Stoichiometry of human recombinant neuronal nicotinic receptors containing the β3 subunit expressed in *Xenopus* oocytes

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(Received 18 April 2000; accepted after revision 30 August 2000)

1. The neuronal nicotinic subunit β3 forms functional receptors when co-expressed with both an α and a β subunit, such as α3 and β4. We examined the subunit stoichiometry of these 'triplet' α3β4β3 receptors by expression in *Xenopus* oocytes of the α3, β4 and β3 subunits, either in wild-type form or after insertion of a reporter mutation.

2. The mutation chosen was the substitution of a conserved hydrophobic residue in the second transmembrane domain of the subunits (leucine or valine 9′) with a hydrophilic threonine. In other ion channels within the nicotinic superfamily, this mutation type consistently increases the potency of agonists. In muscle-type nicotinic receptors, the magnitude of this effect is approximately constant for each mutant subunit incorporated.

3. In α3β4β3 receptors, the ACh EC_{50} was decreased by approximately 17-fold when this mutation was in α3 alone and only by fourfold when β3 alone was mutated. Mutating β4 was equivalent to mutating α3, suggesting that the 'triplet' receptor contains one copy of β3 and two copies each of α3 and β4.

4. Mutating β3 and α3 or β3 and β4 reduced the ACh EC_{50} further, to values two- to threefold lower than those seen when only α3 or β4 carried the mutation.

5. In 'pair' α3β4 receptors (known to contain two α and three β subunits), mutating β4 had a greater effect on the ACh EC_{50} than mutating α3, in agreement with an α:β ratio of 2:3 and a constant and independent effect of each copy of the mutation.

6. Our results suggest that α3β4β3 neuronal nicotinic receptors contain one copy of β3 and two copies each of α3 and β4 and confirm that in pair α3β4 receptors the α/β subunits are present in a 2:3 ratio.

The muscle-type nicotinic acetylcholine receptor (nAChR) is the best characterised of all ligand-gated ion channels. For this receptor, we know both its subunit composition (α, β, δ and ε in adult muscle) and subunit stoichiometry (two copies of α and one each of β, δ and ε). Despite the homology between neuronal and muscle receptor subunits and the likely similarity in the pentameric structures of the assembled muscle and neuronal receptors, the stoichiometry and subunit composition of neuronal nAChRs are still open questions. This is because we have to deal not with one, but with several distinct types of native neuronal nAChRs, and also because the number of combinations theoretically possible (starting from the twelve types of neuronal nicotinic subunits cloned, α2 to α10 and β2 to β4) is very high (McGehee & Role, 1995).

Some simplifying assumptions can be made, as only some of the possible combinations can form functional channels when expressed in heterologous systems; for instance, in *Xenopus* oocytes some α subunits (α7 to α9) can form homomeric channels, whereas most of the other α subunits (namely α2–α4 and α6) can form functional channels only if co-expressed with a β subunit (either β2 or β4). The latter type of receptor (which we shall refer to as a 'pair' receptor) is a pentamer with an α:β stoichiometry of 2:3, as shown by radiolabelling and single channel studies on chick α4β2 receptors expressed in oocytes (Anand *et al.* 1991; Cooper *et al.* 1991).

Nevertheless, extrapolating the 'either homomer or pair' rule to native receptors may be a serious over-simplification; the fact that one (or two) subunits are *sufficient* to produce a functional nicotinic receptor does not mean that native receptors are *necessarily* made in this minimally functional fashion, by just one or two types of subunit. For instance, the main synaptic nAChR in chick parasympathetic ciliary ganglia is made up of three or four different subunits, α3, β4 and α5 (with or

