

Blockade and Activation of the Human Neuronal Nicotinic Acetylcholine Receptors by Atracurium and Laudanosine

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Background: Curaremimetic nondepolarizing muscle relaxants are widely used in clinical practice to prevent muscle contraction either during surgery or during intensive care. Although primarily acting at the neuromuscular junction, these compounds can cause adverse effects, including modification of cardiac rhythm, arterial blood pressure, and in the worst cases, triggering of seizures. In this study, we assessed the interaction of atracurium and its metabolite, laudanosine, with neuronal nicotinic receptors.

Methods: The human neuronal nicotinic receptors $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 3\alpha 5\beta 4$, and $\alpha 7$ are heterologously expressed in *Xenopus laevis* oocytes, and the effect of atracurium and its degradation product, laudanosine, were studied on these receptors.

Results: Atracurium and laudanosine inhibited in the micromolar range the major brain $\alpha 4\beta 2$ receptor and the ganglionic $\alpha 3\beta 4$ or $\alpha 3\beta 4\alpha 5$ and the homomeric $\alpha 7$ receptors. For all four receptors, inhibition was rapid and readily reversible within less than 1 min. Atracurium blockade was competitive at $\alpha 4\beta 2$ and $\alpha 7$ receptors but displayed a noncompetitive blockade at the $\alpha 3\beta 4$ receptors. Inhibition at this receptor subtype was not modified by $\alpha 5$. Laudanosine was found to have a dual mode of action; first, it competes with acetylcholine and, second, it blocks the ionic pore by steric hindrance. At low concentrations, these two drugs are able to activate both the $\alpha 4\beta 2$ and the $\alpha 3\beta 4$ receptors.

Conclusion: Adverse effects observed during atracurium administration may be attributed, at least partly, to an interaction with neuronal nicotinic receptors.

THE benzyloquinoline¹ derivative atracurium (ATR) is a widely used nondepolarizing neuromuscular blocking agent, with a relatively short half-life of 20–40 min, and a rapid elimination period.^{2–4} Although well-tolerated, atracurium can cause different adverse reactions,^{5,6} such as cardiovascular effects, that are thought to be mediated by the ganglionic neuronal nicotinic acetylcholine receptor (nAChR).⁷ In addition, atracurium and its degradation product laudanosine were found in cerebrospinal fluid,

indicating that this compound may cross, in some circumstances, the blood-brain barrier^{8,9} and could, depending on the concentrations, affect brain function. In agreement with this hypothesis, it has been shown in animal models that, when administered at high concentration, neuromuscular blocking agents can trigger seizures.^{10,11} Furthermore, atracurium application to hippocampal slices modifies synaptic transmission.¹²

The recent availability of complementary DNA (cDNA) coding for human neuronal nAChRs (reviewed in Bertrand and Changeux¹³ and Lindstrom *et al.*,¹⁴) opens new possibilities for assessment of the possible effects of atracurium on these receptors. The aim of this study was to evaluate the effects of atracurium and laudanosine on the functional properties of the major brain and ganglionic human nAChRs reconstituted in *Xenopus* oocytes. The $\alpha 4\beta 2$ subunits, which are thought to constitute the major brain nicotinic receptors, were chosen as a model of central nAChRs.^{13,14} Receptors corresponding to those found in ganglia were obtained by expression of $\alpha 3\beta 4$ or $\alpha 3\beta 4\alpha 5$ subunits.¹⁵ In addition, we evaluated atracurium and laudanosine effects on the homomeric $\alpha 7$ nAChR that is expressed centrally and peripherally.^{13,16–18}

Methods

Oocyte Preparation and cDNA Injection

Xenopus oocytes were isolated and prepared as previously described.¹⁹ Oocytes were intranuclearly injected with 2 ng cDNA. All subunits were injected with an equal concentration. Oocytes were kept separately in a 96-well microtiter plate (NUNC) at 18°C in Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄·7H₂O, 0.33 mM Ca(NO₃)₂·4H₂O, 0.41 mM CaCl₂·6H₂O, at pH 7.4 adjusted with NaOH, and supplemented with 20 µg/ml kanamycin, 100 U/ml penicillin, and 100 µg/ml streptomycin). Atracurium (Tracrium[®]) was purchased from Glaxo Wellcome (London, UK). All other drugs, including ACh, laudanosine and atropine were purchased from Sigma (Buchs, Switzerland).

Electrophysiology

Current recordings from oocytes were performed at 18°C, 2–4 days after cDNA injections. During the recording, cells were continuously superfused with original Ringer 2 (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, pH

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7.4 adjusted with NaOH) with either Ca^{2+} or Ba^{2+} (2.5 mM). All drugs were diluted in an original Ringer 2 medium. The flow rate was approximately 6 ml/min and the volume chamber was less than 100 μl . To prevent possible activation of endogenous muscarinic receptors, 0.5 μM atropine was added. Electrophysiologic recordings were performed with a two-electrode voltage clamp (GENECLAMP amplifier; Axon Instruments, Foster City, CA). Electrodes made from 1.2 mm borosilicate Q tubes were pulled using a BB CH PC puller (Mecanex, Nyon, Switzerland), and filled with 3 M KCl. Unless specified, cells were clamped at a holding potential of -100 mV and the current was measured at the peak current. All experiments were performed at 18°C . Current-voltage relations were determined by a linear voltage ramp. To best show open channel blockade, cells were first briefly held at $+40$ mV and the voltage was then ramped down within 500 ms to 100 mV. Current-voltage relation curves were obtained by reporting the current values measured every 4 mV. Subtraction of the currents determined in control conditions from those measured during ACh exposure allowed determination of the nAChR current-voltage relations in isolation.

Data Analysis

Concentration-response curves were adjusted using the empirical Hill equations;

$$Y = 1/(1 + (\text{EC}_{50}/x)^{nH}) \quad (1)$$

where Y is the fraction of activated current, EC_{50} is concentration of half-activation, nH is the apparent cooperativity, and x is agonist concentration.

$$Y = 1/(1 + (x/\text{IC}_{50})^{nH}) \quad (2)$$

where Y is the fraction of remaining current, IC_{50} is concentration of half-inhibition, nH is the apparent cooperativity, and x is antagonist concentration.

Values indicated throughout the text are given with their respective standard deviations (SD).

