, interestingly, glutamatergic spontaneous transmission resulted significantly depressed following edrophonium application (about 4 min from the start; Fig. 14A). This effect consisted in a significant reduction in event frequency without change in event amplitude (Fig. 14B), and was reversible on washout (not shown).

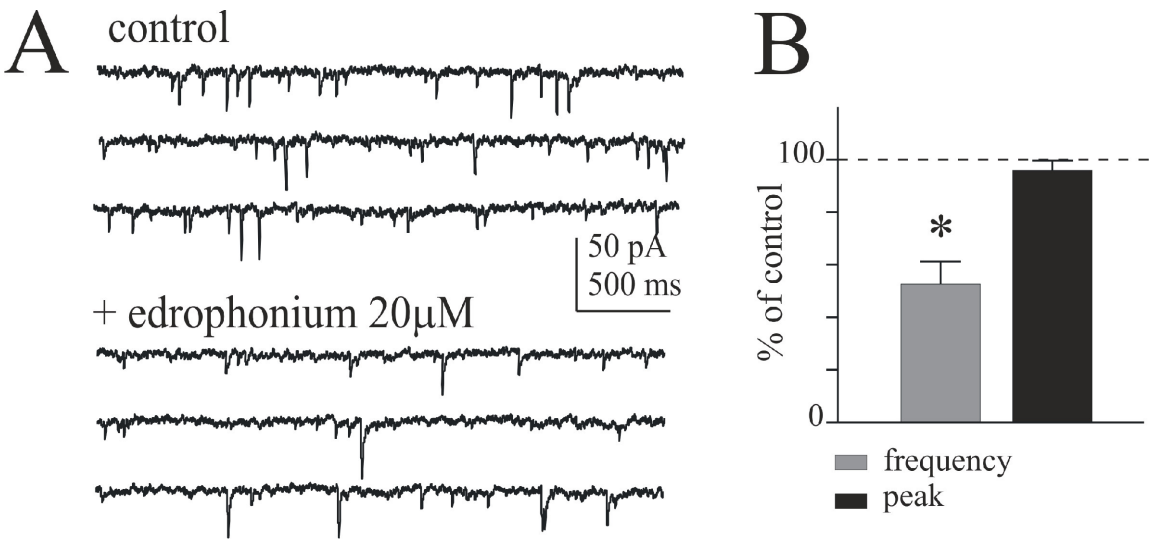


Fig.14. Edrophonium indirect effect on spontaneous glutamatergic transmission. (A) Sample of glutamatergic sPSCs recorded before and during bath-application of 20 µM edrophonium (upper and lower traces, respectively). (B) Histograms showing the resulting effect of edrophonium: 20µM edrophonium significantly reduces the frequency of sPSCs to 52.8 ±9 % (n=9, $p<0.05$) of control value, while leaves unchanged their peak amplitude (99.8 ±4 %, n=9).

Overall, spontaneous transmission is made up of network dependent and network independent events. To isolate synaptic terminal activity, recordings are usually performed in the presence of TTX (1 μM). In these conditions it was possible to record miniature glutamatergic synaptic events (mPSCs), which were unaffected in their frequency and amplitude by edrophonium (N. Lamanauskas, personal communication). Thus, the changes in glutamatergic activity observed during edrophonium application, were the consequence of enhanced cholinergic activity at network level rather than at presynaptic sites.

2. EFFECTS OF NACHR ANTAGONISTS ON GLUTAMATERGIC CURRENTS

High concentrations of di-hydro-β-erythroidine (DHβE) are commonly used for broad spectrum, non-selective antagonism of nAChRs (see review by Dwoskin and Crooks, 2001). Bath application of 200 μM DHβE reduced the peak amplitude of glutamatergic sPSCs and ePSCs recorded from HMs (Fig. 15A, B), without significantly changing either the current kinetics (Fig. 15B) or the frequency of sPSCs (Fig. 15 C). Baseline current was unaffected.

On the other hand, submicromolar concentrations of DHβE possess strong selectivity towards the α4β2 nAChR subtype (Harvey and Luetje, 1996). Because α4β2 receptors are widely expressed in the brainstem (Zaninetti *et al.*, 1999), I tested whether DHβE, at 0.5 μM concentration, was still able to modify glutamatergic currents. Fig. 16A shows that, at this concentration, DHβE significantly depressed the peak amplitude of sPSCs (see also histograms in Fig. 16D). Analogous observations were obtained when examining the action of 0.5 μM DHβE on mPSCs (Fig. 16B, D) and ePSCs (Fig. 16C, D). The right panel of Fig. 16 shows that, after scaling and superimposing, ePSCs retained similar kinetics. No significant change was observed in terms of event frequency either for sPSCs or mPSCs (Fig. 16D). It is noteworthy that the peak amplitude reductions in the presence of 0.5 μM DHβE were quantitatively smaller than those achieved with 200

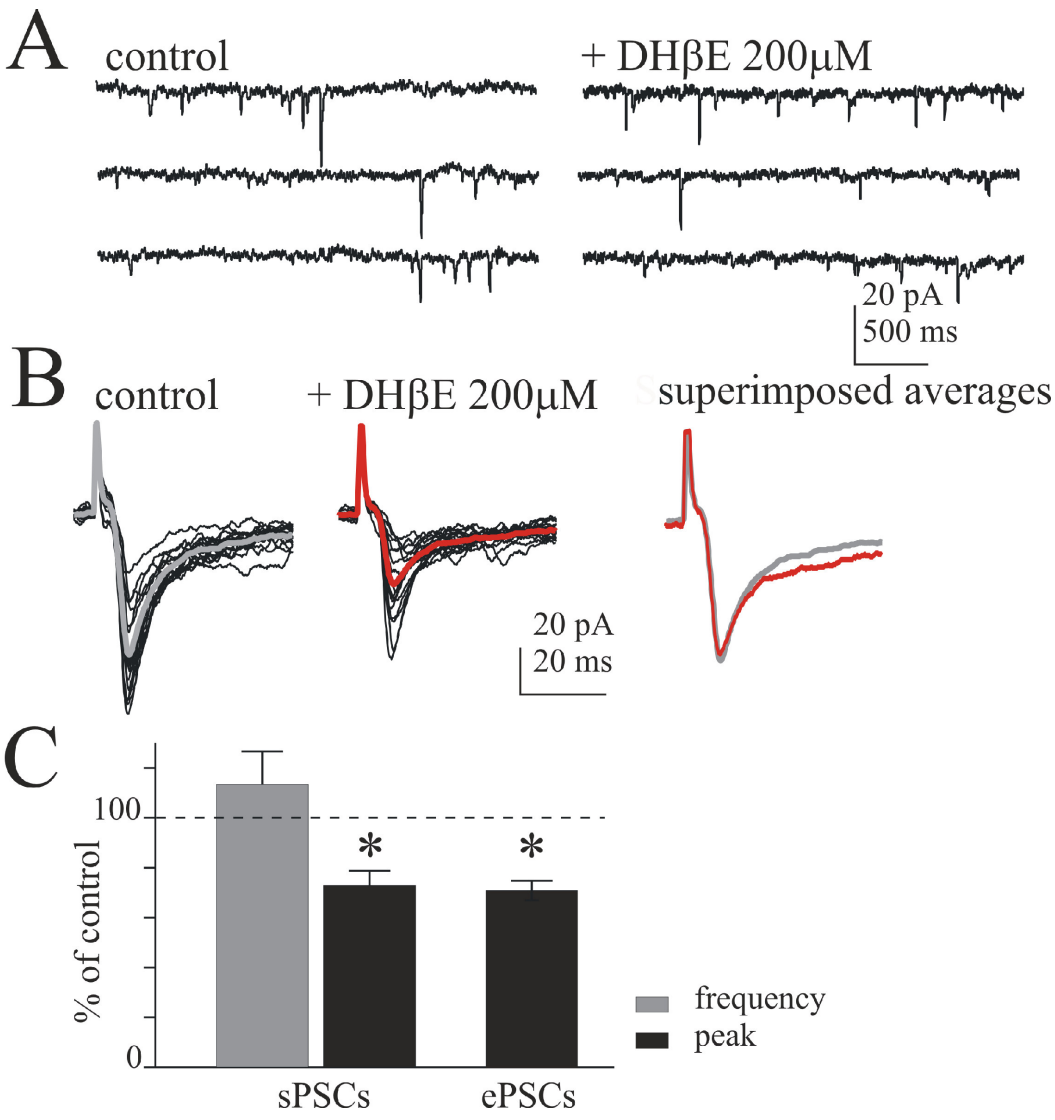


Fig.15. Effect of a massive blockage of nAChRs, on glutamatergic transmission. (A) Recordings of sPSCs in control condition (bicuculline and strychnine in the bath, see methods), and in the presence of 200 µM DHβE (right). Data from the same cell. (B) Seventeen superimposed ePSCs (0.1 Hz; black traces) with their average (coloured trace) were collected in control condition (left) and in the presence of DHβE (middle). Synaptic failures have been omitted. Averaged traces normalized and superimposed show no changes in the kinetics of glutamatergic ePSCs (right). (C) Summary of the effect of 200 µM DHβE on glutamatergic transmission: the peak amplitude of both sPSCs and ePSCs resulted substantially depressed to 72.9 ±6 % (n=9, $p<0.02$) and 71.0 ±4 % (n=7, $p<0.002$), respectively. On the contrary, the frequency of sPSC is not affected (113.4 ±13 %, n=9).

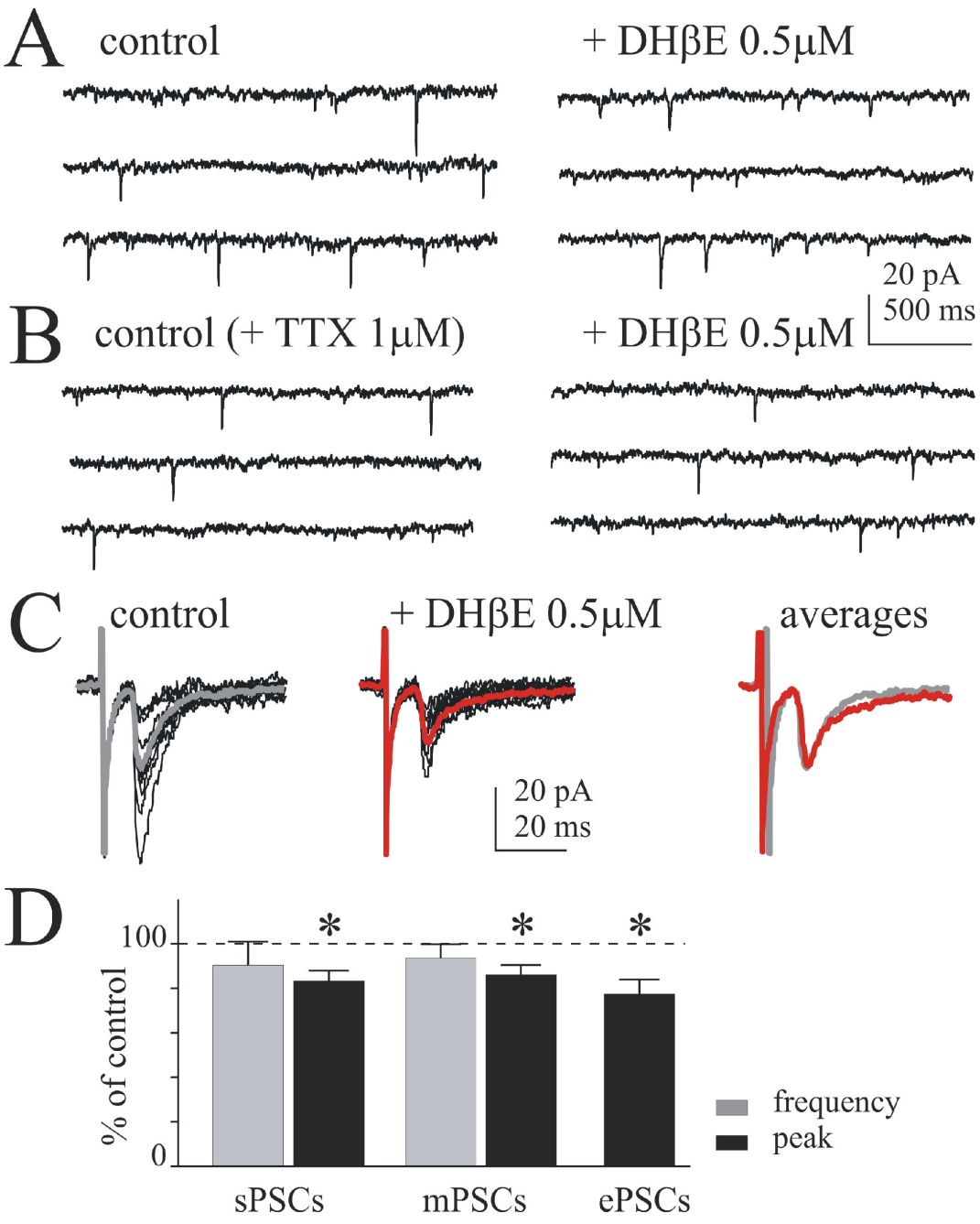


Fig.16. Effect of 0.5 μM DHβE on glutamatergic transmission. (A) Effect of DHβE on glutamatergic sPSCs (right), compared to control condition (left). (B) Glutamatergic mPSCs isolated in the presence of 1 μM TTX (left), and their responsiveness to the application of DHβE (right). (C) Superimposed glutamatergic ePSCs (0.1 Hz stimulation) recorded in control solution (left, nine traces) and in the presence of DHβE (center, twelve traces); their relative averages, shown in coloured line, are normalized and superimposed (right). (D) Histogram summarizes the effect of 0.5 μM DHβE on glutamatergic transmission: sPSCs, mPSCs and ePSCs resulted all reduced in peak amplitude (83.4 ±5 %, 85.8 ±4 % and 77.3 ±7 % of control values, respectively; n ≥ 5, $p<0.05$). However neither sPSCs nor mPSCs appeared significantly changed in their frequency (90.5 ±10 % and 92.8± 6 %, respectively).

