NICOTINIC TRANSMISSION AND DRUGS IN ANESTHESIA

Neuromuscular blocking agents and propofol - consequences for carotid body function

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ABSTRACT

The carotid body is the global oxygen sensor of the human body. Acute hypoxia elicits instant hyperventilation mediated from the carotid body type I cells, where nicotinic transmission is a key component in oxygen sensing and signalling. Neuromuscular blocking agents (NMBAs) reduce this acute hypoxic ventilatory response in humans, and the depression seems to originate from impaired oxygen sensing and signalling in the carotid body. Notably, the carotid bodies are situated outside the blood-brain barrier, and thus accessible for NMBAs. The general anesthetic agent propofol is a potent respiratory depressant and reduces the ventilatory response to hypoxia; however, the site of action for this depression is still not known.

The overall aim of this thesis was to investigate whether NMBAs and propofol impair nicotinic transmission in the carotid body, and furthermore to characterize the pharmacological properties of NMBAs at neuronal nicotinic acetylcholine receptors (nAChRs).

In order to achieve this, we used an isolated carotid body preparation for electrophysiological recordings of the afferent carotid sinus nerve activity in response to either step reductions in PO₂ or nicotine administration. In addition, mRNA for human muscle (α1β1δ) and neuronal (α3β2, α3β4, α4β2 and α7) nAChR subtypes were expressed in *Xenopus* oocytes and studied with a two-electrode voltage clamp setup, the OpusXpress™.

We demonstrate that atracurium and vecuronium reduce the nicotine-induced carotid sinus nerve activity in a concentration-dependent manner. Equi-potent concentrations of NMBAs attenuate the nicotine-induced carotid sinus nerve activity to the same degree. The inhibition is dependent of the nicotine dose, thus suggesting a competitive mechanism of block. Propofol impairs carotid body chemosensitivity to various reductions in PO₂ in a dose-dependent manner. Furthermore, propofol reduces nicotine-induced chemoreceptor activity, most likely by an inhibition of nAChRs in the carotid body. Clinically used non-depolarizing NMBAs inhibit neuronal nAChRs, both by competitive and non-competitive mechanisms, but no receptor activation was seen. Succinylcholine does not activate neuronal nAChRs in concentrations up to 1 mM, and is furthermore a weak antagonist at these subtypes.

We conclude that both non-depolarizing NMBAs and propofol reduce nicotinic transmission in the carotid body, and furthermore that non-depolarizing NMBAs in contrast to depolarizing NMBAs inhibit neuronal nAChRs in a clinically relevant concentration range. This provides a molecular explanation for the reduced hypoxic ventilatory response in humans during residual effects of non-depolarizing NMBAs and propofol. The finding of a distinct action of non-depolarizing NMBAs on the neuronal nAChR subtypes, while succinylcholine had very low affinity to these subtypes, provides interesting insights into the molecular background for neuromuscular transmission.

Keywords: Carotid body, chemoreceptors, neuromuscular blocking agents, propofol, acetylcholine, nicotinic acetylcholine receptors
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


IV. Jonsson M, Gurley D, Dabrowski M, Larsson O, Johnson EC, Eriksson LI. Distinct pharmacological properties of neuromuscular blocking agents on human neuronal nicotinic acetylcholine receptors - a possible mechanism for the train-of-four fade. *Submitted*

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PAPER I-V
ABBREVIATIONS

α-BTX  α-Bungarotoxin
ACh   Acetylcholine
AChR  Acetylcholine receptor
A₁α, A₂α Adenosine receptors
ATP   Adenosine triphosphate
[Ca²⁺]ᵢ Intracellular calcium concentration
C.I.   Confidence interval
CNS   Central nervous system
EC₅₀   The concentration of an agonist that produce 50 % of the maximal possible effect of that agonist
GABA  Gamma amino butyric acid
AHVR  Acute hypoxic ventilatory response
IC₅₀   The concentration of an antagonist that reduce the response to an agonist by 50 %
IV    Intravenous
LGIC  Ligand gated ion channels
mAChR Muscarinic acetylcholine receptor
nAChR Nicotinic acetylcholine receptor
NMBA  Neuromuscular blocking agent
P2X   Purinergic ATP receptor
PCO₂  Carbon dioxide partial pressure
PO₂   Oxygen partial pressure
PNS   Peripheral nervous system
TM    Transmembrane domain
TOF   Train-of-four
5-HT  5-hydroxy tryptamine (serotonin)
**Introduction**

Nicotinic transmission is involved in important physiological vital functions as well as in pathophysiological processes. The neurotransmitter acetylcholine (ACh) exerts its effect both in the central and peripheral nervous systems as well as in extraneuronal tissues through two types of receptors, namely muscarinic (mAChRs) and nicotinic (nAChRs) acetylcholine receptors. Muscarinic AChRs belong to the superfamily of G-protein coupled receptors and mediate slow responses to ACh via a second messenger system. In contrast, nAChRs mediate fast (ms) synaptic transmission in response to ACh, and belong to the superfamily of ligand-gated ion channels (LGIC).

Nicotinic transmission is important in both peripheral and central control of breathing and nicotine, which mimics ACh at the nAChRs, is a risk factor for sudden-infant-death syndrome and sleep-disordered breathing (Cohen, Han, 2002, Fitzgerald, 2000, Prabhakar, 2006, Shao and Feldman, 2005). In the neuromuscular junction AC1 mediates neuromuscular transmission, resulting in skeletal muscle contraction and tone. Furthermore, nicotinic receptors are essential for the transmission in the autonomic nervous system; they are present on all preganglionic, parasympathetic and some sympathetic postganglionic nerves as well as on sympathetic preganglionic nerves innervating the adrenal medulla. Nicotinic receptors are also widespread in the central nervous system (CNS), and involved in drug addiction (i.e. nicotine), as well as in cognition, learning and memory, motor control, arousal, analgesia and regulation of breathing (Cohen, Han, 2002, Gotti and Clementi, 2004). Moreover, nicotinic receptors are important as modulators of the release of other neurotransmitters in the CNS, such as GABA, glutamate and serotonin.

**Nicotinic transmission**

*Structure and distribution of nicotinic acetylcholine receptors*

The “nicotinic receptor substance” in the neuromuscular junction was the first receptor to be recognized and named, the first to be studied electrophysiologically, and the first to be biochemically characterized (Katz and Thesleff, 1957, Langley, 1907).
The nAChRs belong to the superfamily of Cys-loop ligand-gated ion channels (LGIC), which have a common architecture of four transmembrane domains building up each subunit, and also include glycine, 5-HT, and GABA \textsubscript{A} receptors (Figure 1). To date, 17 nicotinic subunits have been cloned in vertebrates: the muscle α1, β1, δ, γ, and ε subunits, and the neuronal α2-10 and β2-4 subunits (Lukas, Changeux, 1999, Hogg, Raggenbass, 2003). The nAChR in fetal muscle consists of two α1, and one of each β1, δ and γ subunit, whereas in the adult muscle nAChR the γ-subunit is being replaced by ε. The neuronal nAChRs include both homomeric and heteromeric receptors, with the α7-9 subunits forming homomeric nAChRs. The heteromeric receptors are formed by a combination of α2-6 and β2-4, and most of these receptors are formed by a single α and a single β subunit, with a stoichiometry of 2 α and 3 β. Although there are many potential combinations of neuronal nAChRs, only a few have been found to be of biological importance.

**Figure 1:** The structural arrangement of a nicotinic acetylcholine receptor (nAChR)

The nAChRs belongs to the Cys-loop LGIC superfamily, which have the cys-loop signature, that is a loop of 13 amino acids between two cysteine residues forming a disulfide bridge within the extensive extracellular N-terminal in each subunit. This N-terminal is involved in the formation of the ligand-binding domain. Furthermore, there are four transmembrane regions (TM1-4) in each subunit, a large cytoplasmatic loop between TM3 and TM4 containing amino acid sequences that are absolutely unique to each subunit, and an extracellular C-domain. In addition, there is a short TM1-TM2 cytoplasmatic loop functioning as an ion selectivity filter. The subunits, which are built up on the TM1-4, surround the central ion pore, where the TM2 region in each subunit lines the pore. Within the this superfamily there is typically a 70 % homology within subunits of the same receptor, and a 30-40 % homology among subunits between different receptors. The sequence homology is high in the ligand-binding domain and in the channel pore. (Ahssalom, Lewis, 2004, Connolly and Wafford, 2004).
The muscle nAChRs have two distinct agonist binding sites, one high affinity binding site between the α1 and δ, and one low affinity binding site at the interface between α1 and ε or γ (Arias, 2000). Heteromeric neuronal nAChRs have two binding sites situated at the interface between an α- and a β-subunit, and both subunits contribute to the pharmacological specificity within each receptor subtype (Luetje and Patrick, 1991). The neuronal homomeric subtypes have five potential binding sites; however, the number of agonists needed for receptor activation is not known (Hogg, Ragganbass, 2003).

In addition to the classification of muscle vs. neuronal nAChRs, the receptors have for a long time been defined by their ability to bind a component of snake venom, α-bungarotoxin (α-BTX), thus as α-BTX sensitive (i.e. α1β1δ, α1β1εδ and α7-9) vs. α-BTX insensitive (i.e. heteromeric neuronal nAChRs). Upon activation of each individual nAChR the ion pore opens and becomes permeable for Na⁺, K⁺ and Ca²⁺ ions, although different subtypes have unique permeability characteristics (Figure 2) (Fucile, 2004).

*Figure 2: Diversity of human nAChRs. α-bungarotoxin binding properties and the Ca²⁺ to Na⁺ permeability ratio (P_{Ca}/P_{Na}) are shown to the left. Stoichiometry, agonist binding sites as well as representative ACh currents for muscle and neuronal nAChRs are displayed.*
Our knowledge of neuronal nAChRs has virtually exploded during the last decade, most likely due to improved molecular techniques. In addition, these receptors are involved in regulation of vital functions and frequently impaired in common neurodegenerative and psychiatric disorders and thus potential targets for pharmacological interventions.

The neuronal nAChRs act both pre- and postsynaptically in the CNS, PNS and in extraneuronal tissues such as the muscles, carotid bodies, lymphocytes, macrophages and skin (Figure 3). Many nAChRs are located presynaptically to modulate the release of ACh as well as other neurotransmitters such as dopamine, norepinephrine, 5-HT, glutamate and GABA.