Results

Evidence, including results obtained from biochemical and electrophysiologic studies, has shown that *d*-tubocurarine produces multiple effects at the neuromuscular nAChR junction. Effects caused by this molecule are (1) competitive inhibition,^{20,21} (2) open channel blockade,^{22,23} and (3) direct activation of the receptor.²⁴⁻²⁶ Therefore, when evaluating possible effects of the structurally related atracurium molecule on neuronal nAChRs, it is necessary to distinguish among these three modes of action.

Effects of atracurium on the Central or Ganglionic nAChRs

To determine atracurium effects in steady state conditions, this compound was pre- and coapplied with ACh.

As shown in figure 1A, the application of 10 μM atracurium markedly inhibits the ACh-evoked current at the $\alpha 4\beta 2$ nAChR. This effect was reversible within 1 min of washout. A comparable inhibition of ACh-evoked current was observed at 4 μM atracurium for the ganglionic $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ nAChRs and at 10 μM atracurium for the $\alpha 7$ receptor. Full recovery was observed within 2 min. For each of these receptor subtypes, the ACh concentration test pulse was adjusted to near their respective EC_{50} . A small inward deflection of the current was observed during atracurium application alone on the $\alpha 4\beta 2$ and $\alpha 3\beta 4$ receptors. To evaluate further this putative receptor activation, currents evoked by low ACh concentrations were compared with those evoked by either atracurium or laudanosine alone (fig. 1B). Increasing the drug concentrations greater than those shown always resulted in a smaller current, indicating that these compounds act as inhibitors at relatively higher concentrations. The comparison of the currents evoked by ACh, atracurium, and laudanosine on a log-log scale, highlights the differences in sensitivity of the $\alpha 4\beta 2$ and $\alpha 3\beta 4$ receptors to these three substances, with ACh always being the most effective agonist.

As shown in figure 1B, data are well-fitted by straight lines and yielded respective slope values of 0.73 and 0.78 for ACh and atracurium on the $\alpha 4\beta 2$ and 1.22, 0.6, and 0.31 for ACh, laudanosine, and atracurium on the $\alpha 3\beta 4$. Offset values were 2.72 and 2.3 for ACh and atracurium on the $\alpha 4\beta 2$ and 1.5, 0.56, and -0.02 for ACh, laudanosine, and atracurium on the $\alpha 3\beta 4$. All correlation factors were superior to 0.95. As expected for a receptor with a lower affinity, all responses on the $\alpha 3\beta 4$ are shifted to the right. Therefore, the relative agonist sensitivities are ACh greater than atracurium on $\alpha 4\beta 2$ receptors and ACh greater than laudanosine greater than atracurium on $\alpha 3\beta 4$ receptors. Laudanosine alone, however, evoked no detectable current at $\alpha 4\beta 2$. Similarly, no currents could be recorded in response to atracurium or laudanosine exposure at the homomeric $\alpha 7$ receptors (data not shown). Given the paucity of $\alpha 5$ expression and the absence of distinguishable atracurium effects, no attempts were made to characterize the activation of this receptor subtype.

Dual Blockade Mechanisms of Atracurium

To further characterize the atracurium inhibition, we first evaluated the atracurium concentration-response inhibition curve at a fixed ACh test condition (fig. 2 and table 1). Afterward, atracurium was kept at a constant concentration while the concentration of ACh was progressively increased. As shown in figure 2A, IC_{50} of the $\alpha 4\beta 2$ nAChR was observed at 1.5 μM atracurium, for 0.1 μM ACh test pulse. Increasing the ACh concentration decreased the IC_{50} value, suggesting that atracurium and ACh may compete for the same binding site at $\alpha 4\beta 2$. The competitive nature of atracurium blockade at the $\alpha 4\beta 2$