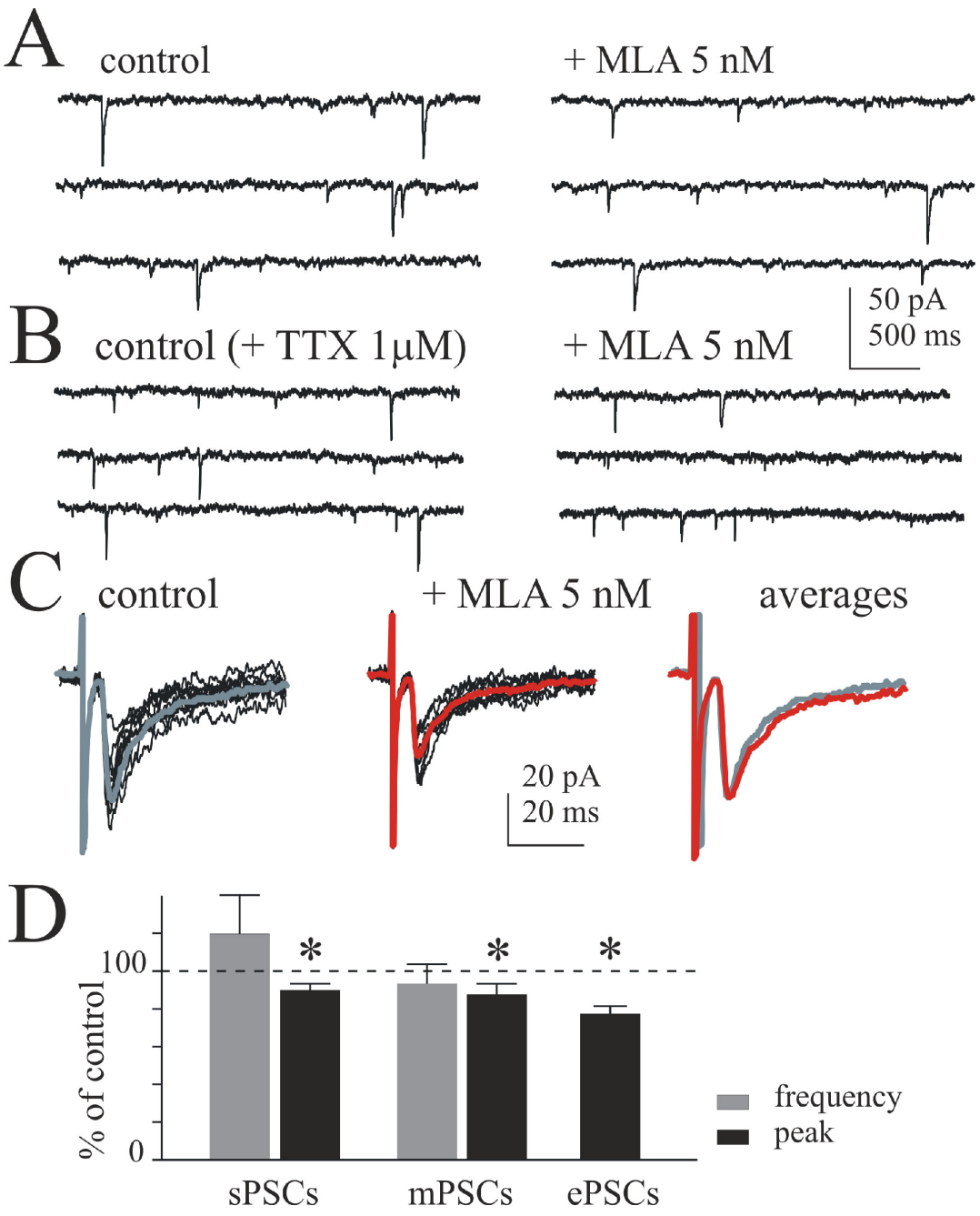


Fig.17. Effect of 5 nM MLA on glutamatergic transmission. (A) Continuous recordings of glutamatergic activity in control (left panel), and after the application of MLA (right panel). (B) mPSCs recorded in control solution and in the presence of 5 nM MLA. (C) Superimposed ePSCs (0.1 Hz) recorded in control (left, eleven traces) and in MLA containing solution (middle, nine traces); their relative averages (coloured) are normalized and superimposed (right). (D) Histogram summarize the effect of 5 nM MLA on glutamatergic transmission: there is a significant depression of the peak amplitude of sPSCs (86.4 ±3 %, n=15, $p \leq 0.001$), mPSCs (84 ±5 %, n=6, $p < 0.05$) and ePSCs (76.7 ±4 %, n=7, $p \leq 0.0001$), with no change on sPSCs (114.9 ±20%) and mPSCs (89 ±10 %) frequencies.

μM DHβE. This result suggests that other nAChR subtypes might have contributed to the regulation of glutamatergic currents.

Because α7 receptor subunits are expressed within the rat brainstem (Zaninetti *et al.*, 1999; E. Fabbretti and N. Lamanauskas, unpublished), I tested the action of the alkaloid MLA, one of the most potent and selective antagonist at the α7-homomeric nAChR subtype (Palma *et al.*, 1996; Bergmeier *et al.*, 1999; Davies *et al.*, 1999; Jones *et al.*, 1999). At the concentration of 5 nM, MLA significantly reduced the peak amplitude of both sPSCs, mPSCs and ePSCs (Fig. 17A-D), without changing either ePSC kinetics (right panel of Fig. 17C, after scaling and superimposing average traces) or sPSCs and mPSCs frequency. I have also tested larger doses of MLA; up to 10 nM, no further reduction in synaptic response amplitude could be observed (data not shown).

3. FUNCTIONAL PROPERTIES OF NACHRS ON HMS

Lack of direct cholinergic synaptic activity on HMs did not preclude the possibility that HMs expressed functional nAChRs. To examine this issue and to minimize problems due to hydrolysis of ACh by AChE and to nAChR desensitization, I applied nicotine to voltage-clamped hypoglossal motoneurons, by means of brief pressure pulses (4-10 psi) delivered through a puffer pipette positioned 20-50 μm far from the soma of the cell (see also Di Angelantonio and Nistri, 2001; Pagnotta *et al.*, 2005). Pressure ejected nicotine was able to generate fast post-synaptic inward currents in the patched cell. The peak amplitude of those currents was directly related to the duration of the pulse, so that increasing the delivery time of nicotine produced increasingly larger inward currents (Fig. 18A, left), because of the larger doses administered. The threshold to get a response to nicotine was at 10 ms, while amplitude saturation was gained with applications lasting approximately 500 ms (Fig. 18A, right). The half-maximal response was calculated to be reached with 160 ms long pulses.

Nicotine-induced inward currents persisted in the presence of CNQX and APV (Fig. 18 Ba; n=7), indicating they were not generated by glutamate released

from presynaptically activated terminals. In fact, 100 μM DHβE was able to fully suppress them (Fig. 18Bb; n=3). Responses to puffer application of nicotine for up to 1 s were not accompanied by changes in sPSCs, showing that a relatively short puffer application of this drug predominantly affected HMs rather than network neurons.

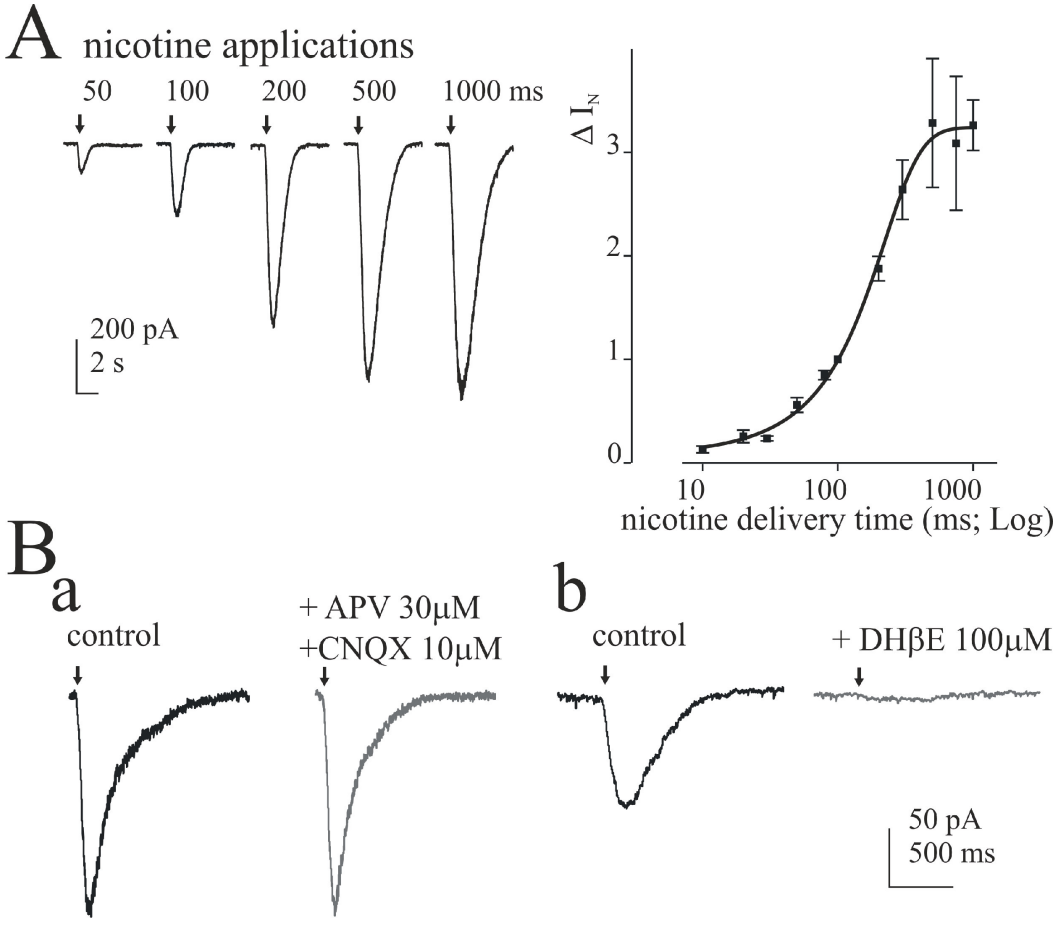


Fig.18. Nicotine generates fast inward currents on HMs. (A) Left: sample of responses to 50, 100, 200, 500 or 1000 ms long pulses of pressure-ejected nicotine. Right: the plot shows the relationship between the normalized peak amplitude of responses (ΔI_N) and the duration of the puffer pulse (normalization is carried out with respect to 100 ms pulse response, n≥ 5). (B) Inward currents generated by nicotine (75 ms) are insensitive to bath application of APV (30 μM) plus CNQX (10 μM, n=6), while are completely antagonized by 100 μM DHβE (n=3).

For pharmacological identification of the nAChR subtypes mediating HM responses to nicotine, DHβE and MLA were again used, at subtype selective concentrations. Fig. 19A

indicates that 0.5 μM DHβE reduced responses at all tested doses of nicotine. Likewise, a similar decrease was obtained in the presence of 5 nM MLA (Fig. 19B). When DHβE and MLA were co-applied, the summative action resulted in an almost complete blockage of the nicotine induced current (Fig. 19C).

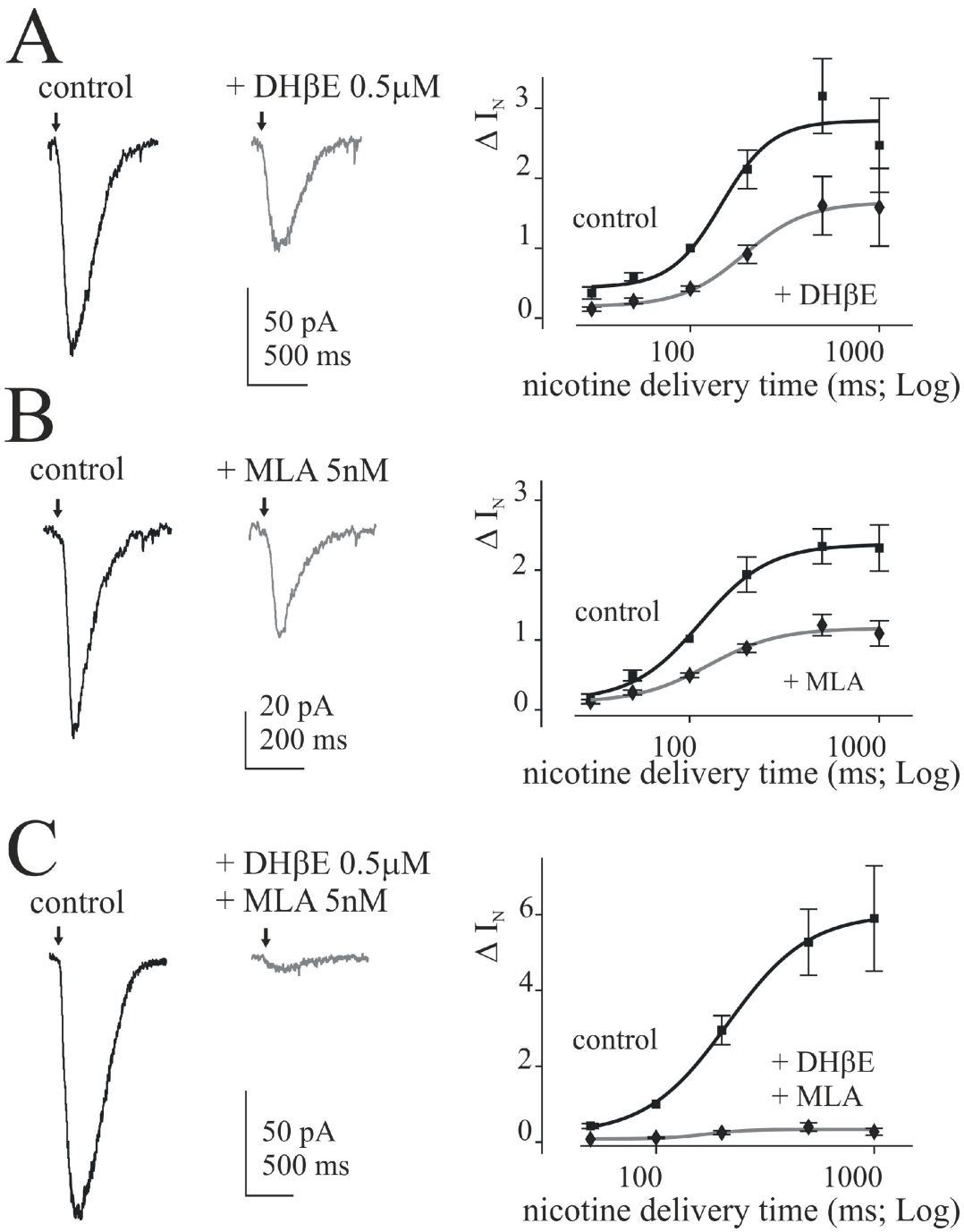


Fig.19. Pharmacological characterization of the nicotine induced inward currents.

4. DESENSITIZATION OF NACHR S ON HMS

To investigate the time constant of nAChR desensitization onset I delivered long pulses of nicotine, in analogy to the protocol used by Katz and Thesleff (1957). HMs responded to such puffer pulses generating inward currents, which peaked and faded back to baseline even though the pulse had not ceased yet, as shown in Fig. 20A. The decay of such currents could be fitted by a mono-exponential time constant (τ) of 211 ±35 ms (n=6). The time necessary for recovering from desensitization was investigated with the paired pulse protocol stimulation: two nicotine puffer pulses of the same duration (50 ms) were delivered at increasing time intervals (Katz and Thesleff, 1957). Recovery was considered complete when response to the second, test pulse reached the same peak amplitude of the first, conditioning pulse (Fig. 20B, left). Full receptors functionality was stably regained with a 60 s long interval between the two consecutive pulses (Fig. 20B, right).