Neuronal nAChRs are important in signalling hypoxia in the peripheral chemoreceptors of the carotid body (see The carotid body). In addition, the neuronal nAChR subunits α4, α7 and β2 have been demonstrated in critical areas of the brain stem involved in central control of breathing (Dehkordi, Haxhiu, 2004, Wada, Wada, 1989), and interactions with nicotinic transmission alter neural activity in the putative site of rhythm generation (the pre-Botzinger complex) (Shao and Feldman, 2005). Furthermore, nicotine is a risk factor for impaired control of breathing during development and sleep (e.g. sudden-infant death and obstructive sleep apnea).

Postsynaptic nAChRs are present in autonomic ganglia. The α3β4 nAChR subtype is believed to be the predominant nAChR in pre- and postsynaptic autonomic ganglia as well as in the adrenal medulla, but functional α3β2 and α7 subtypes are also present. In the CNS, α4β2 and α7 nAChR subtypes are the most common. They are involved in a number of processes connected to cognitive functions, learning and memory, arousal, reward, motor control and analgesia (Gotti and Clementi, 2004).

Interestingly, neuronal nAChRs have been found in the neuromuscular junction in addition to the muscle subtypes. The neuronal α3β2 nAChR is a presynaptic autoreceptor at the motor nerve ending (Tsuneke, Kimura, 1995), and a selective block of this receptor subtype causes tetanic fade (Faria, Oliveira, 2003). In addition, the α7 subtype has been found in muscle during fetal life and denervation (Fischer, Reinhard, 1999, Tsuneke, Salas, 2003). The possible role of the α7 nAChR in the neuromuscular junction and during different disease states was recently reviewed (Martyn and Richtsfeld, 2006).

It is essential to distinguish between the two subtypes of muscle nAChRs since they have individual biophysical and pharmacological properties. The fetal (α1β1γδ) nAChR is sometimes called the extrajunctional or immature receptor and is present in the neuromuscular junction during fetal life. The adult (α1β1εδ) nAChR, sometimes called junctional or mature receptor, replaces the fetal subtype during innervation of the muscles during the first year(s) of life. Throughout life, DNA for both subtypes of muscle receptors is present in the muscle nuclei, but normally only the adult type is synthesized. However, during conditions of reduced muscle activity such as denervation, immobilization, burns, sepsis and protein catabolism, synthesis of the fetal nAChR may start again (Martyn and Richtsfeld, 2006).
**Pharmacology of nAChRs**

The nAChRs are allosteric proteins that can have different conformational states: resting (closed), active (open) or desensitized (closed) (Katz and Thesleff, 1957). Consistent with the allosteric Monod–Wyman–Changeux model, the equilibrium between these different states can be changed by the binding of an agonist or antagonist at the binding site, or by the binding of an allosteric effector to sites distinct from the binding site (Morod, Wyman, 1965). The resting state is when no ligand is bound to the receptor. Binding of two molecules of ACh (for the muscle and heteromeric neuronal nAChRs) creates an active (open) state by isomerization of the receptor. Desensitization is a temporary inactivation of the receptor due to a continuous stimulation with an agonist, but can also occur from the resting state without activation. The “classical” desensitization occurs after stimulation with a high agonist concentration (micro- to millimolar), where the receptor within milliseconds to seconds changes from the active to a desensitized state. At low agonist concentration (nano- to micromolar) the receptor can desensitize directly from the closed state (Giniatullin, Nistri, 2005). Desensitization can be seen as a use-dependent mechanism allowing plasticity in the receptor system and can thereby protect the cell during high excitation. Agonists can be either full agonists (i.e. eliciting full receptor response) or partial agonists (i.e. eliciting a submaximal receptor response) at a particular receptor subtype. Notably, a full agonist at one receptor subtype can be a partial agonist at another, for example, nicotine is a full agonist at the α4β2 subtype, but a partial agonist at the α3β4 receptor. Antagonists can be divided into competitive vs. non-competitive antagonists. A competitive antagonist binds to the ligand-binding site, thus competing for binding with the agonist. The degree of inhibition of a competitive antagonist is therefore dependent on both agonist and antagonist concentration, since a higher antagonist concentration will cause a higher degree of inhibition and *vice versa*. A non-competitive antagonist inhibits the receptor by binding to a different site than the agonist, for example the conductance pathway (i.e. the channel pore). In general, a non-competitive antagonist reduces the maximal response independent of the agonist concentration. Open and closed channel blockers are different types of non-competitive antagonists, both having their own characteristics. An open-channel blocking agent inserts itself into the open receptor pore, thereby blocking the channel, and thus, increased activation of the channel causes an increased degree of block. In contrast, a closed-channel blocking agent prefers the closed channel, consequently, a lower agonist concentration will render fewer open channels and thus a higher degree of block.

**Drugs in anesthesia and nicotinic transmission**

Drugs used in anesthesia (i.e. general anesthetics and neuromuscular blocking agents) have different mechanisms of action, and although neuronal nAChRs are not the primary target, most of the drugs interact with these receptors (Arias and Bhumireddy, 2005, Tassonyi, Charpantier, 2002). One important function involving nicotinic transmission is central and peripheral control of breathing, and the potential interaction of drugs in anesthesia with the regulation of breathing is therefore of relevance. In the following, current knowledge of interactions between drugs in anesthesia and peripheral control of breathing (i.e. oxygen sensing in the carotid body) in relation to nicotinic transmission will be reviewed.

**Neuromuscular blocking agents**

Clinically used NMBAs are classified into either depolarizing or non-depolarizing NMBAs, on the basis of whether they activate the muscle nAChR causing depolarization of the muscle membrane before the onset of neuromuscular block, or not. The non-depolarizing NMBAs are considered to be classical examples of competitive antagonists at the muscle nAChR. Clinically used non-depolarizing NMBAs are synthetic and can be further
classified, based on their chemical structure, into benzisouquinolinium compounds (e.g. atracurium, cis-atracurium, mivacurium) or steroidal compounds (e.g. pancuronium, vecuronium, rocuronium). The common feature of these drugs is a quaternary nitrogen, similar to the quaternary nitrogen found in ACh. This quaternary nitrogen is a key component for the affinity to the nAChRs. Each non-depolarizing NMBA has its own specific effect profile based on chemical structure. For example, the benzisouquinoliniums can cause histamine release, whereas the steroid compound pancuronium has a relatively high affinity to muscarinic receptors (Naguib and Lien, 2005). Notably, rapacuronium, a short-acting steroid compound, was withdrawn from the market because of an unacceptably high incidence of bronchoconstriction, most likely due to inhibition of muscarinic receptors (Jooste, Sharma, 2005). There is evidence that apart from being competitive antagonists, non-depolarizing NMBA also can act as non-competitive antagonists at the muscle nAChR (Garland, Foreman, 1998, Lowenick, Krampfl, 2001). Furthermore, the fetal muscle and some neuronal nAChR subtypes can also be activated by non-depolarizing NMBA (Chiodini, Charpentier, 2001, Fletcher and Steinbach, 1996). While it has been reported that some non-depolarizing NMBA inhibit neuronal nAChRs (Bertrand, Ballivet, 1990, Garland, Foreman, 1998, Chiodini, Charpentier, 2001, Exley, Iturriaga-Vasquez, 2005), we lack information whether this is a pharmacological property among all NMBA in clinical practice. All non-depolarizing NMBA are highly charged molecules, and therefore do not cross the blood-brain barrier under normal circumstances. However, during conditions of disrupted blood-brain barrier, non-depolarizing NMBA have been found in cerebrospinal fluid (Matteo, Pua, 1977, Segredo, Matthey, 1990, Tassonyi, Fathi, 2002), and thus they have the potential to interact with neuronal nAChRs in the CNS.

Succinylcholine was synthesized and described by Bovet in 1949 (Bovet, Bovet-Nitti, 1949). It was introduced in Sweden in 1951 (Thesleff, 1951, Von Dardel and Thesleff, 1951) and is still the only depolarizing NMBA routinely used in clinical practice world-wide. Succinylcholine is composed of two ACh molecules linked together by an ester linkage in the backbone and is rapidly degraded by plasma pseudocholinesterase. The mechanism of action of succinylcholine within the neuromuscular junction is not completely understood at the molecular level, but it is known that succinylcholine depolarizes the muscle nAChR, followed by desensitization (Marshall, Ogden, 1990). The inhibitory effect of succinylcholine on neuronal nAChRs has not been investigated at a molecular level. While succinylcholine does not activate the rat neuronal α3β4 and α7 nAChR subtypes at concentrations lower than 1 mM, insertion of a point mutation into the second transmembrane region (TM2) of the α7 subtype (T244F) dramatically increases its efficacy compared to the wild-type α7 (Placzek, Grassi, 2004). Despite the obvious advantages of a rapid onset and recovery, succinylcholine has a number of side effects, most of them being due to depolarization of the muscle nAChR. Because of the high incidence of serious side effects (i.e. hyperkalemia, arrhythmias and malignant hyperthermia), a replacement for succinylcholine has been sought for decades, but so far alternatives have been unsuccessful (Martyn and Richtsfeld, 2006, Naguib and Lien, 2005).

**General anesthetics**

A variety of ligand gated receptors have been proposed as targets for general anesthesia, but most experimental evidence now indicates that the majority of general anesthetics target the GABA\ receptor complex, thus facilitating GABA-induced inhibition of central neurons (Hemmings, Akabas, 2005). There is also evidence of an interaction between general anesthetics and nicotinic transmission (e.g. regulating breathing, cognition, neurons). In the following, their action on nicotinic transmission will briefly be reviewed.
In general, thiopental, propofol and ketamine non-stereospecifically inhibit neuronal nAChRs in a clinically relevant concentration range. While intravenous anesthetics also inhibit the fetal muscle nAChR, although with a much lower potency compared to the neuronal subtypes (Tassonyi, Charpentier, 2002), the effect of these agents at the adult muscle subtype nAChR is not known. Propofol non-competitively inhibits the α4β2 neuronal nAChR and nicotinic transmission in pheochromocytoma (PC12) cells (Flood, Ramirez-Latorre, 1997, Furuya, Oka, 1999, Violet, Downie, 1997). In contrast, propofol inhibits the α7 nAChR subtype only at very high concentrations (Flood, Ramirez-Latorre, 1997).

Ketamine is an antagonist at the N-methyl-D-aspartate (NMDA) receptor, notably a receptor that is not genetically related to the nAChRs, nonetheless it inhibits the neuronal nAChRs in a non-competitive manner (Arias and Bhumireddy, 2005, Tassonyi, Charpentier, 2002).

In general, inhalational anesthetics act as non-competitive inhibitors at the nAChRs in clinically relevant concentrations and have the highest affinity among all anesthetics to the major brain nAChR, the α4β2 subtype (Arias and Bhumireddy, 2005, Tassonyi, Charpentier, 2002).