without $\beta 2$; Vernallis *et al.* 1993; Conroy & Berg, 1995); the situation is even more complex in chick sympathetic ganglia (Listerud *et al.* 1991; Yu & Role, 1998*a*,*b*). In the central nervous system, an example of complexity comes from cerebellar nAChRs, most of which contain three different β subunits, including $\beta 3$ (Forsayeth & Kobrin, 1997; for a review see Sivilotti *et al.* 2000). Furthermore, the simple homomer or pair scheme does not account for subunits such as $\alpha 5$ and $\beta 3$, which can only be assembled into functional receptors when co-expressed with another *two* different subunits, for instance as $\alpha 4\beta 2\alpha 5$ or $\alpha 3\beta 4\beta 3$ receptors (which we shall refer to as 'triplet' receptors). Incorporation of $\alpha 5$ into functional receptors may manifest itself by changes in the agonist sensitivity, single channel conductance or desensitisation properties of the pair nAChR upon co-expression of $\alpha 5$ in oocytes (Ramirez-Latorre *et al.* 1996; Sivilotti *et al.* 1997; Gerzanich *et al.* 1998; Nelson & Lindstrom, 1999). For $\beta 3$, we have recently shown that insertion of a reporter mutation in $\beta 3$ changes the sensitivity to ACh and the relative maximum response to nicotine of triplet $\alpha 3\beta 4\beta 3$ receptors (Groot-Kormelink *et al.* 1998).

Nothing is known of the stoichiometry of such triplet neuronal nAChRs. We now address this question for recombinant, oocyte-expressed $\alpha 3\beta 4 + \beta 3$ receptors by an extension of the reporter mutation technique that we used to show $\beta 3$ incorporation. The residue chosen for the mutation is the highly conserved leucine (Leu) approximately in the middle of the sequence of the pore-lining second transmembrane domain (TM2) of receptor subunits in the nicotinic superfamily; in the TM2 numbering of Miller (1989), this is the 9th residue downstream from the NH_2-terminal end of TM2 (i.e. 9′; see Fig. 1). The precise role of this residue is as yet controversial; it may provide a gate to the channel which would be kept shut by the hydrophobic interaction between the Leu residues (Unwin, 1993), or may be situated extracellularly to the gate (Akabas *et al.* 1994). Despite this uncertainty of the role of 9′ Leu, the effect of swapping the hydrophobic Leu with a more hydrophilic amino acid, such as serine (Ser) or threonine (Thr), is clear and remarkably consistent across the whole nicotinic superfamily, leading to a decrease in the agonist EC_{50} on the mutated receptor (Revah *et al.* 1991; Yakel *et al.* 1993). The magnitude of this increase in agonist potency depends on the number of mutated subunits incorporated into the muscle-type nAChR (Labarca *et al.* 1995; Filatov & White, 1995), each additional copy of the mutation making a similar contribution that is independent of which subunit carries the mutation. The independent and progressive effect of increasing the number of mutant subunits can be expected to make mutations in 9′ Leu useful for investigating subunit stoichiometry (see for instance Chang *et al.* 1996). As well as the decrease in agonist EC_{50}, there are other changes in receptor properties, namely a slowing of desensitisation (possibly due to a desensitised state becoming conducting) and an increase in spontaneous openings. The magnitude of these effects is also directly related to the number of 9′ Leu mutations carried by the receptor and to the nature of the mutation, being greater for a Leu to Ser than for a Leu to Thr mutation (Revah *et al.* 1991; Chang & Weiss, 1998, 1999).

We have examined the ACh sensitivity of $\alpha 3\beta 4 + \beta 3$ receptors containing mutated subunits and compared it with that of wild-type receptors. We chose to use a Leu to Thr mutation, as our previous work indicated that this mutation produced a sufficient shift in the dose–response curve (Groot-Kormelink *et al.* 1998). An additional advantage of using a Thr mutation is that it produced no observable increase in spontaneous openings. As the mutation was introduced in each of the different subunits or in combinations of more than one subunit, there was a marked, regular progression in the magnitude of the potency shift produced, suggesting that each copy of the mutation had an approximately constant and independent effect. Analysis of our results indicated that triplet receptors formed by $\alpha 3$ and $\beta 4$ plus $\beta 3$ contain one copy of $\beta 3$ and two copies each of $\alpha 3$ and $\beta 4$ and confirmed that in pair receptors the α/β subunits are present in a 2:3 ratio.