Receptor desensitization also occurred in the presence of sustained (4-5 min) bath-application of small concentrations (0.1-1 µM) of nicotine. These nicotine administrations, in 20 out of 55 cells, generated an inward current which on average shift was calculated to be -56 pA. This macroscopic inward current started fading after about 1 min from its peak. Notwithstanding the ability of bath-applied nicotine to induce direct HM responses, the effects of subsequent test pulses of nicotine were always strongly attenuated (Fig. 20C). For instance, the currents elicited by 100 ms nicotine pulses were reduced in the presence of 0.5 µM nicotine to 17.6 ± 4.6 % of control ones (n=5).

Ctd from **Fig.19.**(A) Left: example of the effect of 0.5 µM DHβE on responses produced by brief pulses of nicotine (100ms). Right: dose/response curves representative of control and DHβE enriched medium (n=9). (B) Samples representative of the effect of 5 nM MLA on nicotine generated current, different cells than in (A) Plots of responses to puffer applications of nicotine in control and MLA containing solution (n=10; right). (C) Application of both antagonists resulted in an almost complete blockage of the nicotinic currents (n=9, for right hand side plot). All data are normalized to value of the response to a 100ms pulse, relative to each data set.

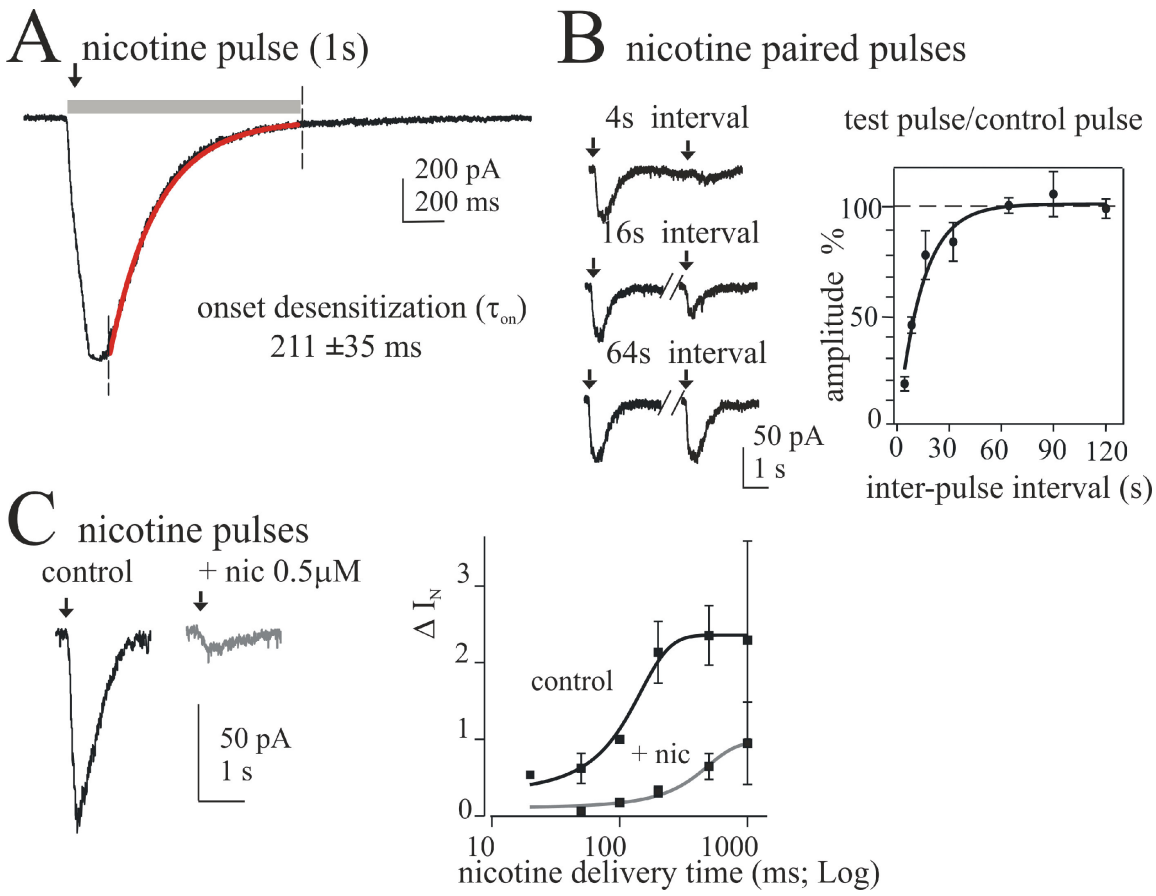


Fig.20. Desensitization affects responsiveness of nAChRs to nicotine. (A) During 1 s long nicotine pulse application the resulting inward current peaks and then decays monoexponentially (average time constant=211 ±35 ms; n=6; vertical dot bars indicate the record used for fitting). (B) Time necessary for recovering from desensitization was investigated with the paired pulse protocol stimulation, where two identical nicotine pulses (50 ms) are delivered at increasing time intervals (4, 8, 16, 32, 64, 90, 120 s). The graph on the right shows the relative amplitude of the second response (as % of the first one) plotted against the inter-pulse interval; complete recovery is regained after about 60 s (n=3). (C) Sample traces showing how currents generated by focally pulsed nicotine (100 ms) are strongly depressed during bath-applied 0.5 µM nicotine. The plot on the right summarizes the depressive effect of bathing nicotine (n≥ 3); values are normalized with respect to the response to 100 ms pulse.

Then I wished to examine if network nAChRs controlling glutamatergic transmission were equally subjected to desensitization. I tested how a much longer application of nicotine, supposed to strongly activate nAChRs, could modulate glutamatergic currents. For this purpose, I applied a long (1 min) puffer pulse of nicotine (in excess of the one eliciting the maximal response, cf. Fig. 18A) and measured both amplitude and frequency of glutamatergic sPSCs. Data were collected before the start of the puffer

administration and after the inward current elicited by nicotine had subsided due to nAChR desensitization, as shown in Fig. 21A. Under these circumstances, there was a significant fall in the sPSC average frequency (analogous to the one found with a small dose of nicotine), plus a significant reduction in sPSC average amplitude (Fig. 21B, n=6).

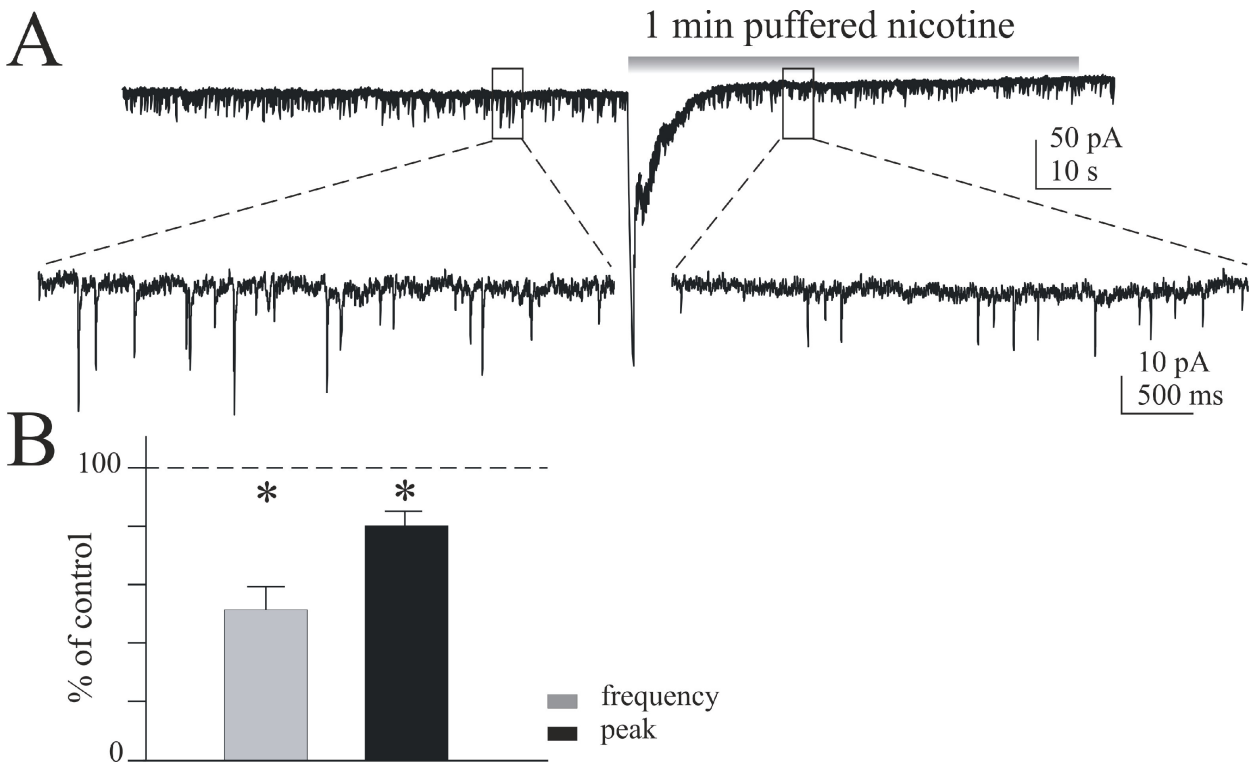


Fig.21. Effect of an acute administration of nicotine on glutamatergic transmission. (A) Top: continuous recording of glutamatergic sPSCs before and during the application (1 min) of nicotine via a puffer pipette. Insets: two fragments runned at a faster time scale. (B) Histograms summarizing the effect of nicotine on sPSCs: both peak amplitude and frequency resulted depressed during nicotine pulse to 80.1 ±4 % and 51.4 ±8 % of control, respectively (n=7, $p<0.01$).

Afterwards I investigated the effect of a long lasting application of nicotine on glutamatergic transmission, using a dose closer to those observed in the brain of smokers, namely 0.5 µM. After 5-6 min of such nicotine bath-application, the frequency of both sPSCs and mPSCs resulted reduced, but no significant changes were observed in their amplitude (Fig. 22A, B, D).

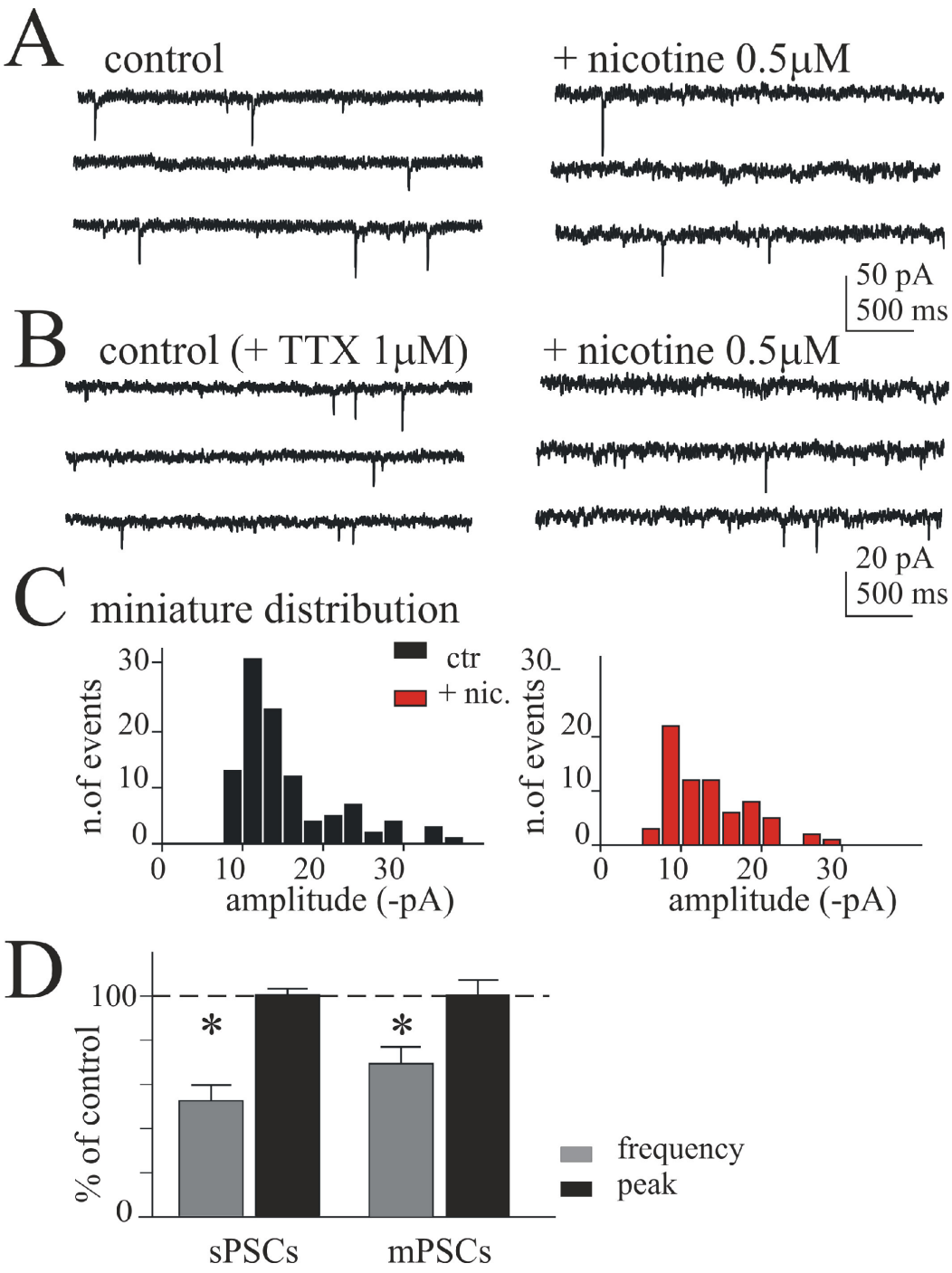


Fig.22. Effect of a low dose of nicotine on spontaneous glutamatergic transmission. (A) Continuous recordings of glutamatergic sPSCs in control condition or in the presence of 0.5 µM bathing nicotine. (B) Continuous recordings of mPSCs in control or in the presence of 0.5 µM nicotine. (C) Example of amplitude distribution histogram for mPSCs. Events are clustered between –8 and –15 pA, even in the presence of nicotine. (D) Indeed 0.5 µM nicotine does not affect the peak amplitude of neither sPSCs or mPSCs (99.6 ±3% and 100.4 ±6 % of control value, respectively, n=7), **.** but it significantly decreases their frequency, resulting 52.1 ±7 % (n=7, $p<0.01$) and 69.1 ±8 % (n=7, $p<0.03$), respectively.