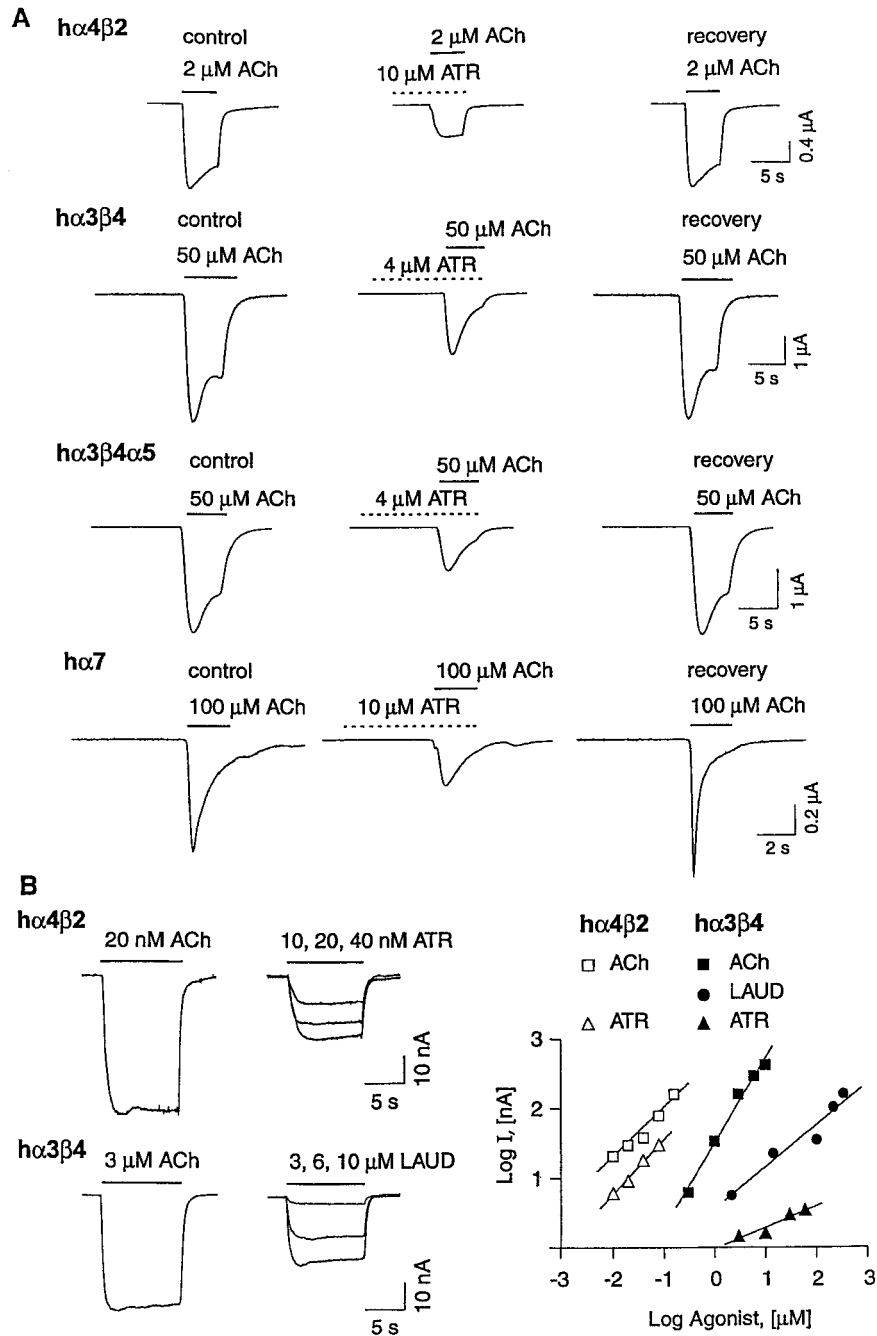


Fig. 1. Blockade and activation of neuronal nAChRs. (A) ACh-evoked currents recorded in $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 7$ receptors expressing oocytes in control conditions (left), during atracurium exposure (middle), and after recovery (right). Dashed lines indicate the atracurium application time, whereas bars indicate the ACh application. (B, Left) Activation of the $\alpha 4\beta 2$ and $\alpha 3\beta 4$ neuronal nicotinic acetylcholine receptors (nAChRs) by low concentrations of ACh, atracurium, or laudanosine. (Right) Plots on a log-log scale ACh- (squares), atracurium- (triangles), and laudanosine (circles)-evoked currents.

nAChR was further confirmed with the evaluation of how atracurium altered the ACh concentration-response relation (fig. 2B). For each cell, data were normalized to the saturating current recorded at maximal ACh concentration (1 mM) in control conditions. The graph shows that 10 μM atracurium caused a shift of the concentration-response curve toward higher concentrations (table 1), without affecting the maximal evoked current.

As shown in figures 2C and 2D, a different pattern of inhibition was observed at the ganglionic $\alpha 3\beta 4$ receptor. First, inhibition was independent of the ACh concentra-

tion (fig. 2C). Second, atracurium caused only a small decrease on the ACh sensitivity, and blockade could not be relieved by increasing the ACh concentration. Because it is known that $\alpha 5$ receptor contributes to a fraction of ganglionic receptors,¹⁵ the effects of atracurium were assessed after coinjection of $\alpha 3$, $\beta 4$, and $\alpha 5$ subunits. As shown in table 1, injection of this subunit caused no detectable changes in atracurium affinity. No differences could be observed on the ACh concentration-response profile either (data not shown). These results suggest that atracurium may act on the ganglionic receptor as an open channel blocker.

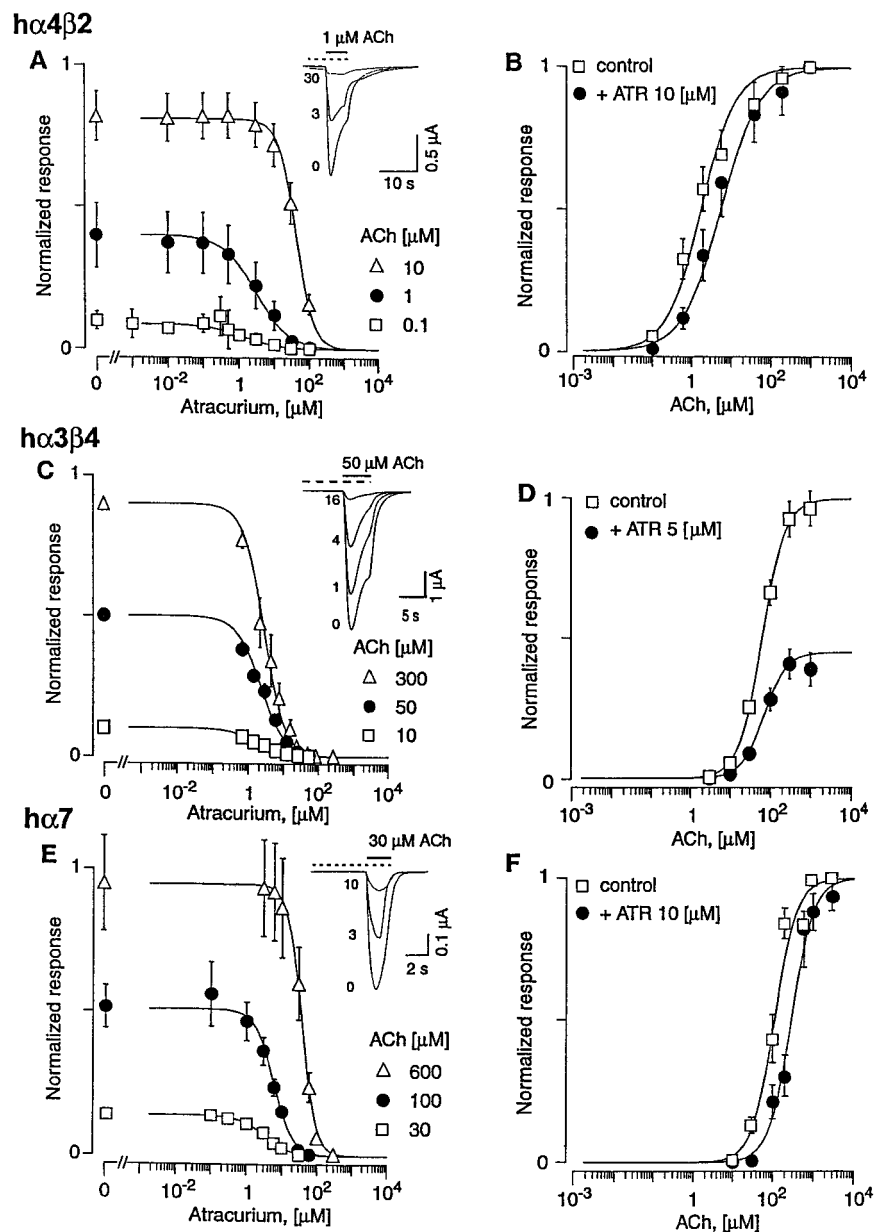


Fig. 2. Mode of action of atracurium inhibition. (Inset) Typical currents recorded for three atracurium concentrations are superimposed. Current amplitudes were scaled with respect to the ACh concentration–response curve. Currents in each cell were normalized to the value measured at 1 mM ACh and plotted on a semilogarithmic scale. The number of cells measured in each condition is indicated in table 1. Continuous lines through the data point are the best fits obtained with the Hill equations (equation 1) for the activation and (equation 2) for the inhibition (table 1).