The hypoxic ventilatory response and drugs in anesthesia

During anesthesia, most general anesthetics influence control of breathing in such a way that assisted or mechanical ventilation is needed to ensure adequate ventilation. In the postoperative period, subanesthetic concentrations of general anesthetics may still affect the control of breathing. Ventilatory depression, aspiration and airway obstruction are the most common critical events in the immediate postoperative period (Cooper, Leigh, 1989, Leigh and Tytler, 1990, Lunn, Hunter, 1983, Rose, Cohen, 1994), and respiratory complications are the most common causes of unplanned ICU admittance in the postoperative period (Rose, Byrick, 1996, Rose, Cohen, 1994).

In 1992, Eriksson and co-workers made the interesting observation that the acute hypoxic ventilatory response (AHVR) in humans was reduced during a partial neuromuscular paralysis with vecuronium (Eriksson, Lenmarken, 1992). Notably, this occurred while the ventilatory response to hypercapnia was maintained, indicating that the respiratory muscle function was not affected. This was a controversial finding, since the respiratory muscles are resistant to NMBAs, and in addition, both static and dynamic resting respiratory parameters as well as the hypercapnic respiratory response are unchanged during partial neuromuscular paralysis (Ali, Wilson, 1975, Dupuis, Martin, 1990, Gal and Goldberg, 1981, Gal and Smith, 1976). However, the finding was confirmed shortly after, now by using an isocapnic AHVR test procedure (Eriksson, Sato, 1993). Furthermore, the AHVR during residual paralysis with three different NMBAs (atracurium, pancuronium or vecuronium) was reduced by approximately 30%, thus demonstrating that the reduction in AHVR is a general property of non-depolarizing NMBAs (Eriksson, 1996). On the basis of these studies together with studies of pharyngeal function during partial paralysis (Eriksson, Sundman, 1997, Sundman, Witt, 2000) a TOF ratio >0.90 was set as the new standard for recovery of vital function, and thus resulted in new clinical guidelines for a safe extubation of the patient (Kopman, 1997, Eriksson, 1999).

The AHVR is mediated from the chemoreceptor cells in the carotid body, and in order to find the origin of the reduced AHVR by NMBAs, their effect on carotid body chemoreception was studied. Injection of vecuronium close to the carotid body reduced the efferent phrenic nerve activity to hypoxia (Wyon, Eriksson, 1996). Furthermore, it was demonstrated that vecuronium reduced the hypoxic chemoreceptor response in the single fiber carotid sinus nerve preparation. Interestingly, the time course for the depression of the chemoreceptor response was the same as for the neuromuscular block (Wyon, Joensen, 1998). Thus, non-depolarizing NMBAs reduce the AHVR by
an impairment of carotid body chemoreception; however, the mechanism behind this depression is not known.

Propofol has pronounced effects on respiration, including ventilation during normoxia, hypercarbia and hypoxia (Blouin, Conard, 1991, Blouin, Seifert, 1993, Nagyova, Dorrington, 1995, Nieuwenhuijs, Sarton, 2000). The AHVR seem to be markedly depressed, even during subanesthetic doses of propofol, i.e. sedation (Blouin, Seifert, 1993, Nagyova, Dorrington, 1995, Nieuwenhuijs, Sarton, 2000). The controversy remaining has been whether this is due to an interaction with carotid body oxygen sensing and signalling (Pontie and Sadler, 1989) or due to central inhibition of respiration (Dow and Goodman, 1993, Nieuwenhuijs, Sarton, 2001). Hence, propofol seem to be a potent respiratory depressant, while the anatomical and pharmacological sites of action are not known.

The carotid body

Oxygen is essential for mammalian life, and the carotid body is a global oxygen sensor of the organism. First described by Taube in 1743, later by DeCastro and Luschka, and by the 1938 year Nobel Laureate Corneille Heymans, the nature by which the carotid body senses oxygen is not known, although there is general agreement about the transduction process. Changes in local O₂ tension can induce modifications of electric activity, transcription factors and second messengers in numerous cell types; however, there are only a limited number of excitable cells in specific O₂-sensitive tissues that are directly involved in systemic adjustments to acute hypoxia. The carotid body is the major O₂-sensitive chemoreceptor, but there are other important O₂-sensitive tissues, such as the neuroepithelial bodies (in the lung), smooth muscle in pulmonary and systemic vasculature and the neonatal adrenal medulla.

The carotid body is a bilateral sensory organ located at the bifurcation of the common carotid artery. Although a tiny organ (a total volume of 6 mm³), the carotid body is the tissue with the highest blood flow, 1400 ml/min/100 g (Barnett, Mulligan, 1988), notably, substantially higher than the cerebral blood flow. The type I (glomus) cell is strongly considered to be the chemosensitive unit in the carotid body, and is surrounded by the type II cells (glia-like sustentacular cells). The type I cells are surrounded by a highly vascularized capillary network and by nerve terminals, both from the afferent carotid sinus nerve and autonomic nerves. Due to its favourable location in the bifurcation of the common carotid artery, the carotid body rapidly (within seconds) responds to a low or decreased PO₂ and instantly increases its afferent input via the carotid sinus nerve to the nucleus tractus solitarius in the brain stem, eliciting hyperventilation and sympathetic stimulation. In addition to monitoring the PO₂ in arterial blood, the carotid body also responds to an increased PCO₂ and a reduced pH and blood glucose. A flow reduction, as during low blood pressure, results in increased carotid sinus nerve activity based on activation of both the carotid body and the nearby baroreceptors in the carotid sinus.

For obvious reasons, it has been difficult or even impossible to perform detailed investigations of the human carotid body. However, in the 1940s carotid body resection was a possible therapeutic intervention in patients with asthma. The shortness of breath disappeared promptly, but at the cost of a lower PO₂ and a reduced or abolished response to hypoxia (Honda, 1992, Prabhakar and Peng, 2004). Furthermore, studies of patients going through carotid endarterectomy or bilateral neck dissection, reveal that after a unilateral carotid body denervation the hypoxic response is somewhat blunted, but it is significantly reduced or abolished after a bilateral denervation. Interestingly, the human HVR does not recover with time after denervation or resection, which is in contrast to
animals, where the aortic bodies successively compensate for the loss of carotid body function (Honda, 1992).

**Mechanism(s) of oxygen sensing and signalling and the role of nicotinic transmission**

The nature in which the carotid body senses oxygen is not known, but a number of putative oxygen sensors have been suggested (e.g. heme-containing proteins or O2-sensitive K+ channels). There is also a lack of knowledge about how this "oxygen sensor" links to the transduction process ultimately leading to release of neurotransmitter and activation of the afferent carotid sinus nerve. The carotid body type I cell functions as a presynaptic element that in response to hypoxia releases neurotransmitter in the synaptic cleft and stimulates the postsynaptic element, the carotid sinus nerve (Figure 4).

During the years, there has been a long-standing debate about which neurotransmitter(s) that increase the carotid sinus nerve activity. However, at present ACh and ATP/adenosine seem to be the most important excitatory transmitters, whereas dopamine is a negative modulator (Conde and Monteiro, 2006, Iturriaga and Alcayaga, 2004, Prabhakar, 2006, Xu, Xu, 2006, Zhang, Zhong, 2000). Although there are species differences, both muscarinic and nicotinic AChRs have been found in the carotid body. ACh mimics the carotid body response to hypoxia, hypercapnia and

![Figure 4: Oxygen sensing and signalling in the carotid body type I cell.](image)

**Figure 4: Oxygen sensing and signalling in the carotid body type I cell.**
The transduction process in the carotid body type I cell is triggered by an increased conductance in O2-sensitive K+ channels at the cell membrane of the glomus cell. Several different O2-sensitive K+ channels have been identified in the glomus cell; the large conductance Ca2+-activated K+ channel (BK), slowly inactivating voltage gated K+ channels (Kv), and the two-pore domain "leak" K+ channel. A closure of these K+ channels depolarizes the cell membrane and activates voltage-gated Ca2+ channels (VGCC), leading to an influx of Ca2+ and a subsequent increase in intracellular Ca2+ that is the trigger for neurotransmitter release. Type I cells contain many transmitters, peptides and neuromodulators that are released in response to an increased intracellular Ca2+. CSN=carotid sinus nerve, D2=dopamine receptor. (Conde and Monteiro, 2006, Iturriaga and Alcayaga, 2004, Prabhakar, 2006, Xu, Xu, 2006, Zhang, Zhong, 2000).
metabolic acidosis, and application of both muscarinic and nicotinic antagonists reduce the response. The neuronal nAChR subunit α4 has been demonstrated in the carotid body as well as in the adjacent petrosal ganglion in cat (Ishizawa, Fitzgerald, 1996) while the α7 subunit has been found in the carotid body afferent system (Ishizawa, Fitzgerald, 1996, Shirahata, Ishizawa, 1998). In addition, the mRNA from six different nAChR subunits (α3, α4, α5, α7, β2 and β4) has been detected in carotid body total RNA (Cohen, Han, 2002). Recently, α3, α4 and β2 subunit-containing nAChRs were found to be present and functional in cultured glomus cells (Higashi, McIntosh, 2003). Altogether, most evidence indicates that ACh and ATP/adenosine are largely responsible for the hypoxic signalling in the carotid body by activation of nAChRs and P2X/A1 receptors. This theory is strongly supported by the fact that a simultaneous application of a nicotinic and purinergic blocker inhibits most of the postsynaptic responses to hypoxia (Zhang, Zhong, 2000).

The discovery that a curare-like agent reduces the human ventilatory response to hypoxia indicated a new and unknown property of this class of drugs. Over the years it has become evident that this property most likely applies to all non-depolarizing NMBAs in clinical use, and furthermore, that the reduction of AHVR is due to an impairment of oxygen sensing and/or signalling in the carotid body. Notably, the reduction in carotid body chemoreceptor responses to hypoxia followed the time-course of a vecuronium-induced neuromuscular block (Wyon, Joensen, 1998). While NMBAs target the muscle type nAChR, there are to our knowledge no reports of the presence of these receptors in the carotid body. However, neuronal type nAChRs are present and functional in the carotid body oxygen sensing and signalling machinery. Activation of these receptors increases neural activity to the nucleus tractus solitarius, leading to augmented breathing. Atracurium, d-tubocurarine and pancuronium inhibit neuronal nAChRs, while the interactions with other NMBAs and neuronal nicotinic receptors are poorly understood.