Experiments on glutamatergic ePSCs provided additional clues about the sensitivity of brainstem nAChRs to desensitize in the presence of submicromolar doses of nicotine. In fact, as indicated in Fig. 23A, after about 4 min in the presence of 0.5 µM nicotine ePSCs were depressed without changing their kinetics. Network transmission was deeply impaired as the number of failures increased significantly (Fig. 23B). It was interesting to observe that the smaller ePSCs amplitude (calculated after excluding failures) displayed much smaller fluctuations as indicated by their CV value (Fig. 23C). The input/output curve obtained plotting the ePSC amplitudes as a fraction of their maximum versus the stimulus intensity resulted flattened in the presence of nicotine. The strongest depression was observed for responses elicited by the higher intensity stimuli (Fig. 23D).

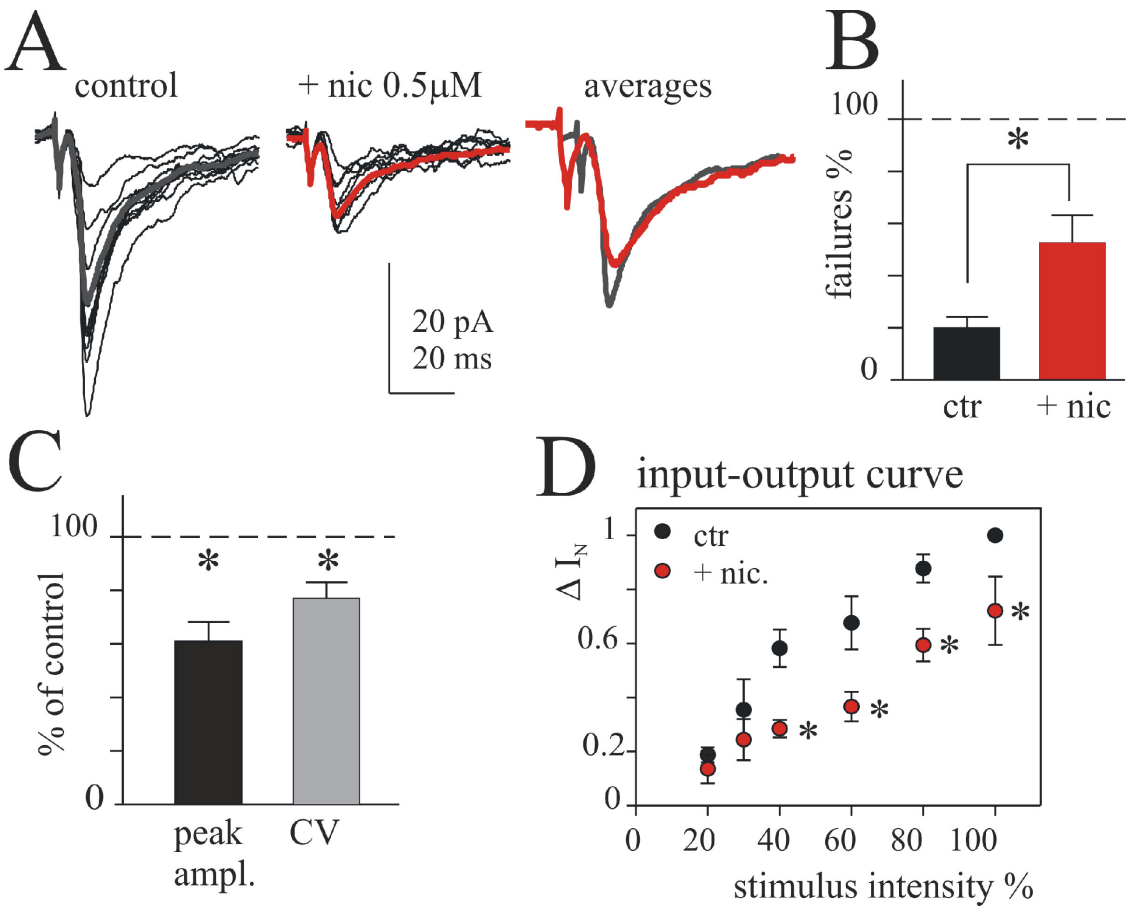


Fig.23. Effect of 0.5 µM nicotine on glutamatergic ePSCs.

In view of the diffuse distribution of glutamatergic afferents to HMs, only part of which were electrically stimulated, and their association with nAChRs, it seemed interesting to compare the dynamics of depression of ePSCs and sPSCs (Fig. 24). So I measured ePSC amplitude following continuous 0.1 Hz stimulation before and following bath-application of 0.5 μM nicotine (Fig. 24A, left; n=8): there was a transient, early facilitation of ePSC amplitude which lasted for about 100 s ($p<0.05$, Tukey test), followed by a significant depression ($p<0.05$) which outlasted the nicotine administration. On average, the facilitation was 136 ±8 % versus the average control amplitude, while the depression amounted to 53 ±5 %. To obtain recovery from nicotine-evoked depression a 5 min long wash-out was necessary. Surprisingly, when an even lower (0.1 μM) concentration of nicotine was used (see Fig. 24A, right), ePSCs were just reduced in amplitude, a phenomenon developing about 2 min from the switch to the nicotine containing solution. The largest reduction in current response (52 ±4 % of control, n=7) was comparable to the one observed during exposure to 0.5 μM nicotine. On the other hand, sPSCs displayed a different sensitivity to bath-application of submicromolar concentrations of nicotine. In particular, as indicated in Fig. 24B, neither 0.5 (left panel) nor 0.1 μM nicotine (right panel) altered the peak amplitude of these currents although both doses facilitated sPSC frequency with dissimilar timecourse. With 0.5 μM nicotine the frequency facilitation (525 ±48 % of control; n=5) peaked at about 2 min and then gradually declined back to baseline. With 0.1 μM nicotine the frequency facilitation (242 ±5 %; n=8) of sPSCs was clearly delayed with development after >4 min from the start of application.

Ctd from **Fig.23.** (A) Ten superimposed glutamatergic ePSCs (black lines) elicited by sub-maximal stimulation of the mrf (0.1Hz), in control solution and after addition of 0.5 μM nicotine (averages are in coloured line). Failures are not shown, but they resulted increased in number (B): 52.7 ±10 % in nicotine containing medium, versus 20 ±4 % of total responses in control medium (n=9, $p<0.01$). (C) Nicotine strongly depresses synaptic transmission, decreasing the mean peak amplitude of ePSCs (60.3 ±7 % of control; n=8, $p<0.02$), and reducing the CV of response amplitude (75 ±6 % of control; n=7, $p<0.02$). (D) In the input-output curves, build up normalizing ePSC peak amplitude to their maximum (ΔI_N), asterisks indicate a significant difference between responses collected in control solution and those recorded in the presence of 0.5 μM nicotine ($p<0.05$, with Tukey test; $n\geq4$).

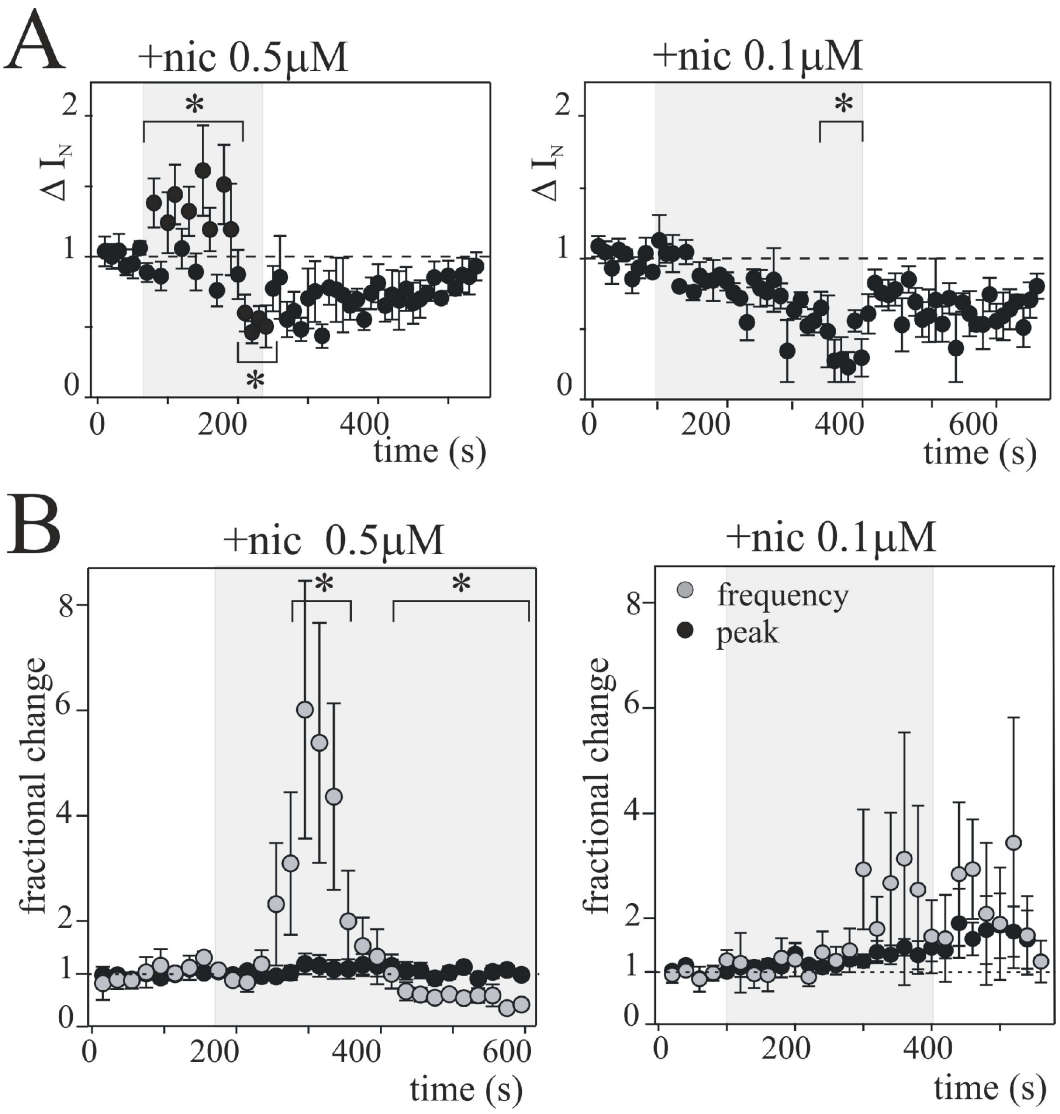


Fig.24. Timecourse of the action of bath-applied nicotine on glutamatergic transmission. (A) Timecourses of the effect of 0.5 (left, n=8) and 0.1 (right, n=7) µM nicotine on glutamatergic ePSCs. Shaded areas indicate the time window relative to nicotine application. Values are normalized with respect to the mean of those recorded before the application of nicotine. Asterisks indicate the datapoints which are signficantly different ($p<0.05$, Tukey test) from control. (B) Timecourses of the effect of 0.5 (left, n=5) and 0.1 (right, n=8) µM nicotine on glutamatergic sPSCs (black dots, peak amplitude; gray dots, frequency). Frequency values were obtained by analyzing 20 s segments of consecutive records. All values are normalized to control ones. To avoid potential interference due to cumulative desensitization, cells were tested with one dose only of bath-applied nicotine.

5. IMMUNOHISTOCHEMISTRY OF nAChR s IN BRAINSTEM SLICES

Molecular biology experiments, performed in our laboratory by E. Fabbretti and N. Lamanauskas, provided further evidence in confirmation of the above reported electrophysiological findings.

As shown in Fig. 25A, within the nucleus hypoglossus, immunoreactive cell bodies, cell processes and fibers were intensely labeled with anti-α4, α7 and β2 antibodies. In general, immunoreactive cells appeared to be more numerous in the caudal end than in the rostral end of the nucleus. In each histological slide the number of motoneurons (20-40 μm somatic diameter) immunoreactive for any nAChR subunit has been counted and divided by the area of the nucleus hypoglossus in same slide (Fig. 25B). The density of neurons immunoreactive to α4, α7 or β2 resulted to be very similar.

Anti-α4, α7 and β2 antibody specificity was evaluated performing western immunoblotting experiments, using lysates derived from P3 rat brainstem. As shown in Fig. 25C (lanes b), single immunoreactive bands corresponding to the expected molecular weights of the rat α4, α7, or β2 nAChR subunits were observed. In particular, the anti-α4 antibody recognized a band of 70 kDa, the anti-α7 highlighted a band of 57 kDa, and the anti-β2 antibody gave a band at approximately 50 kDa, in full agreements with previous studies performed on rat brain tissue (Arroyo-Jiménez *et al.*, 1999; Jones *et al.*, 2001; De Simone *et al.*, 2005). Western blots of comparable amount of protein extracts from rat kidney were used as negative control, showing no detectable signal (Fig. 25C, lanes c).

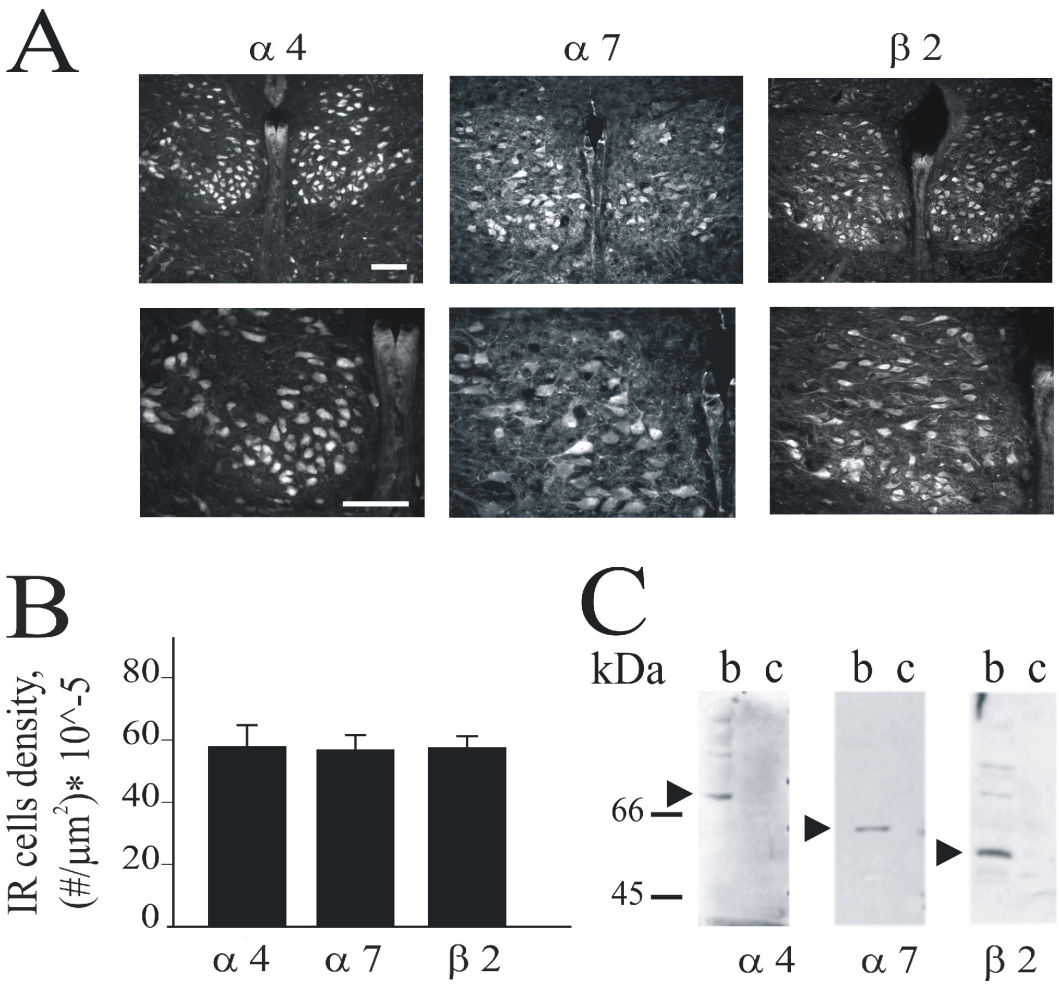


Fig.25. Immunohistochemistry of nAChRs in the nucleus hypoglossus. (A) Immunostaining of brainstem slices (x10 and x20 magnification for top and bottom rows, respectively) with antibodies against α4, α7 or β2 nAChR subunits reveals reactivity in HMs. (Scale bar=100 μm). (B) Bar charts of the density of immunoreactive cells in the hypoglossal nucleus for each type of nAChR subunit. (C) Western immunoblot of α4, α7 or β2 nAChR subunits. Lane "b" refers to brainstem samples while lane "c " refers to kidney samples used as negative control.