Quantification of the atracurium inhibition at the $\alpha 7$ receptor with three ACh test pulse conditions indicates that, as for the $\alpha 4\beta 2$ nAChR, the IC_{50} progressively shifted toward the lower sensitivities as the agonist concentration was increased (fig. 2E, table 1). The IC_{50} dependency of the ACh concentration is indicative of the competitive mode of action of atracurium. This hypothesis was further reinforced by the observation that atracurium inhibition is fully overcome by an increase of the ACh concentration (fig. 2F). Because $\alpha 7$ is highly permeable to Ca^{2+} (its response may be contaminated by calcium-dependent chloride activation), atracurium concentration–response inhibitions were measured in a Ba^{2+} -containing medium, a condition that is known to reduce chloride activation.²⁷ Substitution of extracellular calcium by barium caused a small shift to the left of

the IC_{50} and slightly increased the EC_{50} (300–430 μM , data not show). This indicates that, even when present, chloride contamination plays a minor role in the atracurium blockade. The lower calcium permeability of $\alpha 4\beta 2$ or $\alpha 3\beta 4$ receptors would imply that calcium-dependent chloride contamination might also be neglected for these subtypes. Therefore, all further experiments were performed during normal divalent cation conditions.

Effects of Laudanosine on the Central or Ganglionic nAChRs

To isolate the effects of atracurium from those of laudanosine, experiments were performed using pure laudanosine. When the same experimental protocols as those presented in figure 2 were used, we found that

Table 1. Effects of Atracurium at $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 7$ nAChRs

Human cDNA	EC ₅₀ (μ M)	nH	[ACh] (μ M)	IC ₅₀ (μ M)	nH
$\alpha 4\beta 2$					
Control (n = 8)	1.8 \pm 1.2	0.97 \pm 0.16	0.1 (n = 17)	1.41 \pm 0.34	0.88 \pm 0.08
10 μ M Atracurium (n = 8)	5.8 \pm 4.1	0.87 \pm 0.38	1 (n = 7)	2.98 \pm 1.34	0.93 \pm 0.16
			10 (n = 17)	42.01 \pm 17.3	1.76 \pm 0.52
$\alpha 3\beta 4$					
Control (n = 11)	56.8 \pm 2.75	1.75 \pm 0.04	10 (n = 6)	2.48 \pm 0.48	1.2 \pm 0.07
5 μ M Atracurium (n = 7)	67.6 \pm 2.43	1.73 \pm 0.03	50 (n = 7)	3.17 \pm 0.45	1.33 \pm 0.07
			300 (n = 7)	3.87 \pm 0.4	1.56 \pm 0.1
$\alpha 3\beta 4\alpha 5$					
Control (n = 12)	51.8 \pm 5.70	1.62 \pm 0.08	10 (n = 6)	1.97 \pm 0.2	1.23 \pm 0.04
5 μ M Atracurium (n = 7)	85.8 \pm 10.3	1.61 \pm 0.05	50 (n = 7)	5.03 \pm 0.34	1.61 \pm 0.04
			300 (n = 7)	5.63 \pm 1.11	1.76 \pm 0.08
$\alpha 7$					
Control (n = 5)	108 \pm 17.9	1.56 \pm 0.13	30 (n = 7)	3.13 \pm 0.67	1.21 \pm 0.21
10 μ M Atracurium (n = 5)	284 \pm 66.4	1.56 \pm 0.13	100 (n = 15)	5.83 \pm 1.99	1.63 \pm 0.42
			600 (n = 7)	35.55 \pm 12.28	2.16 \pm 0.46

Acetylcholine (ACh) concentration of half activation (EC₅₀) measured in control and during atracurium exposures (10 or 5 μ M) are given. Mean and SD values corresponding to the best fits obtained with the empirical equation (1) are indicated. Atracurium concentration–response inhibition curves, measured as in figure 2A, were characterized with their respective concentration of half inhibition (IC₅₀) values. Mean and SD values obtained by curve-fitting with the Hill equation (2) are indicated. Numbers in parentheses indicate the number of cells tested in each condition. No statistical difference was observed in EC₅₀, IC₅₀, and Hill coefficient between $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$.

cDNA = complementary DNA; nAChR = neuronal nicotinic acetylcholine receptor; nH = apparent cooperativity.

laudanosine also inhibits the $\alpha 4\beta 2$, $\alpha 3\beta 4$, and $\alpha 7$ receptors (fig. 3, table 2).

Measurement of the fraction of ACh current inhibition at $\alpha 4\beta 2$ as a function of agonist concentration showed that laudanosine blockade was only partially removed by increasing the ACh concentration (fig. 3A). As for atracurium, adequate curve fitting was obtained with the Hill equations (equation 1), providing addition of a scaling factor of 0.65 to account for the laudanosine insurmountable blockade. These data show that, in contrast to atracurium, the mechanism of laudanosine blockade on the $\alpha 4\beta 2$ receptor is competitive, but this compound acts also in a noncompetitive manner for at least 35% of the blockade. Typical ACh-evoked currents recorded in control and during coapplication of laudanosine, are shown in figure 3B. Contrarily to atracurium, laudanosine alone caused no detectable signal. The small rebound observed at the end of the ACh–laudanosine application is compatible with mechanisms of open channel blockade. Concentration–response inhibition measured with a 0.1- μ M ACh test pulse yielded an IC₅₀ of 9.4 μ M (table 2). Here we used low ACh concentration to avoid rapid desensitization of this receptor.