METHODS

Construction of cRNA for oocyte expression

cDNAs for the human $\alpha 3$, $\beta 3$ and $\beta 4$ (GenBank accession numbers Y08418, Y08417 and Y08416, respectively), containing only coding sequences and an added Kozak consensus sequence (GCCACC) immediately upstream of the start codon (Groot-Kormelink & Luyten, 1997), were subcloned into the pSP64GL vector, which contains 5′ and 3′ untranslated *Xenopus* β-globin regions (Akopian *et al.* 1996). Constucts mutated in 9′ ($\alpha 3^{Leu279Thr}$, $\beta 3^{Val273Leu}$, $\beta 3^{Val273Thr}$ and $\beta 4^{Leu272Thr}$, where Val stands for valine) were created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and their full-length sequence verified. These mutants shall be referred to hereafter as $\alpha 3^{LT}$, $\beta 3^{VL}$, $\beta 3^{VT}$ and $\beta 4^{LT}$, respectively. All cDNA/pSP64GL plasmids were linearised immediately downstream of the 3′ untranslated β-globin sequence, and cRNA was transcribed using the SP6 Mmessage Mmachine kit (Ambion). cRNA quality and quantity were checked by gel-electrophoresis and comparison with RNA concentration and size markers.

Xenopus oocyte preparation and electrophysiological recording

Female *Xenopus laevis* frogs were anaesthetised by immersion in neutralised ethyl *m*-aminobenzoate solution (tricaine, methane-sulphonate salt; 0.2% solution weight/volume; Sigma Chemical Co.), and killed by decapitation and destruction of the brain and spinal cord (in accordance with Home Office guidelines) before removing the ovarian lobes. Clumps of stage V–VI oocytes were dissected in a sterile modified Barth's solution of composition (mM): NaCl 88; KCl 1; $MgCl_2$ 0.82; $CaCl_2$ 0.77; $NaHCO_3$ 2.4; Tris-HCl 15; with 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin; pH 7.4 adjusted with NaOH. The dissected oocytes were treated with collagenase (type IA, Sigma Chemical Co; 65 min at 18°C, 245 collagen digestion units ml^{-1} in Barth's solution, 10–12 oocytes per ml), rinsed, stored at 4°C overnight, and manually defolliculated the following day before cRNA injection (46 nl per oocyte). The oocytes were incubated for approximately 60 h at 18°C in Barth's solution containing 5% heat-

inactivated horse serum (Gibco BRL; Quick & Lester, 1994), then stored at 4°C. Experiments were carried out at a room temperature of 18–20°C between 2.5 and 14 days from injection.

cRNA was injected at a ratio of 1:1 in order to express $\alpha3\beta4$ receptors, and at a ratio of 1:1:20 ($\alpha3$:$\beta4$:$\beta3$) in order to express $\alpha3\beta4\beta3$ receptors. We previously found (Groot-Kormelink *et al.* 1998) that when a 1:1:1 ratio of cRNA was used for the $\alpha3\beta4$ and $\beta3$ subunits, the major part of the agonist-induced current was carried through $\beta3$-containing receptors; when a 1:1:20 ratio was used, the proportion of current carried through non-$\beta3$-containing receptors was too low to be quantified by fitting multiple-component Hill curves. Consequently, in the present study we chose a 1:1:20 ratio for triplet expression. The amount of cRNA to be injected (in 46 nl of RNase-free water) for each combination was determined empirically, with the aim of achieving a maximum ACh-evoked current of 1.5–2 μA. The maximum amount of cRNA injected per oocyte was 5–10 ng (wild-type combinations and triplet combinations containing more than one Thr-mutant subunit and the $\alpha3\beta4\beta3^{VT}$ combination). Lower amounts were sufficient for other combinations (0.25–1 ng).

Oocytes, held in a 0.2 ml bath, were perfused at 4.5 ml min^{-1} with modified Ringer solution (mM: NaCl 150, KCl 2.8, Hepes 10, $MgCl_2$ 2, 0.5 μM atropine sulphate, Sigma Chemical Co.; pH 7.2 adjusted with NaOH) and voltage clamped at −70 mV, using the two-electrode clamp mode of an Axoclamp-2B amplifier (Axon Instruments). Electrodes were pulled from Clark borosilicate glass GC150TF (Warner Instrument Corp) and filled with 3 M KCl. The electrode resistance was 0.5–1 MΩ on the current-passing side. The bath was grounded via an Ag–AgCl pellet. Experiments were terminated if the total holding current exceeded 2 μA, in order to reduce series resistance errors; the typical grounding and access resistance of our recording conditions (< 1 kΩ) would cause a worst-case clamp error smaller than 2 mV. We chose a nominally Ca^{2+}-free solution in order to minimise the contribution of Ca^{2+}-gated chloride conductance which is endogenous to the *Xenopus* oocyte and may be activated by Ca^{2+} entry through the neuronal nicotinic channels (see for an example Sands *et al.* 1993).