DISCUSSION

1. USE OF HMs TO INVESTIGATE MODULATION OF EXCITATORY SYNAPTIC TRANSMISSION

Previous reports have demonstrated the usefulness of recording from HMs to investigate the neuronal properties of integrated input signals (Bellingham and Berger, 1996; Rekling *et al.,* 2000). Moreover, it has been shown how the efficiency of excitatory or inhibitory synaptic transmission could be modulated by activation of certain receptor populations. For instance, activation of metabotropic glutamate receptors is known to facilitate glutamatergic transmission and to constrain HMs into an oscillatory pattern (Sharifullina *et al.,* 2004, 2005). Recently, we have observed that muscarinic receptors for ACh strongly depress inhibitory neurotransmission mediated by glycine or GABA (Pagnotta *et al.,* 2005), while they facilitate glutamatergic transmission (Bellingham and Berger, 1996).

Recent experiments have indicated that HMs possess functional nAChRs (Chamberlin *et al.*, 2002). Furthermore, within the brainstem network impinging upon HMs, there are nAChRs that appear to upregulate the activity of inspiratory interneurons projecting glutamatergic inputs to HMs (Shao and Feldman, 2005). Hence, rat HMs offer the special advantage of studying both network and postsynaptic nAChRs in the same preparation.

Nevertheless, it is unclear if nAChRs can actually modulate glutamatergic synaptic transmission directly on HMs and whether such effects can be functionally distinguishable from those due to activation of postsynaptic nAChRs of HMs. In fact, although it is clearly established that one important action of nAChRs on mammalian brain neurons is to favour the release of the excitatory transmitter glutamate (McGehee *et al.*, 1995; Gray *et al.*, 1996; Wonnacott, 1997; Mansvelder and McGehee, 2000; Genzen and McGehee, 2003; Ge and Dani, 2005; Guo *et al.*, 2005), it remains to be ascertained the

relative contribution of pre and postsynaptic nAChRs to HM activity mediated by glutamate.

For these reasons the present study was focused on the functional and pharmacological properties of nAChRs, and in particular on how nAChRs can modulate glutamatergic transmission, their locus of action, and sensitivity to nicotine, so mimicking any potential changes in HM activity induced by exposure to tobacco smoke.

2. nAChRs EXPRESSED BY HMs

HM nAChRs could be rapidly activated by focal application of nicotine, generating inward currents insensitive to glutamate, glycine and GABA receptor blockers. Although a previous report cast doubt on the expression of α7 receptors by HMs (Chamberlin *et al.*, 2002), recent immunocytochemical data, confirmed in our laboratory, support the expression of both α4, α7 and β2 subunits by HMs (Dehkordi *et al.*, 2005). Moreover, the present study demonstrates that they are also functionally active.

The pharmacological profile of HM nAChRs was investigated using specific antagonists, namely DHβE and MLA, in subtype selective doses. Their combined application generated virtually full suppression of nicotine-induced responses, suggesting that the vast majority of nAChRs on motoneurons comprised homomeric α7 and heteromeric α4β2 receptors, in analogy with most other mammalian brain regions (Gotti and Clementi, 2004).

3. ARE NATIVE nAChRs ENDOGENOUSLY ACTIVATED?

The brain slice technique facilitates investigations of synaptic transmission, because it preserves both healthy neurons and good connections, overcoming other technical troubles encountered in “in vivo” experiments, such as mechanical instability, and difficulties in modifying the extracellular environment.

Anyway, it should also be acknowledged that the brain slice preparation is not ideally suited to demonstrate the role of projection neurons: connections are preserved only to a certain extent, because of the unavoidable injury occurring during slicing. The size of the network contributing to a certain physiological function results inevitably limited, but at the same time, the simplified circuitry helps understanding certain basic mechanisms under investigation. This condition thus enables to formulate theories to be confirmed with "in vivo" experiments.

These limitations, intrinsic to the use of brain slices, probably are the explanation for the impossibility of observing any random release of ACh, in the presence of blockers of glutamate, $GABA_A$ and glycine receptors, i.e. no spontaneous or electrically evoked synaptic events could be detected. Even following inhibition of AChE activity with edrophonium, there was no unmasking of cholinergic events. These observations suggest that cholinergic synapses on HMs were sparse and/or with low release probability (note lack of cholinergic miniature events). Thus, in the slice preparation, cholinergic terminals on HMs (Gilmor *et al.*, 1996; Arvidsson *et al.*, 1997; Schäfer *et al.*, 1998; Ferreira *et al.*, 2001) had little functional impact on motoneuron membrane properties. It is possible that such projections may require activation by direct stimulation of neurons located outside the slice preparation. This hypothesis will require future experiments with "in vivo" preparations.

However, the administration of edrophonium, to boost endogenous ACh levels, significantly reduced the frequency of glutamatergic sPSCs. Because this effect was absent in TTX solution, it probably took place at network level. Actually the effect of edrophonium application appears to be complex. In fact, raised ACh concentrations, beside activation and desensitization of nAChRs on glutamatergic premotoneurons, would be expected to activate muscarinic receptors as well, which can modulate HMs excitability (Shao and Feldman, 2000; Pagnotta *et al.*, 2005).

To simplify the resolution of this issue, rather than enhancing the concentration of endogenous ACh to study its potential action on glutamatergic transmission, I investigated whether blocking nAChRs with selective antagonists could help to reveal

any discrete change in excitatory synaptic transmission. It is noteworthy that the nicotinic antagonists used in the present study do not have non-selective actions on glutamate AMPA receptors (Alkondon *et al.*, 1999; Santos *et al.*, 2002).

Application of a large dose of DHβE to antagonize the entire population of nAChRs decreased the amplitude of sPSCs and ePSCs. Similar, though less intense, effects were also found with submicromolar concentrations of DHβE (to selective inhibit the β2 subunit containing receptor subpopulation), or MLA (to antagonize the α7 subtype). Adding up the inhibition produced by each antagonist gave an effect analogous to the one produced by a non-selective, large dose of DHβE, indicating that nAChRs controlling glutamatergic transmission probably belonged to those two receptor subtypes. Their location presumably included some glutamatergic terminals (in view of the fact that the size of mPSCs were reduced by such antagonists), as well as network premotoneurons.

Although the precise location of such nAChRs within the brainstem network remains still incompletely understood, it is worth considering the possibility that some of them were expressed at the level of projecting axons to regulate spike activity and hence transmitter release.

In summary then, the present data suggest a significant contribution by nAChRs activated by endogenous ACh to glutamatergic transmission on HMs in the present experimental model. Although the multiplicity of glutamatergic synapses and their releasing sites make it difficult to perform a quantal analysis of excitatory synaptic transmission, further complexity may originate from the role of nAChRs at such synapses, thus determining quantal parameters at various sites.

It was somewhat surprising to observe that nAChR blockers decreased the amplitude of mPSCs (beside sPSCs and ePSCs) without affecting their frequency. Previous studies have shown that, on rat HMs, such events are mediated by AMPA receptors (Essin *et al.*, 2002; Sharifullina *et al.*, 2004). At mammalian central synapses the amplitude of mPSCs depends on pre and postsynaptic factors. In the case of glutamatergic synapses, although co-activation of nAChRs and AMPA receptors can synergistically excite

interneurons (Alkondon *et al.*, 2003), there is no evidence for a fast, direct cross-interaction between these classes of ionotropic receptors on the postsynaptic membrane. It seems therefore more probable that nAChR activity can facilitate glutamatergic transmission presynaptically. Because nAChR activation mediates influx of Ca^{2+} at presynaptic level (Dajas-Bailador and Wonnacott, 2004), one possibility is that augmented levels of this divalent cations led to release of larger pools of glutamate (Voronin, 1993, 1994; Llano *et al.*, 2000). Reducing the nAChR contribution with selective antagonists might therefore desynchronized vesicle release so that the amplitude of the postsynaptically recorded events was correspondingly decreased. Indeed, Sharma and Vijayaraghavan (2003) have suggested that nAChR activity strongly contribute to the amount of glutamate released at individual synapses of the rat hippocampus.

4. DESENSITIZATION OF NACHRS ON HMS

Currents generated by HMs, in response to focal application of nicotine, showed a relatively fast desensitization which developed with a time constant of approximately 200 ms. Anyway, recovery occurred promptly upon nicotine washout, indicating that nAChRs could rapidly respond to changes in agonist concentrations.

In accordance with the pioneering work by Katz and Thesleff (1957) on the frog neuromuscular junction, I also observed that, on HMs, even bath application of low concentrations of nicotine largely desensitized the currents generated by brief puffer-delivery of nicotine. For these experiments I used bath concentrations of nicotine comparable to those found in the plasma of smokers (Gotti and Clementi, 2004). Interestingly, such concentrations of nicotine elicited a transient inward current in a minority of cells only, leaving a residual ability of the HM membrane to respond (albeit with significantly smaller amplitude) to focal application of large concentrations of nicotine. This finding can be related to the existence of multiple desensitized states with differential agonist sensitivity (Giniatullin *et al.*, 2005).

Administration of nicotine also affected glutamatergic transmission. In fact, a large dose of this drug strongly (and reversibly) decreased the amplitude and frequency of sPSCs as expected as a consequence of full desensitization of nAChRs at prejunctional level. However, smaller (sub-micromolar) concentrations of bath-applied nicotine induced more complex effects on synaptic transmission. On the one hand, the frequency of sPSCs and mPSCs was significantly reduced with no loss in amplitude; on the other hand, ePSCs were depressed, their amplitude fluctuations minimized, and the number of failures increased. It seems likely that these effects originated from broad activation and subsequent desensitization of nAChRs at network and presynaptic level. To clarify this issue, I analyzed the dynamics of changes in synaptic transmission after applying very low doses of nicotine, monitoring both sPCSs and ePSCs. As far as sPSCs were concerned, there was a transient facilitation of synaptic event frequency (without any change in event amplitude) whose occurrence was related to the dose of nicotine. The amplitude of ePSCs was transiently enhanced by 0.5 μM nicotine and subsequently depressed, whereas 0.1 μM nicotine consistently decreased the ePSC size.

It is noteworthy that, also in the case of metabotropic glutamate receptor activation, there is a differential modulation of spontaneous and electrically evoked events which is accounted for by assuming dissimilar receptor distribution (Sharifullina *et al.*, 2004). In analogy with this result, the present data obtained with nicotine could be explained by assuming a differential distribution of nAChRs between neurons stimulated electrically and those spontaneously active. On spontaneously active cells, low doses of nicotine first facilitated event frequency, suggesting initial enhancement of glutamate release. Lack of change in event amplitude argues against a direct interaction between nAChRs activated by nicotine and glutamatergic receptors on the HM membrane. On neurons stimulated electrically, nAChRs were more susceptible to desensitization, which arose rapidly after activation (with increased response amplitude) causing subsequent depression. Very small concentrations (0.1 μM) of nicotine could actually induce synaptic depression with no evidence for prior facilitation, probably because nAChRs can be desensitized without being activated at low agonist concentrations

(Paradiso and Steinbach, 2003). This phenomenon is termed "high affinity desensitization" (Giniatullin *et al.*, 2005) and, when occurring with ambient ACh, it can shape synaptic transmission efficacy (Lester, 2004).

5. A SCHEME TO ACCOUNT FOR THE EFFECTS OF NACHR ACTIVITY ON HMS EXCITABILITY

The complexity of the observed effects with agonist and antagonist applications is not easily resolved, even working on a reduced brain preparation. To aid future experiments targeted at clarifying the precise mode of action of nAChRs, it might, however, be useful to devise a simple scheme, as shown in Fig. 26, which is built on the reported experimental observations with the purpose of combining all available data into a unifying hypothesis.

Since there was close analogy in the functional consequence of applying either α7 or β2 subunit blockers and it was found analogous immunohistochemical expression of these subunits, it seems likely that α7 and α4β2 receptors shared similar distribution and function at network and HM level. When selective nicotinic blockers were applied in the absence of an agonist like nicotine, their effect would be manifested only where endogenously released ACh could activate nAChRs. Because the result was depression of synaptic transmission (including mPSCs), it seemed feasible that background activation of nAChRs, at network and presynaptic levels, facilitated glutamatergic currents perhaps by a dual phenomenon, namely membrane depolarization and increase in their quantal size. Postsynaptic nAChRs were unavailable to ambient ACh because the antagonists did not change the baseline current of HMs. The reason why despite histological evidence for cholinergic synapses on HMs (Gilmor *et al.*, 1996; Arvidsson *et al.*, 1997; Schäfer *et al.*, 1998; Ferreira *et al.*, 2001), I failed to detect their electrophysiological activity remains a matter of conjecture. It is possible that due to the postnatal age of the animals the majority of such synapses was still immature.