Propofol is an anesthetic agent with a pronounced depressant effect on ventilatory regulation during both hypoxia and hypercarbia. Although facilitation of the GABA<sub>γ</sub> receptor mediated inhibition is believed to be the mechanism behind the hypnotic properties of propofol, it has affinity to a multitude of ligand-gated ion channels, and the actual mechanism behind the depression of AHVR is controversial.
The overall aim of this thesis was to investigate the effects of NMBAs and propofol on nicotinic transmission in the carotid body, and furthermore to characterize the pharmacological properties of NMBAs on neuronal nAChRs. The specific aims were:

- To determine the effect of atracurium and vecuronium on nicotinic transmission in the carotid body
- To characterize the concentration–response relationships for atracurium and vecuronium on nicotine-induced carotid sinus nerve activity
- To determine the effect of propofol on carotid sinus nerve activity at lowered PO₂, and if this effect is dependent on the level of PO₂ reduction
- To characterize concentration–response relationships for propofol on the nicotine-induced carotid sinus nerve activity, and to find out if the GABA₅ receptor system is involved in a putative reduction of the nicotinic response by propofol
- To characterize the pharmacological properties of non-depolarizing NMBAs on human neuronal nAChRs
- To investigate the effect of the depolarizing NMA, succinylcholine, on human neuronal nAChRs
MATERIALS AND METHODS

The following sections briefly describe the materials and methods used, followed by a discussion of general aspects of the methodology used in the different papers. Detailed information about materials and methods is found in each paper (I-V).

Animals: anesthesia and surgery
All studies (I-V) were approved by the local animal ethics committee at Karolinska Institutet. In paper I-III, adult male New Zealand White rabbits were used. Anesthesia was provided with a continuous IV infusion of thiopental. The animals were mechanically normoventilated via a tracheotomy and kept at normothermia. No neuromuscular blocking agents were used. The carotid body with its arterial supply and the carotid sinus nerve was removed en bloc from the rabbit and put into a perfusion chamber. The animals were sacrificed after removal of the carotid bodies.

Oocytes from Xenopus laevis were used in paper IV-V. Xenopus laevis were kept in a water tank at 19 °C. The frogs were anesthetized with 3-aminobenzoic acid ethyl ester, and the oocytes were thereafter isolated by partial ovariectomy. The incision was sutured and the animals were monitored during the recovery period before being returned to their tank.

The isolated carotid body preparation (paper I-III)
After removal from the anesthetized rabbit, the carotid body with the carotid sinus nerve was immediately placed into a chamber bath (Figure 5). Carotid sinus nerve activity above a selected threshold was counted as previously described with a frequency meter to measure \( f_s \) expressed in Hz (Iturriaga, Rumsey, 1991). The response was defined as the peak chemoreceptor discharge frequency compared to a baseline recording immediately prior to \( P_O_2 \) reduction or administration of nicotine (\( \Delta f_s = \text{maximal } f_s - \text{basal } f_s \)).
**Figure 5: The isolated carotid body preparation**
The common carotid artery was continuously perfused and superfused by gravity at a constant pressure (45 cm H₂O) with modified Tyrode’s solution. The perfusate was equilibrated with 5% CO₂ and 95% O₂ and maintained at a temperature of 37.0 ± 0.5 °C. After desheathing, the whole carotid sinus nerve was placed onto a platinum electrode and chemosensory discharges were recorded.

**The Xenopus oocyte expression system and OpusXpress™ (paper IV-V)**

**Cloning**
Human DNA for various nAChR subunits was cloned from a human cDNA library and thereafter subcloned into different expression vectors (Table 1). The mRNA was transcribed in vitro and analyzed using a bioanalyzer.

After removal from the frog, the ovaries were mechanically dissected and digested in a collagenase-containing OR-2 buffer in order to remove the follicular epithelia from the oocytes. After 1-24 hours the oocytes were injected with mRNA and thereafter maintained in Leibovitz L-15 medium. Oocytes were incubated at 18-19°C for 2-7 days after injection before being studied.

**Table 1: An overview of GenBank access numbers for the cDNA nucleotide sequences (www.ncbi.nlm.nih.gov) and the vectors used for expression.**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>GenBank access number</th>
<th>Expression vector</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>α1</td>
<td>NM 000079</td>
<td>pKGem</td>
<td>AstraZeneca, Wilmington, DE, USA</td>
</tr>
<tr>
<td>α3</td>
<td>HSU62432</td>
<td>pKGem</td>
<td>AstraZeneca, Wilmington, DE, USA</td>
</tr>
<tr>
<td>α4</td>
<td>L35901</td>
<td>pBSTA</td>
<td>University of California, Irvine, CA, USA</td>
</tr>
<tr>
<td>α7</td>
<td>Y08420</td>
<td>pBluescript II SK(-)</td>
<td>Stratagene, La Jolla, CA, USA</td>
</tr>
<tr>
<td>β1</td>
<td>NM 000747</td>
<td>pKGem</td>
<td>AstraZeneca, Wilmington, DE, USA</td>
</tr>
<tr>
<td>β2</td>
<td>Y08415</td>
<td>pKGem</td>
<td>AstraZeneca, Wilmington, DE, USA</td>
</tr>
<tr>
<td>δ</td>
<td>NM 000750</td>
<td>pBSTA</td>
<td>University of California, Irvine, CA, USA</td>
</tr>
<tr>
<td>ε</td>
<td>NM 000080</td>
<td>pKGem</td>
<td>AstraZeneca, Wilmington, DE, USA</td>
</tr>
</tbody>
</table>
Two-electrode voltage clamp – OpusXpress™

OpusXpress™ 6000A (Molecular Devices, Union City, CA, USA) is a semi-automated two-electrode voltage clamp set-up, that makes it possible to investigate eight oocytes in parallel (Figure 6). Buffers were delivered by automatic fluid pumps to ensure a pre-set perfusion rate. Drugs were delivered through disposable pipettes, thus avoiding cross-contamination of potent drugs, such as ACh. Electrodes were made from borosilicate tubes and filled with 3 M KCl. The oocytes were voltage clamped at −60 mV. All recordings were performed at room temperature (20-22°C). During recordings, the oocytes were continuously perfused with ND-96.

Changes in currents were studied both as peak and net charge responses (area under the curve), except in the α7 subtype, where net charge analysis was used (Papke and Porter Papke, 2002, Papke and Thinschmidt, 1998). The baseline current immediately before drug application was subtracted from the response, and the analysis region for peak and net charge analysis was 20 s, i.e. during the time of agonist application.

Figure 6: The Xenopus oocyte expression model, two-electrode voltage-clamp (TEVC) and OpusXpress™.

Data analysis and statistics

Concentration–response relationships for the drugs tested were fitted by non-linear regression to the one-site competition (paper II-III) or the four parameter logistic (paper IV-V) equation. Unless otherwise stated, data are given as mean ± S.D. or S.E.M, as indicated, alternatively as 95 % confidence interval (95 % C.I.) when appropriate.

Detailed information about the statistical methods used can be found in each individual paper.
Methodological considerations
The isolated carotid body preparation

Carotid body chemoreceptor activity is difficult to measure in humans, and so far we have to rely on animal experiments. Investigations of hypoxic responses as well as more pharmacological studies in the carotid body can be achieved by the usage of several different set-ups (Prabhakar, 2006). The most common approaches for these studies are 1) neural recordings from the afferent carotid sinus nerve 2) measurement of neurotransmitter release and 3) registrations of ion channel activity or Ca++ levels in the type I cells. It is clear that hypoxia ultimately leads to an increased carotid sinus nerve activity. Conflicting data exist, however, about the nature and numbers of neurotransmitters being released during hypoxia, and also about the importance of different ion channels. Given these alternatives we chose to start evaluating whether NMBA impair nicotinic transmission in the carotid body by recording the carotid sinus nerve activity in an isolated carotid body preparation, which is commonly used in physiological and pharmacological studies of carotid body function (Iturriaga, Rumsey, 1991). In order to obtain stable and reliable results the carotid bodies were used directly after removal.

Key events in the chemotransduction process involve opening and closure of ion channels, and thus a close monitoring of the ion content in the perfusate was necessary. In addition, temperature and pressure as well as PO2 and PCO2 were continuously assessed. By application of a constant perfusion pressure governed by gravity, coexisting baroreceptor activity was controlled, making it easier to interpret carotid sinus nerve impulse traffic and single out chemosensation. We are aware that our carotid sinus nerve recordings show the entire nerve action and that the quality of recording may vary over time due to recruitment or loss of nerve fibers. Therefore, each preparation was tested for viability and stability by switching the gas mixture from 95% O2/5% CO2 to 95% N2/5% CO2 before and after each experiment, and the corresponding increase in carotid sinus nerve activity was measured to check for an intact chemoreceptor response. In addition, the absolute carotid sinus nerve activity varied among preparations. In order to normalize the data, we compared the changes in chemoreceptor activity for each concentration of NMBA/propofol with the preceding control, using their ratios. Between each perfusion period with NMBA/propofol, a 20-30 minute washout period and subsequent nicotine injections aimed also to ensure and demonstrate stable experimental conditions over time.

While the initial attempts to develop an isolated carotid body preparation perfused with saline failed due to a rapid loss of chemoreceptor activity, perfusing the preparation with 95% O2 yields a preparation that is stable for over 5 hours (Iturriaga, Rumsey, 1991). Clearly, in vivo, such high O2 level is considered as hyperoxia and is not necessary for maintenance of viability. However, this preparation does not contain any O2-carrying molecules, and it can therefore be speculated that this high O2 level actually represents physiological “normoxia” in the carotid body. The PO2-reduction was achieved by mixing the perfusate with 95% N2 and 5 % CO2, giving a PO2 of approximately 7-8 kPa in the perfusate going into the tubings, while the PO2 values reported were measured in the open chamber bath after going through the preparation. We have not been able to measure the PO2 in the carotid body tissue or inside the arteries and can therefore not present any data about the PO2 in the preparation. However, under resting conditions the preparation shows a constant baseline activity in the carotid sinus nerve, indicating that a local deficit of O2 might exist in the carotid body, since the carotid sinus nerve is silent during normoxia (Wyon, Joensen, 1998). This is furthermore supported by Fitzgerald and co-workers who speculate that a switch in gas mixture from 95% O2/5% CO2 to 40% O2/5% CO2 may well represent a hypoxic challenge in the isolated carotid body preparation (Fitzgerald, Shirahata, 2004). I strongly believe that in the future, with
the development of better PO2 electrodes, measurement of the PO2 level in the carotid body tissue will become possible. Such studies would certainly be both interesting and necessary.