A difference in the mode of action between atracurium and laudanosine was also identified at the ganglionic $\alpha 3\beta 4$ nAChR (figs. 3C and D). Figure 3C shows the dual mode of blockade caused by laudanosine with a shift in the ACh EC₅₀ (table 2) and an insurmountable blockade. Another difference was the inward currents observed during the prepulse of laudanosine alone (figs. 1B and 3D). An important rebound of current was observed at the end of the ACh–laudanosine application (fig. 3D). This rebound was observed in every cell tested. Concen-

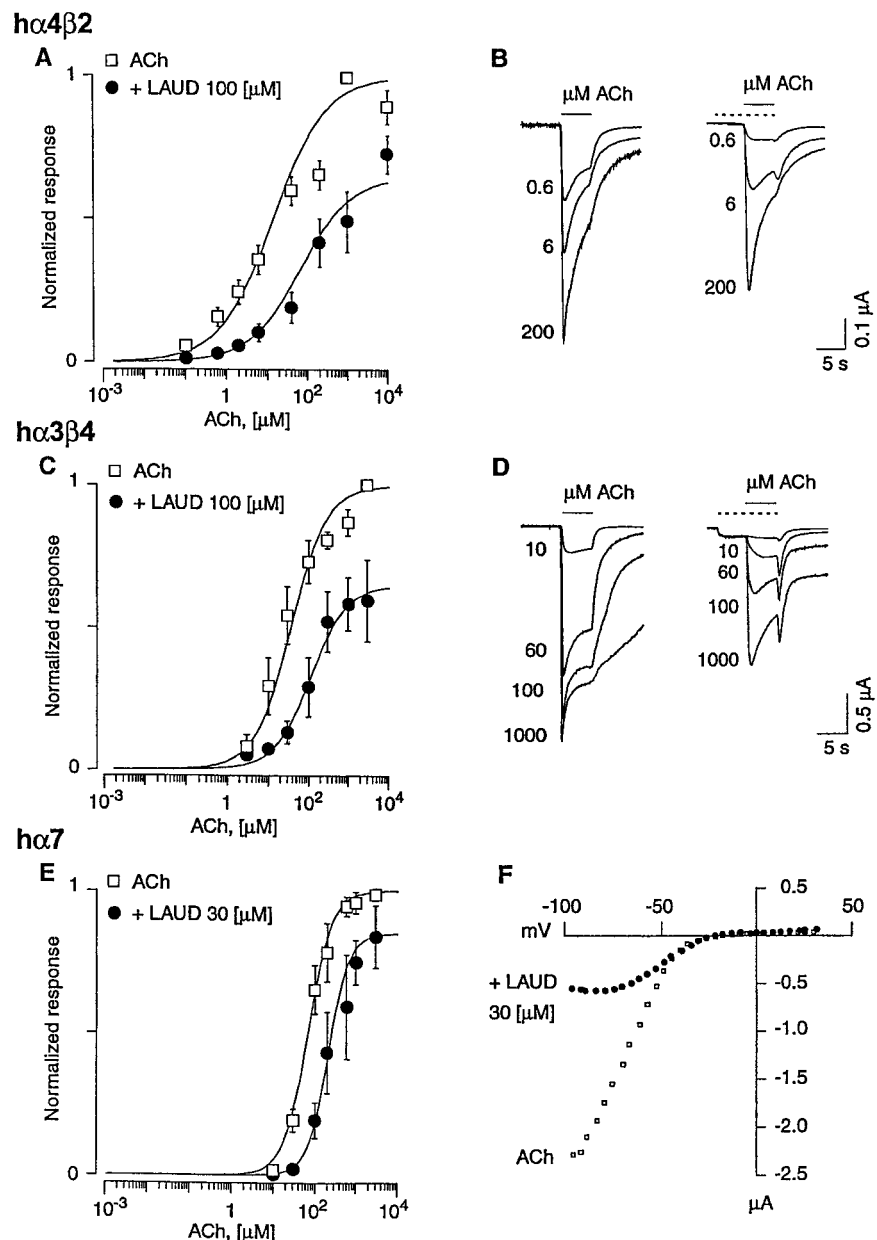
tration–response curves to laudanosine yielded an IC₅₀ of approximately 38 μ M for an ACh test pulse of 50 μ M (table 2).

A dual mode of action of laudanosine was also observed on the $\alpha 7$ receptor but with a smaller fraction of insurmountable blockade (fig. 3E). The Hill equation 1 was used with a scaling factor of 0.85, introduced into the curve fitting to account for this small fraction of blockade. The ACh EC₅₀ increased from 80 to 240 μ M during exposure to 30 μ M laudanosine (table 2). It is well-documented that when charged molecules enter and block the ionic pores of a ligand-gated channel, its fraction of blockade will depend on the transmembrane potential.^{28,29} Therefore, if laudanosine causes a blockade by steric hindrance in the channel pore, its inhibition may be voltage dependent. Typical current–voltage relations recorded in control and during laudanosine exposure showed a marked voltage dependency of laudanosine blockade (fig. 3F). The small rebound observed at the end of the ACh–laudanosine application is coherent with a mechanism of open channel blockade. Determination of the concentration–response inhibition profile with an ACh test pulse of a 100 μ M yielded an IC₅₀ of 18.3 μ M (table 2).

Discussion

Recent advances in molecular biology and DNA cloning have identified the nAChR subtypes expressed in various regions of the central and peripheral nervous system (reviewed in Bertrand and Changeux¹³ and Lindstrom *et al.*¹⁴). Central nicotinic receptors mainly con-

Fig. 3. Laudanosine is a competitive inhibitor and open channel blocker on neuronal nicotinic acetylcholine receptors (nAChRs). Concentration–response curves to ACh were determined for oocytes expressing the $\alpha 4\beta 2$ (A), $\alpha 3\beta 4$ (C), and $\alpha 7$ (E) nAChR in controls (open squares) and during pre- (5 s) and coapplication of a fixed concentration laudanosine (filled symbols). Currents measured in at least 6 cells (see also table 2) were averaged and normalized to the maximal value recorded in control conditions. Typical currents recorded in an oocyte expressing the $\alpha 4\beta 2$ (B) and $\alpha 3\beta 4$ (D) are shown. Responses evoked by a 5 s ACh application in control (left) or during laudanosine exposure (100 μM , right) are superimposed. Note the important rebound at the end of the coapplication of ACh and laudanosine. (F) $\alpha 7$ nAChR current–voltage relations recorded first in control conditions (open squares) and then during exposure to laudanosine (30 μM ; filled circles). Current–voltage relation was determined using a voltage ramp as described in Methods. The $\alpha 7$ receptor was activated by 100 μM ACh application.



tain the $\alpha 4$ and $\beta 2$ subunits, whereas ganglionic receptors result from the assembly of $\alpha 3$ and $\beta 4$. Coimmunoprecipitation experiments have shown that a fraction of ganglionic receptors also contain the $\alpha 5$ subunit.¹⁵ Finally, it has been shown that the homomeric $\alpha 7$ receptor is expressed centrally and peripherally. To evaluate the possible interaction between neuronal nAChRs and atracurium and its first degradation product laudanosine, these two substances were applied alone or with ACh on *Xenopus* oocytes expressing the human $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 3\beta 4\alpha 5$, and $\alpha 7$ receptors.