The global contribution by nAChRs to the efficiency of network glutamatergic transmission might be inferred from the data with a large, receptor saturating concentration of DHβE: the facilitation of glutamatergic transmission amounted to about 25 %. From immunohistochemical results and electrophysiological data concerning nicotine response block, it is apparent that nAChRs were predominantly localized on HMs, a fact that may explain the relatively limited contribution by nAChRs to glutamatergic transmission. Analogous inference could not be obtained simply on the basis of the experiments with edrophonium, because this AChE inhibitor exerted complex effects including boosting ACh actions on muscarinic receptors, the action of which could not be prevented by atropine that also blocks a wide variety of nAChRs (Zwart and Vijverberg, 1997). When nicotine was applied, a much wider population of nAChRs within the slice could be activated. Thus, recruitment of network receptors probably depolarized glutamatergic neurons and increased their frequency of release. This was manifested via transient facilitation of spontaneous events, which were then depressed presumably because of widespread desensitization of nAChRs. Non-linear summation of direct and indirect effects subsequent to nAChR activity over a large neuronal network could perhaps have cancelled out any changes in event amplitude, otherwise observed when nicotinic antagonists were applied. Synaptic currents evoked by focal electrical pulses applied to the reticular formation region were probably monosynaptic (see Essin *et al.*, 2002; Sharifullina *et al.*, 2004), thus requiring activation of a much smaller circuit that the one responsible for spontaneous activity. In this case, the action of nicotine was first facilitatory and then depressant when 0.5 μM was applied, or outright inhibitory when 0.1 μM was used. Because the latter dose is below threshold for activating α4β2 or α7 receptors (Chavez-Noriega *et al.*, 1997), it is suggested that depression was due to high affinity desensitization proceeding from bound-closed receptors (Giniatullin *et al.*, 2005).

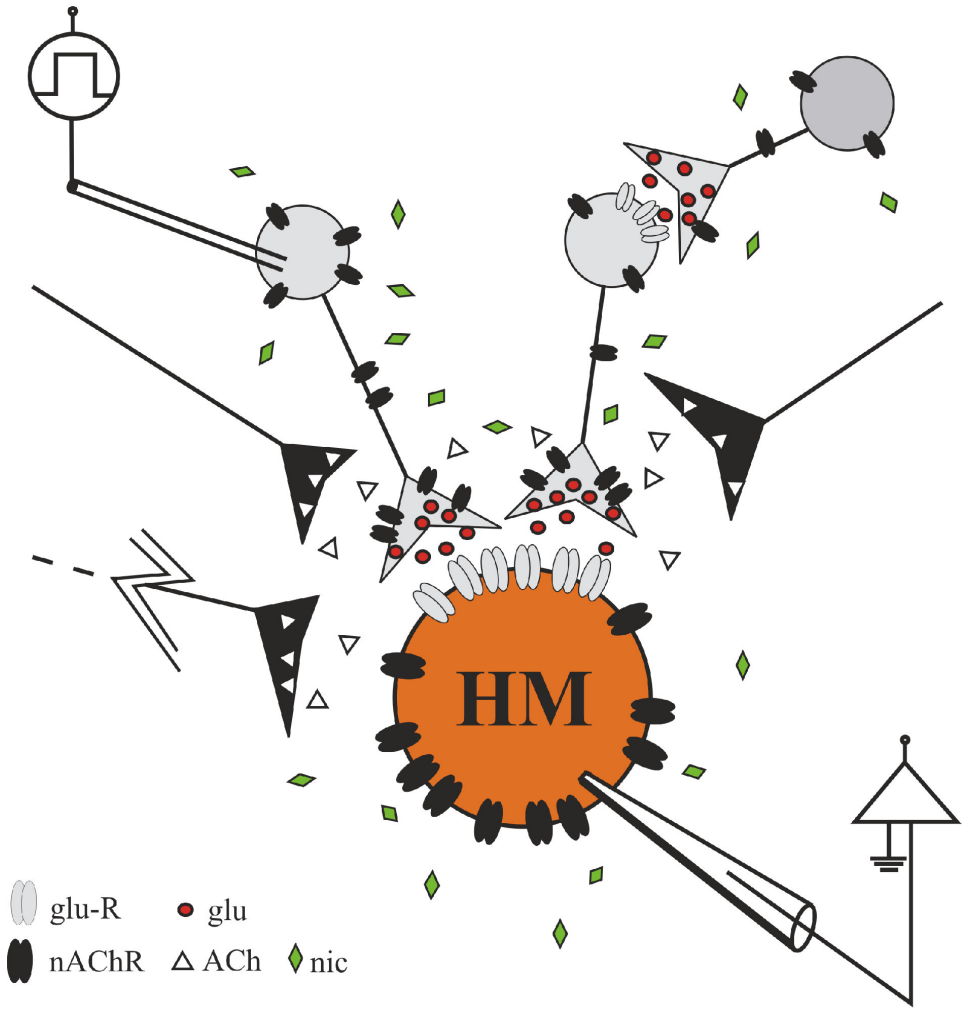


FIG. 26. Schematic diagram to account for the action of nAChRs on glutamatergic transmission in the nucleus hypoglossus. Glutamatergic premotoneurons (grey) impinging upon a HM (orange, under patch clamp condition) release glutamate (red dots) from endogenous pools following either electrical stimulation (see electrode) or spontaneously via local circuits. Glutamate receptors are indicated as grey ellipsoids. Because of the sparse cholinergic afferents (black) presumed to have been largely severed during slicing, cholinergic synaptic events are not detectable on HM. However, background release of ACh (open triangles) takes place and facilitates glutamatergic transmission mainly by acting on presynaptic terminals. HM is endowed with extrasynaptic nAChRs (of at least two subtypes) which are readily activated and then desensitized by nicotine (green diamonds). nAChRs are also expressed by glutamatergic premotoneurons at axonal or somatic level. Such network receptors, normally not accessible to endogenous ACh, unless AChE is inhibited, are activated and desensitized by nicotine with dual facilitation/depression of excitatory synaptic transmission. Note that very small concentrations of nicotine are likely to evoke nAChR desensitization without prior application (Paradiso and Steinbach, 2003).

6. FUNCTIONAL IMPLICATIONS

The observation of strong responses mediated by nAChRs on HMs together with immunohistochemical demonstration of such receptors concur to suggest that the excitability of HMs can be powerfully upregulated by cholinergic agonists, including nicotine. Although nicotine is known to stimulate respiration via an action on respiratory brainstem neurons (Shao and Feldman, 2001, 2002), the present data show that direct stimulation by nicotine of nAChRs on HMs, which are phasically activated during inspiration, could contribute to changes in the respiratory network output. This phenomenon would be particularly important when considering the effects of tobacco smoke because even small doses of nicotine evoked clear alterations in excitatory synaptic transmission. In such a case nicotine would generate a more widespread action involving brainstem circuits and modulating their efficiency. These effects may have dramatic consequences during a crucial period such as development, resulting in impaired network connections and altered HMs responsiveness.

Finally, the on-going contribution by nAChRs to glutamatergic transmission reveals an additional mechanism for interaction between distinct transmitters at similar synaptic sites. In fact, rather than co-release of diverse transmitters like in the case of glycine and GABA (Jonas *et al.*, 1998), the release of the excitatory transmitter glutamate would be subjected to finely tuned, continuous control by ACh, making this process amenable to gain-setting via selective modulation of cholinergic receptors. In this context it is interesting that, in the hippocampus, activation of nAChRs by nicotine can up or down-regulate, in a persistent fashion, the activity of glutamatergic synapses with presumably functional consequences for memory storage at molecular level (Ge and Dani, 2005).

REFERENCE LIST

Agulhon C., Abitbol M., Bertrand D., and Malafosse A. (1999a) Localization of mRNA for CHRNA7 in human fetal brain. ***Neuroreport***. *10:* *2223-7.*

Agulhon C., Charnay Y., Vallet P., Abitbol M., Kobetz A., Bertrand D., and Malafosse A. (1999b) Distribution of mRNA for the alpha4 subunit of the nicotinic acetylcholine receptor in the human fetal brain. ***Brain Res. Mol. Brain Res.*** *58:* *123-31.*

Alkondon M., Pereira E.F., Wonnacott S., and Albuquerque E.X. (1992) Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. ***Mol. Pharmacol.*** *41: 802-8.*

Alkondon M., Pereira E.F., Cortes W.S., Maelicke A., and Albuquerque E.X. (1997) Choline is a selective agonist of alpha7 nicotinic acetylcholine receptors in the rat brain neurons. ***Eur. J. Neurosci.*** *9(12): 2734-42.*

Alkondon M., Pereira E.F., and Albuquerque E.X. (1998) α-bungarotoxin- and methyllycaconitine-sensitive nicotinic receptors mediate fast synaptic transmission in interneurons of rat hippocampal slices. ***Brain Res.*** *810: 257-63.*

Alkondon M., Pereira E.F., Eisenberg H.M., and Albuquerque E.X. (1999) Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. ***J. Neurosci.*** *19: 2693-705.*

Alkondon M., Pereira E.F., and Albuquerque E.X. (2003) NMDA and AMPA receptors contribute to the nicotinic cholinergic excitation of CA1 interneurons in the rat hippocampus. ***J. Neurophysiol.*** *90(3): 1613-25.*

Arroyo-Jiménez M.M., Bourgeois J.P., Marubio L.M., Le Sourd A.M., Ottersen O.P., Rinvik E., Fairen A., and Changeux J.P. (1999) Ultrastructural localization of the α4-subunit of the neuronal acetylcholine nicotinic receptor in the rat substantia nigra. ***J. Neurosci.*** *19: 6475-87.*

Arvidsson U., Riedl M., Elde R., and Meister B. (1997) Vesicular acetylcholine transporter (VAChT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. ***J. Comp. Neurol.*** *378(4):454-67.*

Badio B., and Daly J.W. (1994) Epibatidine, a potent analgetic and nicotinic agonist. ***Mol. Pharmacol.*** *45: 563-9.*

Ballanyi K., Onimaru H., and Homma I. (1999) Respiratory network function in the isolated brainstem-spinal cord of newborn rats. ***Prog. Neurobiol.*** *59(6): 583-634.*

Bellingham M.C., and Berger A.J. (1996) Presynaptic depression of excitatory synaptic inputs to rat hypoglossal motoneurons by muscarinic M2 receptors. ***J. Neurophysiol.*** *76(6): 3758-70.*

Bellingham M.C., and Funk G.D. (2000) Cholinergic modulation of respiratory brain-stem neurons and its function in sleep-wake state determination. ***Clin. Exp. Pharmacol. Physiol.*** *27(1-2): 132-7.*

Berg D.K., and Conroy W.G. (2002) Nicotinic alpha 7 receptors: synaptic options and downstream signaling in neurons. ***J. Neurobiol.*** *53(4): 512-23.*

Berger A.J. (2000) Determinants of respiratory motoneuron output. ***Respir. Physiol.*** *122(2-3): 259-69.*

Bergmeier S.C., Lapinsky D.J., Free R.B., and McKay D.B. (1999) Ring E analogs of methyllycaconitine (MLA) as novel nicotinic antagonists. ***Bioorg. Med. Chem. Lett.*** *9(15): 2263-6.*

Boyd N.D. (1987) Two distinct kinetic phases of desensitization of acetylcholine receptors of clonal rat PC12 cells. ***J. Physiol.*** *389: 45-67.*

Boone T.B., and Aldes L.D. (1984) The ultrastructure of two distinct neuron populations in the hypoglossal nucleus of the rat. ***Exp. Brain Res.*** *54(2): 321-6.*

Borke R.C., Nau M.E., and Ringler R.L.Jr. (1983) Brain stem afferents of hypoglossal neurons in the rat. ***Brain Res.*** *269: 47-55.*

Broide R.S., and Leslie F.M. (1999) The alpha7 nicotinic acetylcholine receptor in neuronal plasticity. ***Mol. Neuobiol.*** *20(1): 1-16.*

Cartier G.E., Yoshikami D., Gray W.R., Luo S., Olivera B.M., and McIntosh J.M. (1996) A new α-Conotoxin which targets α3β2 nicotinic acetylcholine receptors. ***J. Biol. Chem.*** *271: 7522-8.*

Chamberlin N.L., Bocchiaro C.M., Greene R.W., and Feldman J.L. (2002) Nicotinic excitation of rat hypoglossal motoneurons. ***Neuroscience.*** *115(3): 861-70.*

Changeux J.P. (1990) The TiPS lecture. The nicotinic acetylcholine receptor: an allosteric protein prototype of ligand-gated ion channels. ***Trends Pharmacol. Sci.*** *11(12): 485-92.*

Changeux J.P., Devillers-Thiery A., and Chemouilli P. (1984) Acetylcholine receptor: an allosteric protein. ***Science.*** *225(4668): 1335-45.*

Chavez-Noriega L.E., Crona J.H., Washburn M.S., Urrutia A., Elliott K.J., and Johnson E.C. (1997) Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h-alpha2-beta2, h-alpha2-beta4, h-alpha3-beta2, h-alpha3-beta4, h-alpha4-beta2, h-alpha4-beta4 and h-alpha7 expressed in Xenopus oocytes. ***J. Pharmacol. Exp. Ther.*** *280(1): 346-56.*

Clarke P.B.S., Schwartz R.D., Paul S.M., Pert C.B., and Pert A. (1985) Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H]-acetylcholine and [^{125}I]-alpha bungarotoxin. ***J. Neurosci.*** *5: 1307-15.*

Clarke P.B. (1993) Nicotinic receptors in mammalian brain: localization and relation to cholinergic innervation. ***Prog. Brain Res.*** *98: 77-83.*

Collingridge G.L., and Lester R.A. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. ***Pharmacol. Rev.*** *41(2): 143-210.*

Colquhoun L.M., and Patrick J.W. (1997) Alpha3, beta2, and beta4 form heterotrimeric neuronal nicotinic acetylcholine receptors in Xenopus oocytes. ***J. Neurochem.*** *69(6): 2355-62.*

Connaughton M., Priestley J.V., Sofroniew M.V., Eckenstein F., and Cuello A.C. (1986) Inputs to motoneurones in the hypoglossal nucleus of the rat: light and electron microscopic immunocytochemistry for choline acetyltransferase, substance P and enkephalins using monoclonal antibodies. ***Neuroscience.*** *17(1): 205-24.*

Conroy W.G., and Berg D.K. (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. ***J. Biol. Chem.*** *270: 4424–31.*

Cordero-Erausquin M., Marubio L.M., Klink R., and Changeux J.P. (2000) Nicotinic receptor function: new perspectives from knockout mice. ***Trends Pharmacol. Sci.*** *21(6): 211-7.*

Corringer P.J., Le Novere N., and Changeux J.P. (2000) Nicotinic receptors at the amino acid level. ***Annu. Rev. Pharmacol. Toxicol.*** *40: 431-58.*

Cunningham E.T. Jr., and Sawchenko P.E. (2000) Dorsal medullary pathways subserving oromotor reflexes in the rat: implications for the central neural control of swallowing. ***J. Comp. Neurol.*** *417(4): 448-66.*

Dajas-Bailador F., and Wonnacott S. (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signalling. ***Trends Pharmacol. Sci.*** *25(6): 317-24.*

Dani J.A., and Heinemann S. (1996) Molecular and cellular aspects of nicotine abuse. ***Neuron.*** *16(5): 905-8.*