The agonist solution (acetylcholine chloride, Sigma Chemical Co.; freshly prepared from frozen stock aliquots) was applied via the bath perfusion for a period sufficient to obtain a stable plateau response (at low concentrations) or the beginning of a sag after a peak (at the higher concentrations); the resulting inward current was recorded on a flat bed chart recorder (Kipp & Zonen) for later analysis. A minimum interval of 5 min was allowed between ACh applications, as this was found to be sufficient to ensure reproducible responses. In order to compensate for possible decreases in agonist sensitivity throughout the experiment, a standard concentration of ACh (approximately EC_{20} for the particular combination used) was applied every third response. The experiment was started only after checking that this standard concentration gave reproducible responses. The average changes in the response to this ACh standard concentration observed by the end of the experiment for the different combinations ranged from a 46 ± 6% decrease ($\alpha3^{LT}\beta4$) to a 19 ± 11% increase ($\alpha3\beta4^{LT}\beta3^{VT}$; data expressed as a percentage of the initial response). All the data shown in the paper are compensated for the response rundown; however, we found that applying this compensation did not affect the conclusions of our work, as the results of analysing the original data (without compensation) were similar (data not shown).

Experimental controls

A series of control experiments showed that injection of one subunit alone (wild-type or mutant, namely $\alpha3$, $\alpha3^{LT}$, $\beta3$, $\beta3^{VL}$, $\beta3^{VT}$, $\beta4$, $\beta4^{LT}$) or of pair combinations of $\beta3$ with either $\alpha3$ ($\alpha3\beta3$, $\alpha3\beta3^{VL}$, $\alpha3\beta3^{VT}$, $\alpha3^{LT}\beta3$, $\alpha3^{LT}\beta3^{VL}$, $\alpha3^{LT}\beta3^{VT}$) or $\beta4$ ($\beta4\beta3$, $\beta4\beta3^{VL}$, $\beta4\beta3^{VT}$, $\beta4^{LT}\beta3$, $\beta4^{LT}\beta3^{VL}$, $\beta4^{LT}\beta3^{VT}$) did not result in the expression of functional nAChRs (i.e. current responses to 1 mM ACh were less than 5 nA; $n = 5$–8 for each combination tested; total cRNA injected 10 ng; 1:1 ratio for the pairwise combinations).

Holding current in oocytes expressing mutant receptors

The introduction of 9′ hydrophilic mutations into receptors of the nicotinic superfamily can increase the rate of spontaneous openings, i.e. openings of the channel in the absence of agonist (Chang & Weiss, 1998, 1999). We failed to observe such an effect: there was no significant increase in the average oocyte holding current (I_H) at the start of the experiment (Table 1) with the introduction of progressively higher numbers of mutant subunits. This lack of correlation between holding current and putative number of mutated subunits per receptor was maintained even if the holding current was normalised to the maximum agonist-induced current in each oocyte (data not shown). Clearly application of a selective channel blocker (or an inverse agonist if available) is the only reliable way to measure the proportion of holding current caused by spontaneous nAChR channel openings, especially against a background of variability in holding current between one oocyte and the next and between different batches of oocytes. Nevertheless, it would seem that any effect on spontaneous channel openings of the introduction of up to three 9′ hydrophilic substitutions is too small to be detected in the *Xenopus* oocyte system.

Curve fitting

All dose–response curves were fitted with the Hill equation:

$$I = I_{max}\frac{[A]^{n_H}}{[A]^{n_H} + EC_{50}{}^{n_H}}, \quad (1)$$

where I is the response, measured at its peak, $[A]$ is the agonist concentration, I_{max} is the maximum response, EC_{50} is the agonist concentration for 50% maximum response and n_H is the Hill coefficient. We used least squares fitting by the program CVFIT, (courtesy of D. Colquhoun and I. Vais, available from http://www.ucl.ac.uk/pharmacology/dc.html).

Fitting was done in stages, as follows. Each dose–response curve was fitted separately, individual responses being equally weighted, in order to obtain estimates for I_{max}, EC_{50} and n_H. The means and standard deviation of the mean for each combination are shown in Table 1.