Incubation with atracurium or laudanosine caused a marked inhibition of these four receptor subtypes, with IC_{50} s in the micromolar range (tables 1 and 2). Blockade of the ACh-evoked current was fast, and complete recovery was obtained within 1 min, indicating rapid onset

and offset kinetics. In addition, as expected from data obtained on muscle nAChRs, atracurium and laudanosine effects are multiple: (1) competitive inhibition, (2) open channel blockade, and (3) activation of the receptor. The latter effect was observed only for the central $\alpha 4\beta 2$ and ganglionic $\alpha 3\beta 4$ receptors. It is noteworthy to recall that the $\alpha 5$ subunit does not contribute to the pharmacologic profile of the ganglionic receptor.³⁰ Therefore, it is not surprising that injection of this subunit caused no detectable changes in atracurium sensitivity of the $\alpha 3\beta 4$ receptor.

One of the difficulties in the study of the effects caused by atracurium is the instability of this product. It is well-documented that one molecule of atracurium quickly degrades into two laudanosine molecules. The Hoffman degradation of atracurium is independent of

Table 2. Effects of Laudanosine on $\alpha 4\beta 2$, $\alpha 3\beta 4$, and $\alpha 7$ nAChRs

Human cDNA	EC ₅₀ (μ M)	nH	[ACh] (μ M)	IC ₅₀ (μ M)	nH
$\alpha 4\beta 2$					
Control (n = 6)	14.1 \pm 7.47	0.66 \pm 0.10			
100 μ M Laudanosine (n = 6)	65.0 \pm 33.46	0.68 \pm 0.10	0.1 (n = 7)	9.41 \pm 2.23	1.1 \pm 0.14
$\alpha 3\beta 4$					
Control (n = 6)	36 \pm 20.73	0.98 \pm 0.13			
100 μ M Laudanosine (n = 6)	110 \pm 16.33	0.97 \pm 0.09	50 (n = 8)	38.37 \pm 15.28	1.56 \pm 0.27
$\alpha 7$					
Control (n = 11)	80.22 \pm 14.14	1.54 \pm 0.16			
30 μ M Laudanosine (n = 11)	239.8 \pm 86.48	1.78 \pm 0.27	100 (n = 7)	18.34 \pm 5.08	1.88 \pm 0.35

This table summarizes the concentration of half activation (EC₅₀) and concentration of half inhibition (IC₅₀) values obtained as in table 1, but for laudanosine. Values are expressed as mean and SD. The number of cells tested is indicated in parentheses.

cDNA = complementary DNA; nAChR = neuronal nicotinic acetylcholine receptor; nH = apparent cooperativity; ACh = acetylcholine.

biologic processes and therefore precludes an evaluation of the effects of atracurium alone. A few percent contamination of laudanosine in the atracurium solution cannot be excluded. A comparison of the atracurium and laudanosine effects is mandatory to understand their respective contribution. As shown in figures 2 and 3, where atracurium and laudanosine caused a comparable inhibition, a marked difference between these two compounds was observed when comparing the fraction of current they can activate. The major brain $\alpha 4\beta 2$ nAChR was activated by low atracurium concentrations but was unresponsive to laudanosine (data not shown). In contrast, the ganglionic $\alpha 3\beta 4$ nAChR was activated by laudanosine but almost unresponsive to atracurium. Plots of the ACh, atracurium, and laudanosine currents evoked by low concentrations of these three compounds on a log-log scale show that neuronal nAChRs are at least five to fifty times more sensitive to ACh than to curaremytics. At higher concentrations, atracurium and laudanosine inhibit the ACh-evoked currents. In the absence of significant differences between the $\alpha 3\beta 4$ and the $\alpha 3\beta 4\alpha 5$ receptor profiles and given the paucity of expression of these latter receptor subtypes, activation experiments were therefore not performed.

Plasma levels of atracurium range between 0.5 and 5.1 μ g/ml (0.4 and 4.1 μ M, respectively), with the lowest values found during surgical anaesthesia and the highest during intensive care conditions. The degradation of one atracurium molecule into two laudanosine molecules implies that a plasma concentration as high as 8 μ M can be reached for the latter compound. Considering these data, it was important to determine whether these compounds can be responsible for adverse effects by direct action on neuronal nicotinic receptors.

The major central nicotinic receptor $\alpha 4\beta 2$ was blocked by atracurium and laudanosine with an IC₅₀ of 1.4 and 9.4 μ M, respectively, when stimulated with 0.1 μ M ACh (tables 1 and 2). The ACh EC₅₀ was shifted toward higher concentration in the presence of a constant atracurium concentration (figs. 2B and 3B). The atracurium blockade was fully reversible by increasing

the ACh concentration, whereas an insurmountable block of approximately 35% persisted with laudanosine. This indicates differences in the mode of action, with these two compounds atracurium inducing a purely competitive blockade and laudanosine a mixed competitive and noncompetitive action. These data are in agreement with previous findings that showed that *d*-tubocurarine, a related chemical structure, is a competitive inhibitor on the chick $\alpha 4\beta 2$ nAChR with an IC₅₀ in the micromolar range.³¹ Evidence for open channel blockade is clearly seen with the rebounds observed at the end of ACh and laudanosine coapplication on the $\alpha 4\beta 2$ receptor (fig. 3B). The difference observed in EC₅₀ for the $\alpha 4\beta 2$ receptor between tables 1 and 2 is attributable to the use of different oocyte batches. A recent report showed that the neuronal nAChR concentration-response curves are best fitted using the two Hill equations.³² However, because of technical limitations, the number of points collected was restricted and does not allow for further conclusion.