The rabbit carotid body has distinctive properties in terms of receptor population, in that it has a relatively high ratio of muscarinic vs. nicotinic receptors (12:1) compared to other species (Hirano, Dinger, 1992). Application of ACh to the rabbit carotid body inhibits the chemoreceptor response. In the presence of muscarinic blockers, ACh acts as an excitatory transmitter that most likely activates nAChRs at the carotid sinus nerve ending and at the glomus cell. This is supported by the fact that nicotine (a selective nAChR agonist) acts as an excitatory transmitter in many species (Eyzaguirre and Monti-Bloch, 1982). Therefore we chose to use nicotine in order to test the nicotinic transmission in the carotid body. The 500 μg nicotine dose was chosen because its effect corresponded to the increase in carotid sinus nerve activity seen after a reduction in PO2. However, 500 μg is also a peak dose in the dose–response curve for nicotine in this preparation. Therefore, in paper II, we also added a lower dose of nicotine (50 μg), which is approximately EC50 in this preparation. The response to both doses of nicotine had an instant onset and wore off quickly. It can be speculated whether this quick termination of the chemoreceptor response is due to the rapid application or if it indicates desensitization.

In all experiments of paper I-III, commercially available substances have been used. Atracurium can to some extent spontaneously be degraded by Hofmann elimination, which is a temperature and pH dependent process that can occur in vitro as well as in vivo (Stenlake, Waigh, 1983), and therefore great care was taken to avoid degradation of atracurium. We did not measure the degradation product, laudanosine, and can therefore no rule out some contamination of laudanosine although we tried always to use freshly prepared atracurium and to keep it cool. Propofol in the commercially available formula together with the vehicle, Intralipid*, was used in paper III. One can argue that using the pure substance would have been more appropriate; on the other hand, propofol is highly insoluble in buffer and a mixture with DMSO or ethanol might equally well interact with the nicotinic response. Experiments with Intralipid* were therefore performed in order to rule out interactions with the nicotinic response, and based on these experiments we can conclude that the propofol vehicle does not interact with the nicotinic transmission of the carotid body.

The Xenopus oocyte expression system and nAChRs

Human nAChR subunits were injected into Xenopus oocytes, a well-known and stable expression system (Brown, 2004, Gotti, Formasari, 1997). The Xenopus oocyte does not express endogenous receptors, thus, by injection of mRNA for the specific subunits of a receptor, the corresponding nAChR subtype is expressed in the Xenopus oocyte (Gotti, Formasari, 1997, Witzemann, Barg, 1987). The obvious limitation of this expression system is that although we are using human mRNA, it is injected into a frog oocyte, thus relying on non-mammalian intracellular machinery. However, comparison of pharmacological properties between receptors expressed in Xenopus oocytes and mammalian cell lines, as well as in tissue preparations, reveals large similarities (Gotti, Formasari, 1997). This system is therefore regarded as a robust tool to study biophysical and pharmacological properties of receptors and ion channels, and it can also potentially be used with mutated receptors for studies of specific properties.

With the human genome cloned and rather easily accessible it seems rational to use not only mammalian mRNA, but also human mRNA. Despite an 80% species homology for the nAChR subunits in general, a small change in amino acid sequence can dramatically change pharmacological and biophysical properties (Papke and Porter Papke, 2002, Placzek, Grassi, 2004). The stoichiometry of the subunits building up the receptor subtypes used in paper IV-V was 1:1, except for
the α4β2 nAChR subtype where the stoichiometry was 1:9. The α4β2 receptor subtype can form at least two distinct functional receptors with different sensitivities and desensitization patterns dependent on the α4β2 ratio when expressed both in the *Xenopus* oocyte and a human cell line (Nelson, Kuryatov, 2003, Zwart and Vijverberg, 1998). A more sensitive receptor is formed when the α4β2 injection ratio is 1:9, whereas a ratio of 1:1 gives a less sensitive receptor. The presence of two distinct forms of the α4β2 subtype explains the two bulks of EC50 values seen in human α4β2 expressed in *Xenopus* oocytes (Chavez-Noriega, Crona, 1997, Chiodini, Charpentier, 2001).

In order to compare our data with the previous atracurium study, we chose to study the more sensitive receptor. Notably, d-tubocurarine has a 10 fold higher affinity for the 1:1 ratio α4β2 subtype; however, it has not been investigated for other modern non-depolarizing NMBAs (Chavez-Noriega, Crona, 1997). Interestingly, there is evidence indicating that also the α3β2 subtype can have different affinities depending on subunit stoichiometry: increasing the β2 content by using a more effective vector or by increasing the amount of mRNA produces a more sensitive α3β2 subtype (Chavez-Noriega, Crona, 1997).

In experiments using *Xenopus* oocytes and cultured cell lines various antibiotics are commonly used to prevent growth of bacteria and fungi, although we know that these drugs, aminoglycosides in particular, can interfere with neuromuscular transmission and nAChRs (Amici, Eusebi, 2005, Nishizaki, Morales, 1994, Ostergaard, Engbaek, 1989).

The voltage clamp technique was first developed by Cole in 1949, and used in 1952 by Hodgkin and Huxley in their classical study of the squid giant axon (Hodgkin and Huxley, 1952, Hodgkin, Huxley, 1952). The two-electrode voltage clamp technique uses electronic feedback to maintain the voltage across the membrane at some constant level ("voltage clamp"), and then measure the current flowing through the membrane. Deviations from the clamped membrane potential will happen if ions start to cross the oocyte membrane for example as the result of ion channels being opened. Two electrodes are inserted into the cell, one monitor the voltage across the membrane, and the other pass current through it in order to keep the clamped potential. Studies of receptor–ligand interactions are usually conducted using ar electrophysiological approach or ligand binding techniques. While electrophysiological studies measure activities in living cells in real time, the ligand binding technique has the limitation that the binding of a drug to a receptor does not discriminate between different receptor states (e.g. resting, activated, desensitized). The major problem with electrophysiological techniques is that they are very user-dependent and time consuming, thus limiting the use of these techniques. Here we have used a semi-automated eight-channel two-electrode voltage clamp set-up, OpusXpress™ (Molecular Devices, USA). The OpusXpress™ is a stable and reliable high throughput two-electrode voltage clamp platform.

The major advantages are 1) a reduction of the cross-contamination risk by use of disposable tips, 2) strictly controlled drug application protocols, 3) tightly controlled flow rates, 4) less user dependence 5) time saving; taken together, the system is a prerequisite for stable and reproducible results. On the other hand, exact studies of kinetics might not be feasible since the possibility of fine-tuning is lost.

Traditionally, ion channel activity has been measured as the peak response to an agonist. This is based on the assumption that the peak response corresponds to a maximal agonist concentration, thus reflecting the highest channel activity. However, there are receptors that rapidly desensitize following activation by an agonist. Thus, the ion flow across the membrane might not reflect the peak current since the actual peak agonist concentration at the receptor occurs after the peak response. It has convincingly been shown that for the α7 nAChR subtype a measurement of net charge (i.e. area under the curve) more accurate reflects ion fluxes through the receptor (Papke and
Porter Papke, 2002, Papke and Thinschmidt, 1998), and here we also find a significant difference between $EC_{50}$ values based on peak current and net charge. For the $\alpha3\beta4$ and $\alpha4\beta2$ receptor subtypes, the $EC_{50}$ values do not differ between peak current and net charge; however, for the $\alpha3\beta2$ subtype, which has an initial rapid decay, there is a small difference between the $EC_{50}$ values.
SUMMARY OF RESULTS

Atracurium and vecuronium reduce nicotine-induced carotid body chemoreceptor responses in the carotid body (Paper I-II)

In paper I, the effects of atracurium and vecuronium on nicotine-induced carotid body chemoreceptor responses were investigated using an isolated carotid body preparation with registrations from the afferent carotid sinus nerve. Bolus administrations of 500 μg nicotine (roughly corresponding to a peak chamber concentration of 360 μM) resulted in a short-lasting increase in carotid sinus nerve activity with a rapid decay. The nicotine-induced response was reduced by 70 ± 30 % (from 291 ± 186 Hz to 107 ± 121 Hz) and 66 ± 19 % (from 176 ± 45 Hz to 56 ± 30 Hz) after 30 min perfusion with 28 μM atracurium and 10 μM vecuronium, respectively. Notably, equipotent concentrations at the neuromuscular junction (28 μM vs. 10 μM) gave the same reduction in carotid sinus nerve activity. The nicotine-induced carotid sinus nerve activity during neostigmine (9 μM) perfusion (i.e. 315 ± 254 Hz and 99 ± 60 Hz, for atracurium and vecuronium, respectively) was similar to the controls. There was no apparent shift in baseline recordings during perfusion with NMBAs or neostigmine. The preparations recovered after the washout period and the nicotinic response was 352 ± 186 and 177 ± 48 Hz, in the atracurium and vecuronium group.

In paper II, we established concentration–response relationships between the inhibition of nicotine-induced carotid body chemoreceptor responses by atracurium and vecuronium, using two different doses of nicotine.

A dose–response relationship for nicotine-induced chemoreceptor responses was first established, revealing a half maximal response at 77 μg (95 % C.I. 46-128 μg) (Figure 7). The nicotine-induced chemoreceptor response was concentration-dependently reduced by atracurium and vecuronium after administration of both 50 and 500 μg nicotine (Figure 8, Table 2). The baseline chemoreceptor responses did not change during perfusion with either concentration of atracurium or vecuronium.
All experiments (Paper I-II) were performed under stable experimental conditions, i.e. constant PO₂, PCO₂, pH, Na⁺ and K⁺. A PO₂ reduction of the perfusate resulted in an increased carotid sinus nerve activity, that was similar before and after the experimental period, thus validating the preparation.

![Graph showing dose-response curve for nicotine-induced increase in carotid sinus nerve activity.]

**Figure 7:** Dose-response curve for nicotine-induced (5-500 μg) increase in carotid sinus nerve (CSN) activity. Each symbol represents mean ± S.E.M., n=4.

**Table 2:** IC₅₀ values and the potency ratio of atracurium vs. vecuronium

<table>
<thead>
<tr>
<th>Nicotine (μg)</th>
<th>Atracurium (μM)</th>
<th>Vecuronium (μM)</th>
<th>Potency ratio (atr/vec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>3.64 (2.22-6.05)</td>
<td>1.66 (1.05-2.57)</td>
<td>1.22</td>
</tr>
<tr>
<td>500</td>
<td>27.00 (18.07-40.33)</td>
<td>7.29 (5.13-10.37)</td>
<td>3.70</td>
</tr>
</tbody>
</table>

Data are presented as mean (95% C.I.)

![Graph showing original registrations of nicotine-induced carotid sinus nerve (CSN) activity in response to vecuronium. Arrows represent administration of 500 μg nicotine.]