Each response in a particular oocyte was then normalised to the fitted I_{max} for that experiment; all the normalised responses for a given combination were then pooled, giving the data points shown by the symbols in *B* and *C* of Figs 2–4. The pooled normalised data points were then fitted again with the Hill equation (with weight given by the reciprocal of their variance), either in a free fit (see Figs 2*C*, 3*C* and 4*C*) or with the curves constrained to be parallel (i.e. to have equal Hill slopes, displayed in Figs 2*B*, 3*B* and 4*B*). The constrained fits to the normalised pooled experiments were used to estimate the horizontal distance between the dose–response curves (i.e. the dose ratio) obtained from receptors carrying different 9′ mutations (Table 1). Dose ratios were expressed relative to either triplet or pair receptors which had only Leu in 9′ (which therefore had dose ratios of 1; as the dose–response curves for the mutant receptors were shifted to the left, dose ratios for the mutant receptors were greater than 1). These constrained fits allowed the estimation of 2.01-unit likelihood intervals for the dose ratios (or the EC_{50} for the two reference combinations, $\alpha3\beta4$ and $\alpha3\beta4\beta3^{VL}$); these correspond to confidence limits (± 2 standard deviations) for a normally distributed variable (Colquhoun & Sigworth, 1995).

Table 1. Effects of 9′ mutations in different subunits on the concentration–response curve to ACh

	EC_{50} (μM)	I_{max} (nA)	n_H	Dose ratio	Square root of dose ratio	Cube root of dose ratio	I_H (nA)	n
$\alpha3\beta4\beta3^{wt}$	242.7 ± 44.1	1333 ± 373	1.42 ± 0.1	—	—	—	300 ± 83.4	6
$\alpha3\beta4\beta3^{VL}$	163.1 ± 13.4 [159.5–189.8]	305.7 ± 69.3	1.47 ± 0.08	1	—	—	230 ± 63.1	4
$\alpha3\beta4\beta3^{VT}$	43.4 ± 2.2	240.9 ± 41.3	1.28 ± 0.04	4.12 [3.77–4.52]	—	—	175 ± 23.5	10
$\alpha3^{LT}\beta4\beta3^{VL}$	10.5 ± 1.4	993 ± 157	1.14 ± 0.06	17.1 [14.9–19.7]	4.14 [3.86–4.44]	—	148 ± 56.3	4
$\alpha3\beta4^{LT}\beta3^{VL}$	9.54 ± 2.1	963.6 ± 107.1	1.12 ± 0.06	21.9 [18.6–25.6]	4.68 [4.31–5.06]	—	90 ± 15.8	4
$\alpha3^{LT}\beta4\beta3^{VT}$	4.49 ± 0.37	1866 ± 276	1.13 ± 0.04	41.6 [37.6–46.0]	—	3.46 [3.35–3.58]	80 ± 20.4	4
$\alpha3\beta4^{LT}\beta3^{VT}$	3.22 ± 0.53	592 ± 177	0.94 ± 0.04	50.3 [43.7–58.0]	—	3.69 [3.52–3.87]	83 ± 23	4
$\alpha3\beta4^{wt}$	180 ± 17.0 [163.6–193.9]	1430 ± 425	1.81 ± 0.09	1	—	—	184 ± 57.2	7
$\alpha3^{LT}\beta4$	5.8 ± 1.0	1043 ± 411	1.15 ± 0.08	37.3 [31.2–44.6]	6.11 [5.58–6.68]	—	85 ± 28.7	6
$\alpha3\beta4^{LT}$	0.75 ± 0.05	562 ± 264	0.92 ± 0.08	292 [267–318]	—	6.63 [6.44–6.83]	200 ± 83	4

EC_{50}, I_{max} and n_H are the means (± S.D. of the mean) of parameter estimates obtained by fitting separately each concentration–response curve with a Hill equation. Dose ratios were estimated from fits in which curves were constrained to have the same slope (Figs 2*B*, 3*B* and 4*B*) and are expressed in relation to receptors containing 5 leucines in 9′ (which consequently have dose ratio values of 1). The same parallel fits allowed estimation of 2.01-unit likelihood intervals (in square brackets under the means of the parameters they refer to; see Methods): these are equivalent to 95% confidence intervals. The superscript wt stands for the wild-type.