Davies A.R., Hardick D.J., Blagbrough I.S., Potter B.V., Wolstenholme A.J., and Wonnacott S. (1999) Characterisation of the binding of [3H]methyllycaconitine: a new radioligand for labelling alpha 7-type neuronal nicotinic acetylcholine receptors. ***Neuropharmacology.*** *38 (5): 679-90.*

Davis M.J., Wu X., Nurkiewics T.R., Kawasaki J., Gui P., Hill M.A., and Wilson E. (2001) Regulation of ion channels by protein tyrosine phosphorylation. ***Am. J. Physiol.*** *281: H1835-H1862.*

Dehkordi O., Millis R.M., Dennis G.C., Coleman B.R., Johnson S.M., Changizi L., and Ovid Trouth C. (2005) Alpha-7 and alpha-4 nicotinic receptor subunit immunoreactivity in genioglossus muscle motoneurons. ***Respir. Physiol. Neurobiol.*** *145(2-3): 153-61.*

De Simone R., Ajmone-Cat M.A., Carnevale D., and Minghetti L. (2005) Activation of α7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures. ***J. Neuroinflammation.*** *2: 4.*

Di Angelantonio S., and Nistri A. (2001) Calibration of agonist concentrations applied by pressure pulses or via rapid solution exchanger. ***J. Neurosci. Methods.*** *110(1-2): 155-61.*

DiFranza J.R., and Lew R.A. (1995) Effect of maternal cigarette smoking on pregnancy complications and sudden infant death syndrome. ***J. Fam. Pract.*** *40(4): 385-94.*

Dobbins E.G., and Feldman J.L. (1995) Differential innervation of protruder and retractor muscles of the tongue in rat. ***J. Comp. Neurol.*** *357(3): 376-94.*

Donato R., and Nistri A. (2000) Relative contribution by GABA or glycine to Cl(-)-mediated synaptic transmission on rat hypoglossal motoneurons in vitro. ***J. Neurophysiol.*** *84: 2715-24.*

Dourado M., and Sargent P.B. (2002) Properties of nicotinic receptors underlying Renshaw cell excitation by alpha-motor neurons in neonatal rat spinal cord. ***J. Neurophysiol.*** *87(6): 3117-25.*

Dwoskin L.P., and Crooks P.A. (2001) Competitive neuronal nicotinic receptor antagonists: a new direction for drug discovery. ***J. Pharmacol. Exp. Ther.*** *298(2): 395-402.*

Eccles J.C., Fatt P., and Koketsu K. (1954) Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. ***J. Physiol.*** *126(3): 524-62.*

Edelstein S.J., and Changeux J.P. (1998) Allosteric transitions of the acetylcholine receptor. ***Adv. Protein Chem.*** *51: 121-84.*

Elgoyhen A.B., Johnson D.S., Boulter J., Vetter D.E., and Heinemann S. (1994) Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. ***Cell.*** *79(4): 705-15.*

Essin K., Nistri A., and Magazanik L. (2002) Evaluation of GluR2 subunit involvement in AMPA receptor function of neonatal rat hypoglossal motoneurons. ***Eur. J. Neurosci.*** *15(12): 1899-906.*

Feltz A., and Trautmann A. (1982) Desensitization at the frog neuromuscular junction: a biphasic process. ***J. Physiol.*** *322: 257-72.*

Fenster C.P., Rains M.F., Noerager B., Quick M.W., and Lester R.A. (1997) Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. ***J. Neurosci.*** *17(15): 5747-59.*

Fenster C.P., Beckman M.L., Parker J.C., Sheffield E.B., Whitworth T.L., Quick M.W., and Lester R.A. (1999) Regulation of alpha4beta2 nicotinic receptor desensitization by calcium and protein kinase C. ***Mol. Pharmacol.*** *55(3): 432-43.*

Ferreira M., Ebert S.N., Perry D.C., Yasuda R.P., Baker C.M., Davila-Garcia M.I., Kellar K.J., and Gillis R.A. (2001) Evidence of a functional alpha7-neuronal nicotinic receptor subtype located on motoneurons of the dorsal motor nucleus of the vagus. ***J. Pharmacol. Exp. Ther.*** *296(2): 260-9.*

Fisher J.L., Pidoplichko V.I., and Dani J.A. (1998) Nicotine modifies the activity of ventral tegmental area dopaminergic neurons and hippocampal GABAergic neurons. ***J. Physiol.*** *92: 209-13.*

Fucile S. (2004) Ca2+ permeability of nicotinic acetylcholine receptors. ***Cell Calcium.*** *35(1): 1-8.*

Funk G.D., Smith J.C., and Feldman J.L. (1993) Generation and transmission of respiratory oscillations in medullary slices: role of excitatory amino acids. ***J. Neurophysiol.*** *70(4): 1497-515.*

Ge S., and Dani J.A. (2005) Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation. ***J. Neurosci.*** *25: 6084-91.*

Genzen J.R., and McGehee D.S. (2003) Short- and long-term enhancement of excitatory transmission in the spinal cord dorsal horn by nicotinic acetylcholine receptors. ***Proc. Natl. Acad. Sci.*** *100(11): 6807-12.*

Ghosh A., and Greenberg M.E. (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. ***Science.*** *268: 239-47.*

Gilmor M.L., Nash N.R., Roghani A., Edwards R.H., Yi H., Hersch S.M., and Levey A.I. (1996) Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. ***J. Neurosci.*** *16(7): 2179-90.*

Giniatullin R., Nistri A., and Yakel J.L. (2005) Desensitization of nicotinic ACh receptors: shaping cholinergic signaling. ***Trends Neurosci.*** 28(7): 371-8.

Gothe B., Strohl K.P., Levin S., and Cherniack N.S. (1985) Nicotine: a different approach to treatment of obstructive sleep apnea. ***Chest.*** *87(1): 11-7.*

Gotti C., Fornasari D., and Clementi F. (1997) Human neuronal nicotinc receptors. ***Prog. Neurobiol.*** *53: 199-237.*

Gotti C., and Clementi F. (2004) Neuronal nicotinic receptors: from structure to pathology. ***Prog. Neurobiol.*** *74: 363-96.*

Grady S., Marks M.J., Wonnacott S., and Collins A.C. (1992) Characterization of nicotinic receptor-mediated [^{3}H]dopamine release from synaptosomes prepared from mouse striatum. ***J. Neurochem.*** *59: 848-56.*

Gray R., Rajan A.S.Radcliffe K.A., Yakehiro M., and Dani J.A. (1996) Hippocampal synaptic transmission enhanced by low concentrations of nicotine. ***Nature.*** *383(6602): 713-6.*

Groot-Kormenlink P.J., Luyten W.H., Colquhoun D., and Silviotti L.G. (1998) A reporter mutation approach shows incorporation of the "orphan" subunit beta3 into a functional nicotinic receptor. ***J. Biol. Chem.*** *273(25): 15317-20.*

Guo J.Z., Tredway T.L., and Chiappinelli V.A. (1998) Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus. ***J. Neurosci.*** *18: 1963-9.*

Guo J.Z., Liu Y., Sorenson E.M., and Chiappinelli V.A. (2005) Synaptically released and exogenous ACh activate different nicotinic receptors to enhance evoked glutamatergic transmission in the lateral geniculate nucleus. ***J. Neurophysiol.*** [Epub ahead of print]

Hamill O.P., Marty A., Neher E., Sakmann B., and Sigworth F.J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. ***Pflugers Arch.*** *391: 85-100.*

Harvey S.C., and Luetje C.W. (1996) Determinants of competitive antagonist sensitivity on neuronal nicotinic receptor beta subunits. ***J. Neurosci.*** *16(12): 3798-806.*

Harvey S.C, Maddox F.N., and Luetje C.W. (1996) Multiple determinants of dihydro-beta-erythroidine sensitivity on rat neuronal nicotinic receptor alpha subunits. ***J. Neurochem.*** *67(5): 1953-9.*

Harvey S.C., McIntosh J.M., Cartier G.E., Maddox F.N., and Luetje C.W. (1997) Determinants of specificity for α -Conotoxin MII on α3β2 neuronal nicotinic receptors. ***Mol. Pharmacol.*** *51: 336-42.*

Hopfield J.F., Tank D.W., Greengard P., and Huganir R.L. (1988) Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. ***Nature.*** *336:677-80.*

Holstege G., Kuypers H.G.,and Dekker J.J. (1977) The organization of the bulbar fibre connections to the trigeminal, facial and hypoglossal motor nuclei. II. An autoradiographic tracing study in cat. ***Brain.*** *100(2):264-86.*

Hsiao B., Dweck D., and Luetje C.W. (2001) Subunit-dependent modulation of neuronal nicotinic receptors by zinc. ***J. Neurosci.*** *21(6): 1848-56.*

Huganir R.L., Delcour A.H., Greengard P., and Hess G.P. (1986) Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. ***Nature.*** *321(6072): 774-6.*

Itier V., and Bertrand D. (2001) Neuronal nicotinic receptors: from protein structure to function. ***FEBS Lett.*** *504(3): 118-25.*

Jean A. (2001) Brain stem control of swallowing: neuronal network and cellular mechanisms. ***Physiol. Rev.*** *81(2): 929-69.*

Jonas P., Bischofberger J., and Sandkuhler J. (1998) Corelease of two fast neurotransmitters at a central synapse. ***Science.*** *281(5375): 419-24.*

Jones I.W., Bolam J.P., and Wonnacott S. (2001) Presynaptic localisation of the nicotinic acetylcholine receptor β2 subunit immunoreactivity in rat nigrostriatal dopaminergic neurones. ***J. Comp. Neurol.*** *439: 235-47.*

Jones S., Sudweeks S., and Yakel J.L. (1999) Nicotinic receptors in the brain: correlating physiology with function. ***Trends Neurosci.*** *22(12): 555-61.*

Kaiser S.A., Soliakov L., Harvey S.C., Luetje C.W., and Wonnacott S. (1998) Differential inhibition by α-Conotoxin-MII of the nicotinic stimulation of [^{3}H]dopamine release from rat striatal synaptosomes and slices. ***J. Neurochem.*** *70(3): 1069-77.*

Katz B., and Thesleff S. (1957) A study of the 'desensitization' produced by acetylcholine at the motor endplate. ***J. Physiol.*** *138: 63-80.*

Khiroug L., Sokolova E., Giniatullin R., Afzalov R., and Nistri A. (1998) Recovery from desensitization of neuronal nicotinic acetylcholine receptors of rat chromaffin cells is modulated by intracellular calcium through distinct second messengers. ***J. Neurosci.*** *18(7): 2458-66.*

Khiroug S.S., Harkness P.C., Lamb P.V., Sudweeks S.N., Khiroug L., Millar N.S., and Yakel J.L. (2002) Rat nicotinic alpha7 and beta2 subunits co-assemble to form functional heteromeric nicotinic receptor channels. ***J. Physiol.*** *540: 425-34.*

Kouznetsova M., and Nistri A. (2000) Facilitation of cholinergic transmission by substance P methyl ester in the mouse hippocampal slice preparation. ***Eur J Neurosci.*** *12(2): 585-94.*

Krammer E.B., Rath T., and Lischka M.F. (1979) Somatotopic organization of the hypoglossal nucleus: A HRP study in the rat. ***Brain Res*** *170: 533–7.*

Lena C., and Changeux J.P. (1993) Allosteric modulations of the nicotinic acetylcholine receptor. ***Trends Neurosci.*** *16(5):181-6.*

Lena C., Changeux J.P., and Mulle C. (1993) Evidence for "preterminal" nicotinic receptors on GABAergic axons in the rat interpeduncular nucleus. ***J. Neurosci.*** *13: 2680-8.*

Le Novere N., Zoli M., and Changeux J.P. (1996) Neuronal nicotinic receptors alpha6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. ***Eur. J. Neurosci.*** *8: 2428-39.*

Lester R.A.J. (2004) Activation and desensitization of heteromeric neuronal nicotinic receptors: implications for non-synaptic transmission. ***Bioorg. Med. Chem. Lett.*** *14(8): 1897–900.*

Lewis P.R., Flumerfelt B.A., and Shute C.C.D. (1971) The use of cholinesterase techniques to study topographical localization in the hypoglossal nucleus of the rat. ***J. Anat.*** *110:203–13.*

Li Y.Q., Takada M., and Mizuno N. (1993) Identification of premotor interneurons which project bilaterally to the trigeminal motor, facial or hypoglossal nuclei: a fluorescent retrograde double-labeling study in the rat. ***Brain Res.*** *611(1): 160-4.*

Li Y.Q., Takada M., Kaneko T., and Mizuno N. (1997) Distribution of GABAergic and glycinergic premotor neurons projecting to the facial and hypoglossal nuclei in the rat. ***J. Comp. Neurol.*** *378(2): 283-94.*

Li X., Rainnie D.G., McCarley R.W., and Greene R.W. (1998) Presynaptic nicotinic receptors facilitate monoaminergic transmission. ***J. Neurosci.*** *18: 1904-12.*

Lindstrom J. (2000) The structure of neuronal nicotinic receptors. *In: Neuronal Nicotinic Receptors ed. F. Clementi, C. Gotti, D. Fornasari, Springer, New York,* ***Handbook of Experimental Pharmacology*** *144: 101-62.*

Llano I., Gonzalez J., Caputo C., Lai F.A., Blayney L.M., Tan Y.P., and Marty A. (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. **Nat. Neurosci.** 3(12): 1256-65.
Erratum in: Nat. Neurosci (2001) 4(3): 331.

Lowe A.A. (1980) The neural regulation of tongue movements. ***Prog. Neurobiol.*** *15(4): 295-344.*

Luo P., Dessem D., and Zhang J. (2001) Axonal projections and synapses from the supratrigeminal region to hypoglossal motoneurons in the rat. ***Brain Res.*** *890(2): 314-29.*

Maggi L., Sher E., and Cherubini E. (2001) Regulation of GABA release by nicotinic acetylcholine receptors in the neonatal rat hippocampus. **J. Physiol.** 536(Pt 1): 89-100.