Muscle relaxant drugs have been described to have adverse effects and, in the worst cases, can trigger seizures *in vitro* or in the animal model.^{10,11} Here we report that atracurium and laudanosine can block the major brain nicotinic receptor at concentrations that can be present in the plasma of patients. Recently mutations on the $\alpha 4$ subunit have been shown to induce autosomal dominant nocturnal epilepsy (reviewed in Steinlein³³). It follows that a modification of the $\alpha 4\beta 2$ receptor activity could be the origin of seizures. Even if these receptors are exclusively expressed in the brain and thus protected by the blood-brain barrier, it has been already shown that atracurium and laudanosine can be found in cerebrospinal fluid.^{8,9} This suggests that part of atracurium adverse effects may be caused through its action on the $\alpha 4\beta 2$ receptor.

A different mode of action was observed at the ganglionic $\alpha 3\beta 4$ nAChR with atracurium and laudanosine, causing a noncompetitive blockade. Note that, in agreement with the absence of effects of $\alpha 5$ on ganglionic receptor pharmacology, the addition of $\alpha 5$ caused no

detectable changes on the action of atracurium. The ganglionic $\alpha 3\beta 4$ receptor was inhibited by atracurium and laudanidine with an IC_{50} of 3.2 and 38.4 μM , respectively. In agreement with a noncompetitive blockade, half-inhibition was independent on the agonist test pulse concentration. Moreover, the addition of a constant inhibitor concentration induced a slight shift of the concentration-response curve toward higher ACh concentrations, but the maximal response measured in the presence of an antagonist was reduced by at least 35%. In addition, for laudanidine, an important rebound indicative of an open channel blockade was observed at the end of the test pulse. Results obtained with atracurium on the $\alpha 3\beta 4$ receptor suggest that this compound blocks these receptors by noncompetitive blockade. Finally, the atracurium IC_{50} of 3.2 μM observed in our conditions (table 1) is compatible with the 3 μM dissociation constant reported for *d*-tubocurarine blockade on rat ganglia.³⁴

Recalling that the blood-brain barrier does not isolate autonomic ganglia, it is conceivable that concentrations of atracurium and laudanidine comparable with plasma levels may be reached in their environment. Our data are in agreement with previous hypotheses that adverse cardiovascular effects may be attributed to the direct action of atracurium or laudanidine on cardiac ganglia.⁷

At the homomeric $\alpha 7$ receptor, atracurium acts as a competitive inhibitor. This was shown by the shift in the IC_{50} of the atracurium blockade in function of the ACh test pulse concentration and by the full relief of inhibition observed at saturating ACh. Therefore, as for the $\alpha 4\beta 2$ and $\alpha 3\beta 4$, it can be concluded that the effects caused by laudanidine contaminant on $\alpha 7$ are negligible. Previous studies of the blockade caused by *d*-tubocurarine have shown that this compound acts as a noncompetitive blocker on the chick receptor and that 0.5 μM was already sufficient to reduce by 40% the ACh evoked current.³⁵ Although initially different, these results and ours are not contradictory. The difference in the mode of action between atracurium and *d*-tubocurarine may be attributed to the difference in size between these two molecules. Moreover, experiments performed with the desensitized open L247T receptor have shown the dual mode of action of *d*-tubocurarine, with activation and blockade of this mutant.³⁶ Although it was proposed that, in some cases, perfusion conditions might affect the $\alpha 7$ responses in *Xenopus* oocytes,³⁷ we think that the agreement between our results and those obtained with the same cDNA expressed in human embryonic kidney cells is indicative of adequate experimental conditions. Therefore, no attempts were made to compensate ACh concentration-response curves, and raw data are presented herein.

The $\alpha 7$ receptor is expressed in both the central and the peripheral nervous systems (reviewed in Bertrand and Changeux¹³ and Lindstrom *et al.*¹⁴), but also in

nonneuronal cells such as the embryonic skeletal muscle cells. However, $\alpha 7$ has never been found in adult innervated muscle.³⁸⁻⁴⁰ According to these findings, we can conclude that muscular effect observed during atracurium treatment cannot be caused by interaction with $\alpha 7$ receptors. The atracurium and laudanidine IC_{50} values on this receptor were 3.1 and 18.3 μM , respectively. These data confirmed that atracurium and laudanidine in therapeutic conditions could block $\alpha 7$ receptors. Therefore, we cannot exclude that side effects observed during atracurium administration could be caused by a direct effect on these receptors.

In conclusion, we have shown that atracurium and laudanidine interact with neuronal nicotinic ACh receptors at concentrations that can be present in clinical conditions.