**Figure 8:** Original registrations of nicotine-induced carotid sinus nerve (CSN) activity in response to vecuronium. Arrows represent administration of 500 μg nicotine.
Propofol reduces carotid body chemosensitivity and nicotinic chemotransduction (Paper III)

In this study the effect of propofol on responses to various degrees of PO$_2$ reductions and nicotine-induced carotid body chemoreceptor responses were studied using the isolated carotid body preparation.

Using three levels of PO$_2$ reduction in the chamber bath, we established a dose–response relationship for the chemoreceptor response in the isolated carotid body preparation (i.e. a greater degree of PO$_2$ reduction produced a larger increase in carotid sinus nerve activity). Propofol (100 µM) attenuated the increase in carotid sinus nerve activity at all three levels of PO$_2$ reduction. Interestingly, a greater degree of PO$_2$ reduction resulted in less depression of the chemoreceptor response. Furthermore, propofol concentration-dependently reduced the nicotine-induced carotid body chemoreceptor response to 500 µg nicotine (Figure 9). The vehicle of propofol (Intralipid® 1:560) did not reduce the nicotine-induced responses. In order to investigate whether this reduction of nicotine-induced responses was mediated via the GABA$_A$ receptor system we applied a competitive (bicuculline) and a non-competitive (picrotoxin) GABA$_A$ receptor antagonist. If the reduction of the nicotine-induced response by propofol were mediated via the GABA$_A$ system, application of an antagonist would at least partially reverse the effect of propofol. Notably, addition of 100 µM bicuculline to 100 µM propofol did not reverse the response elicited by only propofol. In contrast, 100 µM picrotoxin with 100 µM propofol further reduced the nicotine-induced carotid sinus nerve activity (P<0.05).

Figure 9: Propofol inhibits the nicotine-induced carotid body chemoreceptor responses to 500 µg nicotine in a concentration-dependent manner with an IC$_{50}$ of 40 µM (95% CI: 8-198 µM). Data are presented as mean ± SEM, n=7-9.
Pharmacological properties of non-depolarizing NMBAs on neuronal nAChRs expressed in Xenopus oocytes (Paper IV)

The purpose of this study was to characterize the properties of atracurium, cis-atracurium, d-tubocurarine, mivacurium, pancuronium, rocuronium and vecuronium on neuronal α3β2, α3β4, α4β2 and α7 nAChR subtypes. Using nAChRs expressed in Xenopus oocytes and the Opus-Xpress™, concentration–response curves for ACh were established for all subtypes studied, each receptor having its own characteristic ACh current and EC50 value. Initially, concentration–response curves for ACh alone and together with 10 μM NMBA for the neuronal subtypes were established (Figure 10). Using a low and a high ACh concentration for each receptor subtype, inhibition curves for all NMBA tested were thereafter performed. Despite different inhibition mechanisms for individual non-depolarizing NMBA and nAChR subtypes, all the non-depolarizing NMBA inhibited the nAChRs in a concentration-dependent manner.

All NMBA tested, except mivacurium, inhibited the α3β2 subtype with IC50 values of 3-20 μM at 50 μM acetylcholine activation and 1-62 μM at 300 μM acetylcholine. Mivacurium had the lowest potency, and did not inhibit the 300 μM ACh response at concentrations lower than 100 μM. In general, the non-depolarizing NMBA competitively inhibit the neuronal nAChRs, while d-tubocurarine and vecuronium act as non-competitive inhibitors.

All non-depolarizing NMBA inhibited the α3β4 nAChR subtype, in a concentration-dependent manner with IC50 values from 2-20 μM and 0.3-2 μM for 50 and 300 μM acetylcholine, respectively. The NMBA non-competitively inhibited the α3β4 subtype and all drugs except cis-atracurium and mivacurium had a higher affinity for the closed channel.

By contrast, the α4β2 nAChR subtype was competitively blocked by the non-depolarizing NMBA. The IC50 values were increased from 1-13 μM to 5-67 μM for 1 and 10 μM acetylcholine, respectively. Notably, 10 μM rocuronium reduced the peak ACh response, indicating a non-competitive mechanism of inhibition.

In general, non-depolarizing NMBA competitively inhibited the α7 subtype, since all the NMBA right shifted the ACh concentration–response curve without a reduction in peak response, and furthermore concentration-dependently inhibited the response to 30 and 100 μM ACh. Notably, rocuronium inhibited 100 μM ACh to a higher degree than 30 μM ACh.

The adult human muscle (α1β1δ) nAChR was used as a reference, and all non-depolarizing NMBA concentration-dependently inhibited the response to 10 μM ACh with IC50 values of 4-97 nM. The selectivity for the adult muscle nAChR in this system was high, with IC50 values ranging from 4-97 nM vs. 0.46-69 μM for the neuronal nAChR. However, the IC50 for a neuromuscular block in vitro nerve-muscle preparations ranges from 2 to 12 μM (Bowman and Webb, 1976; Fortier, Robinaille, 2001), and the clinical relevant concentration range is 1-5 μM (Hou, Hirshman, 1998). Application of the non-depolarizing NMBA tested to both muscle (α1β1δ) and neuronal (α3β2, α3β4, α4β2 and α7) nAChR did not produce any current.
Figure 10: Non-depolarizing NMBAs inhibit human neuronal nAChRs.
The effect of 10 μM non-depolarizing NMBAs on voltage clamped Xenopus oocytes expressing the human α3β2, α3β4, α4β2 and α7 AChR subtypes. Responses in each oocyte were normalized to the maximal ACh peak current and net charge (α7) in each oocyte giving the concentration–response curves. Data are presented as mean ± SEM of 3-14 oocytes. When no error bars are seen, they are smaller than the symbols.
The effect of succinylcholine on neuronal nAChRs (Paper V)

Here, we investigated the effect of the depolarizing \textit{N}MBA succinylcholine on muscle and neuronal nAChRs expressed in \textit{Xenopus} oocytes using OpusXpress\textsuperscript{TM}. Concentration–response relations for acetylcholine and succinylcholine were established for the human \(\alpha1\beta1\gamma\delta\), \(\alpha3\beta2\), \(\alpha3\beta4\), \(\alpha4\beta2\) and \(\alpha7\) nAChR subtypes. ACh concentration-dependently activated all nAChR subtypes, while only the muscle (\(\alpha1\beta1\gamma\delta\)) subtype was activated by succinylcholine at concentrations lower than 1 mM (\textbf{Figure II}). The EC\(_{50}\) for succinylcholine at the \(\alpha1\beta1\gamma\delta\) nAChR was 10.82 \(\mu\)M (95% C.I. 9.81-11.94 \(\mu\)M). No succinylcholine-activated currents were seen in the \(\alpha3\beta2\) and \(\alpha3\beta4\) subtypes, and the \(\alpha4\beta2\) and \(\alpha7\) subtypes produced only minimal currents at succinylcholine concentrations above 1 mM. Thus, there is a huge contrast between the efficacies of succinylcholine as an agonist at muscular vs. neuronal nAChRs. We also made an attempt to investigate the inhibitory effect of succinylcholine on the muscle nAChR. Succinylcholine was pre- and co-applied together with ACh, and not surprisingly, co-application of succinylcholine with 1 or 5 \(\mu\)M ACh did not inhibit the \(\alpha1\beta1\gamma\delta\) nAChR. Preapplication of succinylcholine and the subsequent activation of the receptor made the ACh response difficult to measure; however, an IC\(_{50}\) of 126 \(\mu\)M (95% C.I. 47.8-334 \(\mu\)M) was noted. In order to investigate the inhibitory effect of succinylcholine on the neuronal nAChRs, it was both pre- and co-applied with an EC\(_{50}\) concentration of ACh. Succinylcholine was found to have a low potency as inhibitor at the neuronal nAChRs, and there was no difference whether succinylcholine was pre- or co-applied.

\textbf{Figure II:} Activation by acetylcholine (ACh) and succinylcholine (SuCh) in voltage clamped \textit{Xenopus} oocytes expressing human muscle and neuronal nAChRs. (A) Representative currents activated by various concentrations of \textit{ACh}, and 1 (\(\alpha3\beta2\), \(\alpha3\beta4\), \(\alpha4\beta2\)) or 10 (\(\alpha7\)) \(\mu\)M succinylcholine (B) Corresponding concentration–response curves. Responses in each oocyte were normalized to peak current and maximal net charge response to \textit{ACh} in each oocyte. For the \(\alpha1\beta1\gamma\delta\) subtype, succinylcholine-induced responses were normalized to peak succinylcholine response within each oocyte. Peak and net charge analysis are displayed. Each symbol represents mean ± S.E.M. of 7-17 oocytes. When no error bars are seen they are smaller than the symbols.
In this thesis we have shown that both NMBAs and propofol reduce nicotine-induced chemoreceptor responses in the carotid body. In addition, while non-depolarizing NMBAs inhibit neuronal nAChRs, succinylcholine has a distinct action at the muscle subtype and does not activate the neuronal nAChRs, and is furthermore a weak antagonist at these nAChRs.

**Regulation of breathing during hypoxia and the effect of non-depolarizing NMBAs**

Cholinergic transmission is one of at least three key systems in the transmission of carotid body chemoreceptor sensing of oxygen via the carotid sinus nerve and beyond (Cohen, Han, 2002, Fitzgerald, 2000, Iturriaga and Alcayaga, 2004, Prabhakar, 2006). It is obvious that an inhibition of nicotinic transmission is not sufficient to completely abolish chemoreceptor signalling during hypoxia (Fitzgerald, 2000, Iturriaga and Alcayaga, 2004), which indicates that a multitude of neurotransmitters and ion channels operate during hypoxia to orchestrate a complete transmission of information via the carotid sinus nerve to the brain (Weir, Lopez-Boamé, 2005). Moreover, in animals a full neuromuscular block is followed by a reduced but not abolished chemoreceptor response to hypoxia (Wyon, Joensen, 1998). In these experiments, spontaneous return of chemoreceptor signalling via the carotid sinus nerve occurs simultaneously with a gradual return of neuromuscular transmission to normal (Wyon, Joensen, 1998). We therefore hypothesize that our finding of a reduced AHVR during partial neuromuscular block in humans to a major extent is due to an inhibition of nicotinic transmission of carotid body chemoreceptor activity by the neuromuscular blocking agent. From previous studies it has not been clear to what extent NMBAs interfere with carotid body nicotinic transmission and whether this class of drugs used in anesthesia interact with both muscle and neuronal subtypes of nicotinic acetylcholine receptors.