Maggi L., Le Magueresse C., Changeux J.P., and Cherubini E. (2003) Nicotine activates immature "silent" connections in the developing hippocampus. ***Proc. Natl. Acad. Sci.*** *100(4): 2059-64.*

Maggi L., Sola E., Minneci F., Le Magueresse C., Changeux J.P., and Cherubini E. (2004) Persistent decrease in synaptic efficacy induced by nicotine at Schaffer collateralCA1 synapses in the immature rat hippocampus. ***J. Physiol.*** *559(Pt 3): 863-74.*

Mansvelder H.D., McGehee and D.S. (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. ***Neuron***. *27(2): 349-57.*

Marchetti C., Pagnotta S.E., Donato R., and Nistri A. (2002) Inhibition of spinal or hypoglossal motoneurons of the newborn rat by glycine or GABA. ***Eur. J. Neurosci.*** *15(6): 975-83.*

Marks M.J., Stitzel J.A., Romm E., Wehner J.M., and Collins A.C. (1986) Nicotinic binding sites in rat and mouse brain: comparison of acetylcholine, nicotine, and alpha-bungarotoxin. ***Mol. Pharmacol.*** *30(5): 427-36.*

McGehee D.S., Heath M.J., Gelber S.Devay P., and Role L.W. (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. ***Science.*** *269(5231): 1692-6.*

McGehee D.S., and Role L.W. (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. ***Annu. Rev. Physiol.*** *57: 521-6.*

Millar N.S. (2003) Assembly and subunit diversity of nicotinic acetylcholine receptors. ***Biochem. Soc. Trans.*** *31(Pt 4): 869-74.*

Mulle C., Lena C., and Changeux J.P. (1992) Potentiation of nicotinic receptor response by external calcium in rat central neurons. ***Neuron.*** *8: 937-45.*

Murakoshi T., Suzue T., and Tamai S. (1985) A pharmacological study on respiratory rhythm in the isolated brainstem-spinal cord preparation of the newborn rat. ***Br. J. Pharmacol.*** *86(1): 95-104.*

Nordberg A., Zhang X.A., Fredriksson A., and Eriksson P. (1991) Neonatal nicotine exposure induces permanent changes in brain nicotinic receptors and behaviour in adult mice. ***Brain Res. Dev. Brain Res.*** *63(1-2): 201-7.*

O'Brien J.A., Isaacson J.S., and Berger A.J. (1997) NMDA and non-NMDA receptors are co-localized at excitatory synapses of rat hypoglossal motoneurons. ***Neurosci. Lett.*** *227(1): 5-8.*

O'Brien J.A., and Berger A.J. (1999) Cotransmission of GABA and glycine to brain stem motoneurons. ***J. Neurophysiol.*** *82(3): 1638-41.*

Ochoa E.L., Li L., and McNamee M.G. (1990) Desensitization of central cholinergic mechanisms and neuroadaptation to nicotine. ***Mol. Neurobiol.*** *4(3-4): 251-87.*

Ogier R., Liu X., Tribollet E., Bertrand D., and Raggenbass M. (2004) Identified spinal motoneurons of young rats possess nicotinic acetylcholine receptors of the heteromeric family. ***Eur. J. Neurosci.*** *20(10): 2591-7.*

Oikawa H., Nakamichi N., Kambe Y., Ogura M., and Yoneda Y. (2005) An increase in intracellular free calcium ions by nicotinic acetylcholine receptors in a single cultured rat cortical astrocyte. ***J Neurosci. Res.*** *79(4): 535-44.*

Orr-Urtreger A., Goldner F.M., Saeki M., Lorenzo I., Goldberg L., De Biasi M., Dani J.A., Patrick J.W., and Beaudet A.L. (1997) Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents. ***J. Neurosci.*** *17(23): 9165-71.*

Pagnotta S.E., Lape R., Quitadamo C., and Nistri A. (2005) Pre- and postsynaptic modulation of glycinergic and GABAergic transmission by muscarinic receptors on rat hypoglossal motoneurons in vitro. ***Neuroscience.*** *130(3): 783-95.*

Palma E., Bertrand S., Binzoni T., and Bertrand D. (1996) Neuronal nicotinic alpha7 receptor expressed in Xenopus oocytes presents five putative binding sites for methyllycaconitine. ***J. Physiol.*** *491(Pt 1): 151-61.*

Paradiso KG, and Steinbach JH. (2003) Nicotine is highly effective at producing desensitization of rat alpha4beta2 neuronal nicotinic receptors. ***J. Physiol.*** *553(Pt 3): 857-71.*

Paterson D., and Nordberg A. (2000) Neuronal nicotinic receptors in the human brain. ***Prog. Neurobiol.*** *61(1): 75-111.*

Pereira E.F.R., Alkondon M., Reinhardt S., Maelicke A., Peng X., Lindstrom J., Whiting P., and Albuquerque E.X. (1994) Physiostigmine and galanthamine: probes for a novel binding site on α4β2 subtype of neuronal nicotinic acetylcholine receptors stably expressed in fibroblast cells. ***J. Pharmacol. Exp. Ther.*** *270: 768-78.*

Perrins R., and Roberts A. (1995) Cholinergic and electrical synapses between synergistic spinal motoneurones in the Xenopus laevis embryo. ***J. Physiol.*** *485 (Pt 1): 135-44.*

Perry D.C., Xiao Y., Nguyen H.N., Musachio J.L., Davila-Garcia M.i., and Kellar K.J. (2002) Measuring nicotinic receptors with characteristics of alpha4beta2, alpha3beta2 and alpha3beta4 subtypes in rat tissues by autoradiography. ***J..Neurochem.*** *82:468-81.*

Picciotto M.R., Zoli M., Rimondini R., Lena C., Marubio L.M., Pich E.M., Fuxe K., and Changeux J.P. (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. ***Nature.*** *391(6663): 173-7.*

Pierrefiche O., Bischoff A.M., Richter D.W., and Spyer K.M. (1997) Hypoxic response of hypoglossal motoneurones in the in vivo cat. ***J. Physiol.*** *505 (Pt 3): 785-95.*

Quick M.W., and Lester R.A. (2002) Desensitization of neuronal nicotinic receptors. ***J. Neurobiol.*** *53(4): 457-78.*

Radcliffe K.A., Fisher J.L., Gray R., and Dani J.A. (1999) Nicotine modulation of glutamate and GABA synaptic transmission of hippocampal neurons. ***Ann. NY Acad. Sci.*** *868: 591-610.*

Remmers J.E., de Groot W.J., Sauerland E.K., and Anch A.M. (1978) Pathogenesis of upper airway occlusion during sleep. ***J. Appl. Physiol.*** *44(6): 931-8.*

Rekling J.C., Funk G.D., Bayliss D.A., Dong X.W., and Feldman J.L. (2000) Synaptic control of motoneuronal excitability. ***Physiol. Rev.*** *80(2): 767-852.*

Richmonds C.R., and Hudgel D.W. (1996) Hypoglossal and phrenic motoneuron responses to serotonergic active agents in rats. ***Respir. Physiol.*** *106(2): 153-60.*

Robinson D.M., Peebles K.C., Kwok H., Adams B.M., Clarke L.L., Woollard G.A. and Funk G.D. (2002) Prenatal nicotine exposure increases apnoea and reduces nicotinic potentiation of hypoglossal inspiratory output in mice. ***J. Physiol.*** *538(Pt 3): 957-73.*

Roerig B., Nelson D.A., and Katz L.C. (1997) Fast synaptic signaling by nicotinic acetylcholine and serotonin 5-HT_3 receptors in developing visual cortex. ***J. Neurosci.***17: *8353-62.*

Role LW, and Berg DK. (1996) Nicotinic receptors in the development and modulation of CNS synapses. ***Neuron.*** *16(6): 1077-85.*

Sahara Y., Hashimoto N., and Nakamura Y. (1996) Hypoglossal premotor neurons in the rostral medullary parvocellular reticular formation participate in cortically-induced rhythmical tongue movements. ***Neurosci. Res.*** *26(2): 119-31.*

Sargent P.B. (1993) The diversity of neuronal nicotinic acetylcholine receptors. ***Annu. Rev. Neurosci.*** *16: 403-43.*

Seguela P., Wadiche J., Dineley-Miller K., Dani J.A., and Patrick J.W. (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. ***J. Neurosci.*** *13(2): 596-604.*

Schrattenholz A., Pereira E.F., Roth U., Weber K.H., Albuquerque E.X., and Maelicke A. (1996) Agonist responses of neuronal nicotinic acetylcholine receptors are potentiated by a novel class of allosterically acting ligands. ***Mol. Pharmacol.*** *49(1): 1-6.*

Schäfer M.K., Eiden L.E., and Weihe E. (1998) Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. I. Central nervous system. ***Neuroscience.*** *84(2): 331-59.*

Shao X.M., and Feldman J.L. (2000) Acetylcholine modulates respiratory pattern: effects mediated by M3-like receptors in preBötzinger complex inspiratory neurons. ***J. Neurophysiol.*** *83(3): 1243-52.*

Shao X.M., and Feldman J.L. (2001) Mechanisms underlying regulation of respiratory pattern by nicotine in preBötzinger complex. ***J. Neurophysiol.*** *85(6): 2461-7.*

Shao X.M., and Feldman J.L. (2002) Pharmacology of nicotinic receptors in preBötzinger complex that mediate modulation of respiratory pattern. ***J. Neurophysiol.*** *88(4): 1851-8.*

Shao X.M., and Feldman J.L. (2005) Cholinergic neurotransmission in the preBötzinger Complex modulates excitability of inspiratory neurons and regulates respiratory rhythm. ***Neuroscience.*** *130(4): 1069-81.*

Sharifullina E., Ostroumov K., and Nistri A. (2004) Activation of group I metabotropic glutamate receptors enhances efficacy of glutamatergic inputs to neonatal rat hypoglossal motoneurons in vitro. ***Eur. J. Neurosci.*** *20(5): 1245-54.*

Sharifullina E., Ostroumov K., and Nistri A. (2005) Metabotropic glutamate receptor activity induces a novel oscillatory pattern in neonatal rat hypoglossal motoneurones. ***J. Physiol.*** *563(Pt 1): 139-59.*

Sharma G., and Vijayaraghavan S. (2003) Modulation of presynaptic store calcium induces release of glutamate and postsynaptic firing. ***Neuron.*** *38(6): 929-39.*

Singer J.H., Bellingham M.C., and Berger A.J. (1996) Presynaptic inhibition of glutamatergic synaptic transmission to rat motoneurons by serotonin. ***J. Neurophysiol.*** *76(2): 799-807.*

Slotkin T.A., Epps T.A., Stenger M.L., Sawyer K.J., and Seidler F.J. (1999) Cholinergic receptors in heart and brainstem of rats exposed to nicotine during development: implications for hypoxia tolerance and perinatal mortality. ***Brain. Res. Dev. Brain Res.*** *113(1-2): 1-12.*

Smith J.C., Ellenberger H.H., Ballanyi K., Richter D.W., and Feldman J.L. (1991) Pre-Bötzinger complex: a brainstem region that may generate respiratory rhythm in mammals. ***Science.*** *254(5032): 726-9.*

Strohl K.P., Hensley M.J., Hallett M., Saunders N.A., and Ingram R.H.Jr (1980) Activation of upper airway muscles before onset of inspiration in normal humans**.** ***J. Appl. Physiol.,*** *49: 638-42.*

Suzue T. (1984) Respiratory rhythm generation in the in vitro brain stem-spinal cord preparation of the neonatal rat. ***J. Physiol.*** *354: 173-83.*

Takada M., Itoh K., Yasui Y., Mitani A., Nomura S., and Mizuno N. (1984) Distribution of premotor neurons for the hypoglossal nucleus in the cat. ***Neurosci. Lett.*** *52(1-2): 141-6.*

Takasu N., and Hashimoto P.H. (1988) Morphological identification of an interneuron in the hypoglossal nucleus of the rat: a combined Golgi-electron microscopic study. ***J. Comp. Neurol.*** *271(3): 461-71.*

Travers J.B., and Norgren R. (1983) Afferent projections to the oral motor nuclei in the rat. ***J. Comp. Neurol.*** *220(3): 280-98.*

Tribollet E., Bertrand D., Marguerat A., and Raggenbass M. (2004) Comparative distribution of nicotinic receptor subtypes during development, adulthood and aging: an autoradiographic study in the rat brain. ***Neuroscience.*** *124(2): 405-20.*

Ugolini G. (1995) Specificity of rabies virus as a transneuronal tracer of motor networks: transfer from hypoglossal motoneurons to connected second-order and higher order central nervous system cell groups. ***J. Comp. Neurol.*** *356(3): 457-80.*

Vernino S., Amador M., Luetje C.W., Patrick J., and Dani J.A. (1992) Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. ***Neuron.*** *8(1): 127-34.*

Viana F., Gibbs L., and Berger A.J. (1990) Double- and triple-labeling of functionally characterized central neurons projecting to peripheraltargets studied in vitro. ***Neuroscience.*** *38: 829-41.*

Viana F., Bayliss D.A., and Berger A.J. (1994) Postnatal changes in rat hypoglossal motoneuron membrane properties. ***Neuroscience.*** *59: 131-48.*

Voronin L.L. (1993) On the quantal analysis of hippocampal long-term potentiation and related phenomena of synaptic plasticity. ***Neuroscience.*** 56(2): 275-304.

Voronin L.L. (1994) Quantal analysis of hippocampal long-term potentiation. ***Rev. Neurosci.*** *5: 141-70.*

Wada E., Wada K., Boulter J., Deneris E., Heinemann S., Patrick J., and Swanson L.W. (1989) Distribution of alpha-2, alpha-3, alpha-4, and beta-2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. ***J. Comp. Neurol.*** *284(2): 314-35.*

Wang F., Gerzanich V., Wells G.B., Anand R., Peng X., Keyser K., and Lindstrom J. (1996) Assembly of human neuronal nicotinic receptor alpha5 subunits with alpha3, beta2, and beta4 subunits. ***J. Biol. Chem.*** *271(30): 17656-65.*

Wang H., and Sun X. (2005) Desensitized nicotinic receptors in brain. ***Brain Res. Rev.*** *48(3): 420-37.*

Wonnacott S. (1997) Presynaptic nicotinic acetylcholine receptors. ***Trends Neurosci.*** *20: 92-8.*

Woolf N.J. (1991) Cholinergic systems in the mammalian brain and spinal cord. ***Prog. Neurobiol.*** *37: 475–524.*

Yu C.R., and Role L.W. (1998) Functional contribution of the alpha5 subunit to neuronal nicotinic channels expressed by chick sympathetic ganglion neurons. ***J. Physiol.***509: *667-81.*

Zaninetti M., Tribollet E., Bertrand D., and Raggenbass M. (1999) Presence of functional neuronal nicotinic acetylcholine receptors in brainstem motoneurons of the rat. ***Eur. J. Neurosci.*** *11(8): 2737-48.*

Zhang J., Luo P., and Pendlebury W.W. (2001) Light and electron microscopic observations of a direct projection from mesencephalic trigeminal nucleus neurons to hypoglossal motoneurons in the rat. ***Brain Res.*** *917(1): 67-80.*

Zhang J., and Luo P. (2003) Ultrastructural features of synapse from dorsal parvocellular reticular formation neurons to hypoglossal motoneurons of the rat. ***Brain Res****. 963(1-2): 262-73.*

Zoli M., Le Novere N., Hill J.A. Jr, and Changeux J.P. (1995) Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. ***J. Neurosci.****15: 1912-39.*

Zwart R., and Vijverberg H.P.M. (1997) Potentiation and inhibition of neuronal nicotinic receptors by atropine: competitive and noncompetitive effects. ***Mol. Pharmacol.*** *52(5): 886-95.*