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References

- Fuchs-Buder T: New muscle relaxants. Update on mivacurium, rocuronium and cis-atracurium [German]. *Anaesthesist* 1997; 46:350-9
- Boyd AH, Eastwood NB, Parker CJ, Hunter JM: Comparison of the pharmacodynamics and pharmacokinetics of an infusion of cis-atracurium (51W89) or atracurium in critically ill patients undergoing mechanical ventilation in an intensive therapy unit. *Br J Anaesth* 1996; 76:382-8
- Grigore AM, Brusco L Jr, Kuroda M, Koorn R: Laudanidine and atracurium concentrations in a patient receiving long-term atracurium infusion. *Crit Care Med* 1998; 26:180-3
- Hughes R, Chapple DJ: The pharmacology of atracurium: A new competitive neuromuscular blocking agent. *Br J Anaesth* 1981; 53:31-44
- Goldberg M, Rosenberg H: New muscle relaxants in outpatient anaesthesia. *Dent Clin North Am* 1987; 31:117-29
- Foldes FF, Nagashima H, Boros M, Tassonyi E, Fitzal S, Agoston S: Muscular relaxation with atracurium, vecuronium and duador under balanced anaesthesia. *Br J Anaesth* 1983; 55:978-1035
- Ostergaard D, Engbaek J, Viby-Mogensen J: Adverse reactions and interactions of the neuromuscular blocking drugs. *Med Toxicol Adverse Drug Exp* 1989; 4:351-68
- Fahey MR, Canfell PC, Taboada T, Hosobuchi Y, Miller RD: Cerebrospinal fluid concentrations of laudanidine after administration of atracurium. *Br J Anaesth* 1990; 64:105-6
- Eddleston JM, Harper NJ, Pollard BJ, Edwards D, Gwinnutt CL: Concentrations of atracurium and laudanidine in cerebrospinal fluid and plasma during intracranial surgery. *Br J Anaesth* 1989; 63:525-30
- Cardone C, Szenohradszky J, Yost S, Bickler PE: Activation of brain acetylcholine receptors by neuromuscular blocking drugs. A possible mechanism of neurotoxicity. *ANESTHESIOLOGY* 1994; 80:1155-61;
- Szenohradszky J, Trevor AJ, Bickler P, Caldwell JE, Sharma ML, Rampil JJ, Miller RD: Central nervous system effects of intrathecal muscle relaxants in rats. *Anesth Analg* 1993; 76:1304-9
- Chiodini FC, Tassonyi E, Fuchs-Buder T, Fathi M, Bertrand D, Muller D: Effects of neuromuscular blocking agents on excitatory transmission and γ -aminobutyric acid_A-mediated inhibition in the rat hippocampal slice. *ANESTHESIOLOGY* 1998; 88:1003-13
- Bertrand D, Changeux JP: Nicotinic receptor: An allosteric protein specialized for intercellular communication. *Semin Neurosci* 1995; 7:75-90
- Lindstrom J, Anand R, Peng X, Gerzanich V, Wang F, Li Y: Neuronal nicotinic receptor subtypes. *Ann N Y Acad Sci* 1995; 757:100-16
- Conroy WG, Berg DK: Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. *J Biol Chem* 1995; 270:4424-31
- Seguela P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW: Molecular cloning, functional properties, and distribution of rat brain $\alpha 7$: A nicotinic cation channel highly permeable to calcium. *J Neurosci* 1993; 13:596-604
- Albuquerque EX, Pereira EF, Alkondon M, Schratzenholz A, Maelicke A: Nicotinic acetylcholine receptors on hippocampal neurons: distribution on the

neuronal surface and modulation of receptor activity. *J Recept Signal Transduct Res* 1997; 12:243-66

18. Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T, Ballivet M: A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. *Neuron* 1990; 5: 847-56
19. Bertrand D, Cooper E, Valera S, Rungger D, Ballivet M: Electrophysiology of neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes following nuclear injection of genes or cDNA, *Methods in Neuroscience*. Edited by Conn M. New York, Academic Press, 1991, pp 174-93
20. Del Castillo J, Katz B: A study of curare action with an electrical micro-method. *Proc Royal Soc (Lond)* 1957; B146:339-56
21. Adams PR: Drug blockade of open end-plate channels. *J Physiol (Lond)* 1976; 260:531-52
22. Colquhoun D, Sheridan RE: The effect of tubocurarine competition on the kinetics of agonist action on the nicotinic receptor. *Br J Pharmacol* 1982; 75:77-86
23. Buffer J, Wilhelm R, Parnas H, Franke C, Dudel J: Open channel and competitive block of the embryonic form of the nicotinic receptor of mouse myotubes by (+)-tubocurarine. *J Physiol (Lond)* 1996; 495:83-95
24. Trautmann A: Curare can open and block ionic channels associated with cholinergic receptors. *Nature* 1982; 298:272-5
25. Steinbach JH, Chen Q: Antagonist and partial agonist actions of d-tubocurarine at mammalian muscle acetylcholine receptors. *J Neurosci* 1995; 15:230-40
26. Fletcher GH, Steinbach JH: Ability of nondepolarizing neuromuscular blocking drugs to act as partial agonists at fetal and adult mouse muscle nicotinic receptors. *Mol Pharmacol* 1996; 49:938-47
27. Galzi JL, Devillers-Thiery A, Hussy N, Bertrand S, Changeux JP, Bertrand D: Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. *Nature* 1992; 359:500-5
28. Woodhull AM: Ionic blockage of sodium channels in nerve. *J Gen Physiol* 1973; 61:687-708
29. Buisson B, Bertrand D: Open-channel blockers at the human alpha4beta2 neuronal nicotinic acetylcholine receptor. *Mol Pharmacol* 1998; 53:555-63
30. Wang F, Gerzanich V, Wells GB, Anand R, Peng X, Keyser K, Lindstrom J: Assembly of human neuronal nicotinic receptor alpha5 subunits with alpha3, beta2, and beta4 subunits. *J Biol Chem* 1996; 271:17656-65
31. Bertrand D, Ballivet M, Rungger D: Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes. *Proc Natl Acad Sci U S A* 1990; 87:1993-7
32. Covernton PJO, Connolly JG: Multiple components in the agonist concentration-response relationships of neuronal nicotinic acetylcholine receptors. *J Neurosci Meth* 2000; 96: 63-70
33. Steinlein OK: Neuronal nicotinic receptors in human epilepsy [In Process Citation]. *Eur J Pharmacol* 2000; 393: 243-7
34. Fieber LA, Adams DJ: Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J Physiol (Lond)* 1991; 434: 215-37
35. Bertrand D, Bertrand S, Ballivet M: Pharmacological properties of the homomeric alpha 7 receptor. *Neurosci Lett* 1992; 146: 87-90
36. Bertrand D, Devillers-Thiery A, Revah F, Galzi JL, Hussy N, Mulle C, Bertrand S, Ballivet M, Changeux JP: Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain. *Proc Natl Acad Sci U S A* 1992; 89:1261-5
37. Papke RL, Thinschmidt JS: The correction of alpha7 nicotinic acetylcholine receptor concentration-response relationships in *Xenopus* oocytes. *Neurosci Lett* 1998; 256:163-6
38. Corriveau RA, Romano SJ, Conroy WG, Oliva L, Berg DK: Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development. *J Neurosci* 1995; 15:1372-83
39. Fischer U, Reinhardt S, Albuquerque EX, Maelicke A: Expression of functional alpha7 nicotinic acetylcholine receptor during mammalian muscle development and denervation. *Eur J Neurosci* 1999; 11:2856-64
40. Romano SJ, Pugh PC, McIntosh JM, Berg DK: Neuronal-type acetylcholine receptors and regulation of alpha 7 gene expression in vertebrate skeletal muscle. *J Neurobiol* 1997; 32:69-80