From the present work and that of others (Igarashi, Amagasa, 2002) it is evident that atracurium and vecuronium, despite structural differences, effectively block ACh- and nicotine-induced carotid sinus nerve activity, at concentrations of the NMBA that are seen in clinical practice. Data
from these studies were generated in an isolated carotid body preparation with exogenous administration of a cholinergic agonist. The natural ligand ACh stimulates both muscarinic and nicotinic subtypes of AChRs whereas nicotine stimulates only the nicotinic subtypes. There are several reasons why we chose to use exogenous nicotine rather than the natural ligand ACh to study nicotinic transmission in the carotid body. First, the release of the natural ligand ACh varies to a large extent, depending on the level of hypoxia, and the neurotransmitter release is further modulated by the simultaneous release of other transmitters and modulators such as dopamine, ATP and nitric oxide. Furthermore, nicotine is a selective agonist at the nicotinic subtypes of ACh receptors, leaving the muscarinic subtypes unaffected. On the other hand, it may be argued that we could have applied the natural ligand ACh to the isolated carotid body preparation and simultaneously blocked the muscarinic ACh receptors by application of atropine to the perfusate. However, it has been shown that atropine can both activate and inhibit certain nAChRs (Zwart and Vijverberg, 1997).

There are conflicting data regarding the ACh release during hypoxia and also about whether ACh is an excitatory or inhibitory transmitter in the carotid body oxygen signalling (Monti-Bloch and Eyzaguirre, 1980). Most of this controversy is due to the fact that ACh binds both to nicotinic and muscarinic AChRs at the glomus cell and at the carotid sinus nerve: depending on the degree of binding to each receptor, the ACh release and carotid sinus nerve activity is modified correspondingly. In the rabbit, ACh hyperpolarizes the chemoreceptors, whereas nicotine depolarizes the receptors and increases the carotid sinus nerve activity (Monti-Bloch and Eyzaguirre, 1980). From recent data it is now clear that muscarinic AChRs are present as excitatory M1 receptors both at the glomus cell and at the afferent nerve endings, and as inhibitory M2 receptors at the glomus cell (Shirahata, Hirasawa, 2004). In rabbit, the ratio of muscarinic vs. nicotinic AChRs is 12:1, in contrast to in the cat (2:1) (Hirano, Dinger, 1992). This explains earlier reports that ACh is an inhibitory transmitter in the rabbit carotid body.

Parallel to the increased knowledge about neuronal nAChRs we have gained more insight into the structure and function of nAChRs in the carotid body and this places older data into a new perspective. Twenty-five years ago, α-BTX binding sites were found at the carotid body type I cell (Chen, Mascorro, 1981, Dinger, Gonzalez, 1981), indicating the presence of a nAChR. Having the current knowledge that α-BTX only binds to the α1 (muscle nAChR) and α7-9 nAChR subunits, it now seems reasonable to believe that the authors detected a α7 nAChR at the type I cell. Later, functional nAChRs have been demonstrated on neonatal carotid body type I cells (Wyatt and Peers, 1993). In addition, the neuronal subunits α3, α4, α5, α7, β2 and β4 have been detected in carotid body total mRNA (Cohen, Han, 2002), whereas the α3, α4 and β2 subunits have been found as proteins and also functioning in cultured type I cells (Higashi, McIntosh, 2003). The α7 subtype is present at the afferent carotid sinus nerve (Shirahata, Ishizawa, 1998).

Speculating about which nAChR subtype(s) are present in the carotid body, we consider it likely that the α3β2, α4β2 and α7 subtypes are expressed at the surface of the type I cell, whereas the α7 nAChR subtype is present at the afferent carotid sinus nerve. Notably, atracurium and vecuronium inhibit the α3β4, α4β2 and α7 subtypes expressed in the Xenopus oocytes (paper IV) in the same concentration range as they reduce the carotid sinus nerve activity in paper I-II. Thus, there is a molecular prerequisite for an inhibition by atracurium and vecuronium of the nAChRs in the carotid body. However, the exact location of this inhibition is not known, i.e. whether it is a block of presynaptic nAChRs at the carotid body type I cell, or an inhibition of postsynaptic nAChRs at the carotid sinus nerve. On the basis of results from paper I-II, we can only speculate about whether the block is pre- or postsynaptic, or both. In other synapses presynaptic nAChRs function as autoreceptors that mobilize more ACh in response to stimulation. Whether the same holds true for the type I cell is not known, but it seems reasonable to assume that the same mechanism is present.
inhibition of putative presynaptic nAChRs would then attenuate release of ACh and maybe other transmitters, whereas an inhibition of postsynaptic nAChRs at the carotid sinus nerve definitely would reduce carotid sinus nerve activity.

Finally, we cannot rule out that non-depolarizing NMBAs reduce the nicotinic transmission by interaction with other receptors (dopamine, ATP, adenosine etc) or steps in the transduction process (K+ or voltage-gated Ca++ channels). Certain types of K+ channels (BK, Ks) can be blocked by d-tubocurarine, but only if it is applied inside the cell (Egan, Dagan, 1993, Rossohkin, Teodorescu, 2006), and thus this is not relevant in this context. Notably, the receptor population (e.g. muscarinic and nicotinic ACh-, P2X- and adenosine receptors) in the synaptic unit of the carotid body display large similarities with the neuromuscular junction (see below). D-tubocurarine and vecuronium also reduce central respiratory control in vitro (Sakuraba, Kuyama, 2005; Sakuraba, Kuyama, 2003). However, human studies demonstrate no effect of vecuronium on resting ventilation or the response to hypercapnia (Eriksson, 1996, Eriksson, Lemmark, 1992). Moreover, the concentrations used (Sakuraba, Kuyama, 2005; Sakuraba, Kuyama, 2003) were higher than under clinical conditions. Furthermore, NMBAs are highly charged molecules and therefore do not easily cross the blood-brain barrier. Thus, in the clinical setting the likelihood of getting such high concentrations of NMBAs in the brain is low.

The mechanisms behind the reduced hypoxic ventilatory response by propofol

There has been a controversy about whether propofol reduces the hypoxic ventilatory response, and if so, over the putative site of this reduction. During recent years the first controversy has been resolved and it has become clear that propofol does attenuate the ventilatory response to hypoxia (Blouin, Seifert, 1993, Nieuwenhuys, Storton, 2000), whereas the debate about the origin of this depression is still unsolved (Nieuwenhuys, Storton, 2001). In paper III, we demonstrate that propofol attenuates the carotid sinus nerve activity in response to a reduction in oxygen partial pressure of the perfusate. This observation adds to earlier findings by Ponte and Sadler, that propofol impairs single fiber chemoreceptor activity in response to hypoxia (Ponte and Sadler, 1989). Interestingly, a more pronounced reduction in PO2 seemed to, at least in part, overcome the propofol-induced attenuation in carotid sinus nerve activity, indicating a competitive mechanism behind this depression. In addition, propofol reversibly and concentration-dependently reduced nicotine-induced carotid sinus nerve activity with an IC50 of 40 μM. We believe that this is due to an inhibition of neuronal nAChRs within the carotid body since both ganglionic and α4β2 nAChR subtypes are inhibited by propofol, whereas the α7 subtype is inhibited only at very high concentrations (Flood, Ramirez-Latorre, 1997, Furuya, Oka, 1999, Violet, Downie, 1997). Furthermore, application of a competitive (bicuculline) or a non-competitive (picrotoxin) GABAβ receptor antagonist in addition to propofol does not reverse the attenuated nicotinic transmission in the carotid body; this is also in agreement with the absence of any evidence for the existence of GABAβ receptors in the carotid body so far. In summary, propofol seems to reduce nicotinic transmission in the carotid body by inhibition of nAChRs. We did not study the effect of propofol at the receptor level, but based on the present discussion about the different nAChR subtypes in the carotid body (see above), and previous results concerning the affinity for propofol at different subtypes of nAChRs, propofol most likely inhibit the nAChRs at the type I cell, since the α7 nAChR so far only has been found at the carotid sinus nerve.

Even if our data add to the current knowledge about the mechanism of propofol-induced reduction in AHVR, we have not fully solved the issue about the site of action. While the mechanism of
anesthesia for propofol to a large part is mediated by binding to the GABA\(_\text{A}\) receptor complex, and thus facilitation GABA-ergic transmission, the molecular structure of propofol allows binding to a multitude of other receptors and ion channels (Hemmings, Akbas, 2005, Rudolph and Antkowiak, 2004). Among others, propofol inhibits glutamate, nAChR and NMDA receptors as well as two-pore K\(^+\) channels, voltage-gated Ca\(^{2+}\) and Na\(^+\) channels. Many of these receptors and channels are located both in the carotid bodies as well as in the respiratory center in the brain stem (Burton and Kazemi, 2000, Prabhakar, 2006). Recently, it was shown that propofol inhibits preinspiratory and expiratory neurons in the brain stem, most likely due to a facilitation of the GABA\(_\text{A}\) receptor complex (Kashiwagi, Okada, 2004). In summary, being a drug with a multitude of targets, it is not surprising that propofol interacts with the response to hypoxia at different receptors as well as at different locations.

The presence of both muscle and neuronal nAChR subtypes in the neuromuscular junction

Adequate maintenance of the neuromuscular transmission is vital, and in order to ensure this there is a high safety factor in the neuromuscular junction (i.e. an excess of ACh is released onto an excess of muscle type nAChRs at the postsynaptic muscle membrane). Moreover, there is strong evidence for a presynaptic positive feedback mechanism via nicotinic autoreceptors at the motor nerve ending (Bowman, 1980, Bowman, Prior, 1990, Wessler, 1989, Vizi and Lendvai, 1997). Activation of these autoreceptors and subsequent mobilization of ACh is a way in which the neuromuscular junction can maintain transmitter release during high activity, i.e. during physiological conditions associated with a high frequency of nerve action potentials arriving at the motor nerve ending. These pre- and postsynaptic mechanisms cooperate to transmit chemical information to the muscle membrane, ultimately leading to skeletal muscle contractions.