RESULTS

$\alpha3\beta4\beta3$: comparing $\beta3^{VL}$ with $\beta3^{wt}$

In all subunits belonging to the nicotinic superfamily, the midpoint (9′) residue in the second transmembrane domain is highly conserved, being occupied by a hydrophobic amino acid. This is a Leu in all vertebrate nicotinic subunits, except for $\beta3$ and $\alpha5$, which have a Val in 9′ (position 273 for $\beta3$; see Fig. 1). In homomeric receptors formed by subunits which have Leu in 9′, introducing a Val in this position increases agonist sensitivity, slows desensitisation (see for homomeric $\alpha7$ nAChR, Revah *et al.* 1991) and increases spontaneous openings (for $GABA_C$ $\rho1$ receptors, see Chang & Weiss, 1998). Such effects of a Leu to Val mutation in 9′ are smaller, but similar to those of the Leu to Thr mutation we planned to use in our investigation. Additionally, the crucial assumption for the interpretation of our experiments in terms of stoichiometry is that the main determinant for the effect of the Thr mutations on

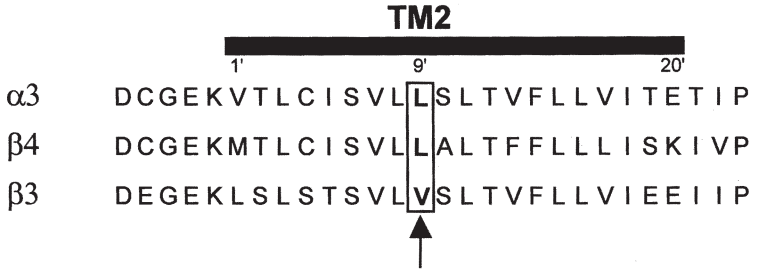

Figure 1. Sequences of the TM2 region for the human neuronal nicotinic $\alpha3$, $\beta4$ and $\beta3$ subunits

In this alignment of the TM2 region the conserved hydrophobic residue in 9′ (Leu or Val) is boxed and marked by an arrow. The numbering of the TM2 domain follows Miller (1989).

agonist EC_{50} should be their copy number in the assembled receptor complex. This assumption is validated by data obtained for receptors that have only Leu in the 9′ position (such as muscle nAChR, see Labarca *et al.* 1995; see also our data on the $\alpha3\beta4$ receptor below and the Discussion). It was therefore considered possible that the presence of one or more non-Leu residues in the 9′ position (which would be contributed by wild-type $\beta3$ subunits to the assembled triplet receptor) might confound the effect of progressively introducing 9′ Thr in the different subunits. This would reduce the sensitivity of our experimental design. In order to avoid this potential problem, we introduced a Val273Leu (i.e. V9′L) mutation in $\beta3$, and used the $\beta3^{VL}$ rather than $\beta3^{wt}$ in all the experiments (unless otherwise specified).

The ACh sensitivity of $\alpha3\beta4\beta3^{wt}$ receptors (see Groot-Kormelink *et al.* 1998) was compared with that of $\alpha3\beta4\beta3^{VL}$ receptors and – contrary to expectations – found to be consistently increased in $\alpha3\beta4\beta3^{VL}$ receptors (see Table 1). As the decrease in the EC_{50} of ACh was relatively small (from 242.7 ± 44.1 to 163.1 ± 13.4 μM), it was still thought preferable to use $\beta3^{VL}$ rather than $\beta3^{wt}$ in the work described below, in order to introduce subunits carrying the 9′ Thr mutation into receptors which only had Leu in the 9′ position.