The level of impairment of neuromuscular transmission, e.g. during a non-depolarizing neuromuscular block, can be assessed by means of repeated nerve stimulation. Many years ago the train-of-four (TOF) response was described for clinical monitoring and has ever since been used to assess a non-depolarizing neuromuscular block during anesthesia (Ali, Utting, 1970). In short, the contraction pattern after a 1.5-second train of four nerve impulses at 2 Hz is described as the change in amplitude (either mechanical or electrical) of the first twitch (T1) or the ratio between the fourth and the first twitch (T4/T1 ratio, or TOF ratio). A classical non-depolarizing neuromuscular block is characterized by a reduction in both the T1 and the TOF ratio, while a depolarizing block shows an almost equal reduction of all four twitches without a fade in the TOF or tetanic response, i.e. a lack of TOF or tetanic fade. Over the duration of a neuromuscular block, these two events, i.e. the T1 twitch depression and the TOF or tetanic fade, display a different time course. Based on the above, it has been speculated whether these two features of a non-depolarizing neuromuscular block represent two (or even more) distinct receptor mechanisms within the neuromuscular junction (Bowman, 1980, Bowman, Prior, 1990, Wessler, 1989, Vizi and Lendvai, 1997). Investigations of the tetanic fade and twitch tension in cats by Bowman and Webb supported the theory of two different mechanisms of block (Bowman and Webb, 1976). Whereas pancuronium and d-tubocurarine, although to different degrees, produced both a reduction in twitch tension and tetanic fade, α-BTX reduced the twitch tension without tetanic fade and hexamethonium (a ganglionic blocker) produced only tetanic fade (Bowman and Webb, 1976).

There can be at least four subtypes of nAChRs that contribute to transmission at the neuromuscular junction, α1β1δ6, α1β1γδ, α3β2 and/or α7 (Figure 12) (Bowman, 2006, Fischer, Reinhardt, 1999, Martyn and Richtsfeld, 2006, Tsuneki, Kimura, 1995; Tsuneki, Salas, 2003). The α3 nAChR subu-
nit has been localized at the motor nerve ending (Tsuneki, Kimura, 1995), and a selective block of the α3β2 subtype in a nerve-muscle preparation causes tetanic fade (Faria, Oliveira, 2003). It is therefore likely that the TOF fade during non-depolarizing neuromuscular block reflects a block of the presynaptic α3β2 nAChR (paper IV) whereas the lack of TOF fade during a neuromuscular block by succinylcholine is explained by the low affinity for succinylcholine at this receptor subtype (paper V). Thus, non-depolarizing NMBAs reduce the “safety factor” in the neuromuscular junction both by a decrease in ACh release (by block of the α3β2 subtype) and by reducing the number of available postsynaptic nAChRs (block of the α1β1δ). However, mivacurium seems to be an exception in this respect, since mivacurium was a poor blocker at the α3β2 nAChR (paper IV). Notably, mivacurium is the most short-acting non-depolarizing NMBA at present, and since TOF fade disappears late in the time course of a neuromuscular block the low affinity to the α3β2 nAChR might, in combination with the rapid breakdown, further curtail the action of mivacurium. In addition to the well-known inhibition of the postsynaptic α1β1δ/α1β1γδ nAChRs, it has been suggested that a low dose of non-depolarizing NMBAs may activate these receptor subtypes. However, this is only true for the α1β1γδ (fetal) subtype (Fletcher and Steinbach, 1996). A generalization between the two muscle subtypes is common, but since these different receptors have distinct properties in terms of channel opening time, conductance and binding affinities, such a generalization is no longer acceptable.

![Diagram of nAChRs in the neuromuscular junction.](image)

Interestingly, recent data indicate that adenosine and ATP interacting with A1/A2A and P2X receptors at the motor nerve ending can be critically involved in presynaptic release and mobilization of ACh (Baxter, Vega-Riveroll, 2005; Giniatullin and Sokolova, 1998; Nitahara, Vizi, 2005; Timoteo, Faria, 2003). The interactions between NMBAs and adenosine/ATP receptors are not fully evaluated, and need more attention for a better understanding of the molecular mechanisms in the neuromuscular junction.

Modern non-depolarizing NMBAs inhibit the human neuronal α3β2, α3β4, α4β2 and α7 nAChR subtypes in the micromolar concentration range, and therefore have the potential to cause a ganglionic block. Penetration of non-depolarizing NMBAs into the central nervous system can alter
synaptic transmission and cause seizures (Szepethyeczky, Trevor, 1993). Although these drugs are highly water soluble there have been reports of d-tabocurarine and vecuronium in the cerebrospinal fluid of patients with an impaired blood-brain barrier (Segredo, Matthey, 1990). Although there is molecular evidence for a potential interaction with nAChRs in the CNS, this will probably not occur during normal dosage and duration of administration. However, a degradation product of atracurium, laudanosine, is lipid soluble, has been found in the human cerebrospinal fluid, can cause seizures in dogs and is a non-competitive inhibitor of neuronal nAChRs (Chiodini, Charpentier, 2001, Exley, Iturriaga-Vasquez, 2005, Tassonyi, Fathi, 2002). Thus, during prolonged infusions with atracurium in the intensive care setting this interaction may be of clinical relevance. It is worth noting that cis-atracurium is also degraded to laudanosine, but to a much lesser extent. Hence, cis-atracurium should be safe to use in this context.

Although succinylcholine efficiently produces neuromuscular block and paralysis, its use is plagued with a high incidence of serious side effects, the majority being attributed to its initial and massive activation of a large number of muscle type nAChR at the neuromuscular junction or in the diseased muscle membrane. As recently reviewed by Martyn and Ritchfelt (Martyn and Richtsfeld, 2006), the consequence of such activation is hyperkalemia with a subsequent risk of life-threatening cardiac arrhythmias or even cardiac arrest and death. Part of succinylcholine-induced tachyarythmias have been attributed to an increased catecholamine release by stimulation of nAChRs in the adrenal medulla (Naguib and Lier, 2005). However, speculations that succinylcholine activates nAChRs in peripheral autonomic ganglia (Naguib and Lien, 2005) seem not to be proven, and are most likely based on the assumption that succinylcholine has chemical similarity with ACh. Based on this and its ability to activate the muscle nAChR have served as the base for the assumption that succinylcholine also would activate other nAChRs. In this thesis, it is demonstrated that succinylcholine does not activate the α3β2 and α3β4 neuronal nAChRs, and although the α4β2 and α7 subtypes were activated, this happened only at concentrations higher than the clinically relevant. Moreover, it is unlikely that the breakdown products of succinylcholine (i.e. choline and succinylmonocholine) would activate the neuronal nAChRs in the clinical concentration range (Fuentesalba, Olivares, 2004, Lee, 2003, Papke and Porter Papke, 2002). Notably, choline is known as a selective α7 nAChR agonist, but as such it has low potency (EC50 ~ 400-500 µM), and is furthermore a weak inhibitor (IC50 ~ 1-10 mM) at this subtype (Fuentesalba, Olivares, 2004, Papke and Porter Papke, 2002). Based on the findings in paper V, it is not likely that succinylcholine activates nAChRs in peripheral autonomic ganglia or in the adrenal medulla.
Despite almost a century of intense research we are still unable to identify the mechanism(s) of oxygen sensing and signalling. In addition, there are contradictory results probably because of use of different drug concentrations, different levels of hypoxia and various animal species. Recent data indicate that the oxygen sensing mechanisms might differ depending on the actual level and duration of hypoxia (Prabhakar, 2006). This is a tempting hypothesis and would certainly explain some of the differences reported. Given the above, improved methods in molecular biology and the use of knockout mice would provide us further information about critical parts of the process in oxygen sensing and signalling. One of the biggest challenges in the future is to identify oxygen sensing and signalling pathways in the human carotid body, which today is an unknown territory.

The inhibition of neuronal nAChRs in the carotid body provides a molecular explanation for the reduction in acute hypoxic ventilatory response in humans during residual paralysis by non-depolarizing NMBAs. This is a very surprising property of non-depolarizing NMBAs, although highly clinically relevant. Impairment of vital functions such as swallowing and the ventilatory response to hypoxia is a consequence of residual paralysis by non-depolarizing NMBAs and may contribute to adverse outcome after anesthesia (Eriksson, Sandman, 1997). While residual paralysis by pancuronium has already been shown to increase postoperative morbidity and mortality (Berg, Roed, 1997), we need to gain more knowledge about how other NMBAs influence the risk of postoperative adverse events. This is illustrated by a recent study where the use of several non-depolarizing NMBAs increased the risk of anesthesia-related mortality and morbidity, and notably, this increased risk was eliminated by reversal of NMBAs by an anticholinesterase (Arbous, Meursing, 2005).

The discovery that a neural stimulation of α7 nAChRs on macrophages alters the pro-inflammatory response reveals a novel type of inflammatory mechanism involving the autonomic nervous system, and thus a potential target for drug development in the treatment of sepsis (Czura and Tracey, 2005, Tracey, 2002). Based on this, and other aspects of nicotinic transmission discussed in this thesis (i.e. regulation of breathing), detailed knowledge about the potential interactions between nAChRs and the drugs used in the practice of anesthesia and intensive care medicine is important. Anesthesia has become safer over the years, and in order to maintain on the winning track, we need to gain more detailed knowledge of underlying molecular mechanisms of the drugs used in anesthesia, since most of them have a multitude of targets and we do not know them all yet!
Conclusions

Based on this thesis it is concluded that both non-depolarizing NMBAs and propofol reduce nicotinic transmission in the carotid body, and furthermore that non-depolarizing NMBAs, in contrast to depolarizing NMBAs, interact with neuronal nAChR and inhibit these receptors. In detail:

- Atracurium and vecuronium reduce nicotine-induced carotid body chemoreceptor responses. This reduction is concentration-dependent. Also, the attenuation is dependent on the nicotine dose, suggesting a competitive mechanism of inhibition. There is a similar degree of reduction at equipotent neuromuscular blocking concentrations.

- Propofol impairs carotid body chemosensitivity at various reductions in PO$_2$ in a dose-dependent manner.

- Propofol reduces nicotine-induced carotid body chemoreceptor responses in a concentration-dependent and reversible manner. Antagonism of the GABA$_A$ receptor complex does not reverse this propofol-mediated inhibition. Hence, it seems likely that propofol impairs nAChRs in the carotid body.

- Clinically used non-depolarizing NMBAs concentration-dependently inhibit the $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$ and $\alpha_7$ neuronal nAChR subtypes. The inhibitory mechanism (competitive and/or non-competitive) depends on receptor subtype and the N MBA. In addition, non-depolarizing NMBAs do not activate the muscle $\alpha_1\beta_1\epsilon\delta$ or any of the neuronal nAChRs studied.

- Succinylcholine does not activate the $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$ and $\alpha_7$ neuronal nAChR subtypes at clinically relevant concentrations (i.e. only at concentration $>1$ mM). Furthermore, succinylcholine is a weak antagonist at all studied neuronal nAChRs. The lack of inhibition at the $\alpha_3\beta_2$ provides a molecular explanation for the lack of TOF fade during a neuromuscular block with succinylcholine in contrast to non-depolarizing NMBAs.
“It is what we think we know already that often prevents us from learning”

Claude Bernard
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