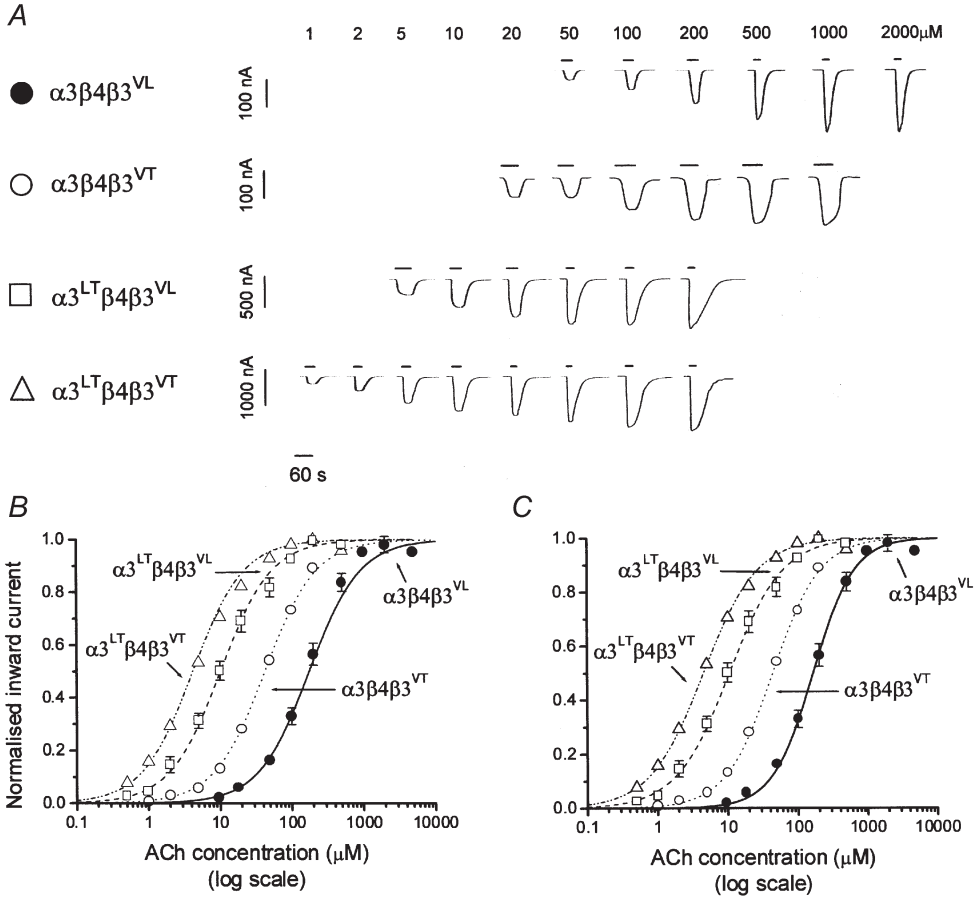

Figure 2. Agonist sensitivity of 'triplet' $\alpha3\beta4\beta3$ nicotinic receptors containing 9′ Thr mutations in $\alpha3$ and/or $\beta3$

A, examples of inward currents elicited by a representative range of ACh concentrations (see values above the application bars) in oocytes expressing $\alpha3\beta4\beta3^{VL}$, $\alpha3\beta4\beta3^{VT}$, $\alpha3^{LT}\beta4\beta3^{VL}$ or $\alpha3^{LT}\beta4\beta3^{VT}$. ACh was bath-applied to oocytes clamped to a holding potential of −70 mV. *B* and *C* show ACh concentration–response curves pooled from experiments such as the ones shown in *A* ($n = 4$–10 oocytes): each response was normalised to the maximum current evoked by ACh in that oocyte (see Methods). Pooled normalised results were fitted with the Hill equation either as a free fit (*C*) or under the constraint of equal slopes (*B*) in order to estimate the horizontal distance between the curves as a dose ratio (see Table 1). The concentration–response curves shown refer to oocytes injected with $\alpha3\beta4\beta3^{VL}$ (●), $\alpha3\beta4\beta3^{VT}$ (○), $\alpha3^{LT}\beta4\beta3^{VL}$ (□) and $\alpha3^{LT}\beta4\beta3^{VT}$ (△); bars show standard deviation of the mean (when larger than the symbol). Note the progressive increase in the potency of ACh as the 9′ Thr mutation was introduced in $\beta3$, $\alpha3$ alone or $\alpha3$ and $\beta3$.

Mutating α3 has a greater effect than mutating β3 in α3β4β3 receptors

Figure 2 show the result of introducing the 9′ Thr mutation in either the β3 or the α3 subunits. Figure 2*A* shows representative responses elicited by a range of ACh concentrations in oocytes injected with the different combinations: the concentration–response curves are shown in Fig. 2*B* and *C*. Figure 2*B* shows fits in which the Hill slope was constrained to be the same for all curves: these were used to obtain estimates of the distance between the curves and the 2-unit likelihood intervals for the EC_{50} values (expressed in Table 1 as dose ratios, see Methods).

As we previously reported (Groot-Kormelink *et al.* 1998), the introduction of $\beta3^{VT}$ shifted the concentration–response curve of ACh for the triplet $\alpha3\beta4\beta3^{VT}$ receptor to the left, relative to the receptor containing $\beta3^{wt}$. When the triplet containing $\beta3^{VT}$ is compared to the triplet containing only Leu in 9′ (i.e. $\alpha3\beta4\beta3^{VL}$), the shift is by a factor of approximately 4 (see Table 1).

The effect of introducing the mutation in α3, rather than in β3, was considerably larger, producing a further fourfold increase in the potency of ACh on $\alpha3^{LT}\beta4\beta3^{VL}$ receptors compared to $\alpha3\beta4\beta3^{VT}$ receptors. When $\alpha3^{LT}\beta4\beta3^{VL}$ receptors were compared to receptors with only Leu in 9′ ($\alpha3\beta4\beta3^{VL}$), they were found to be 17-fold more sensitive to ACh.

If both β3 and α3 carried the 9′ Thr mutation, the concentration–response curve for ACh shifted even further to the left, with an additional decrease in the ACh EC_{50} of about threefold. Thus the introduction of $\beta3^{VT}$ alone, $\alpha3^{LT}$ alone and $\alpha3^{LT}$ together with $\beta3^{VT}$ into the $\alpha3\beta4\beta3^{VL}$ receptor produced a progressive change in ACh sensitivity, with stepwise three- to fourfold increases.

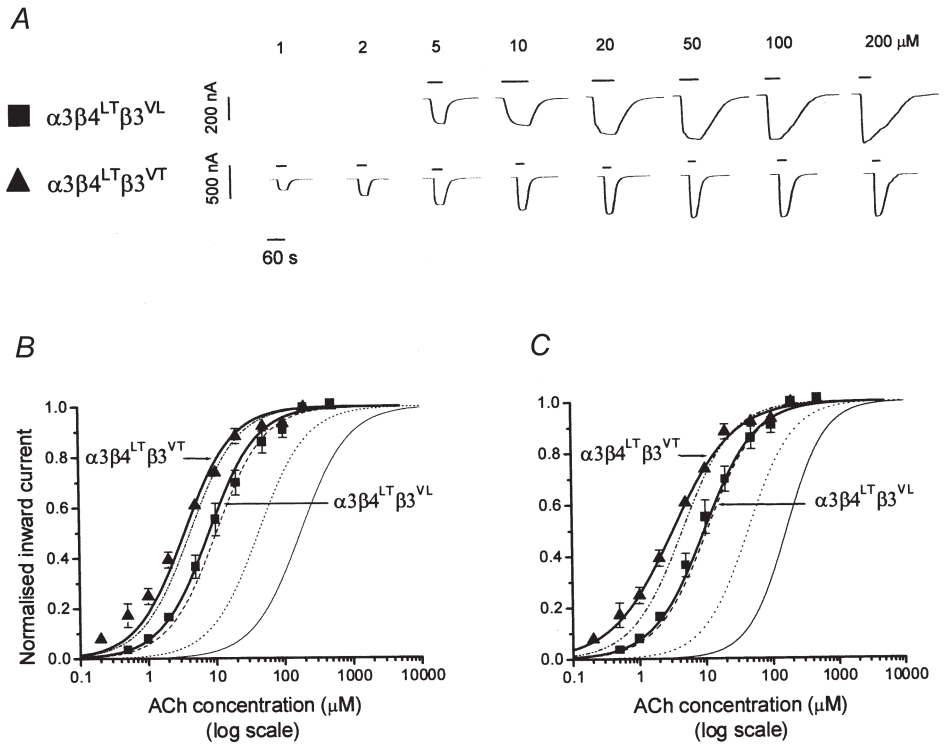

Figure 3. Agonist sensitivity of 'triplet' α3β4β3 nicotinic receptors containing 9′ Thr mutations in β4 and/or β3

A, examples of inward currents elicited by a representative range of ACh concentrations in oocytes expressing $\alpha3\beta4^{LT}\beta3^{VL}$ or $\alpha3\beta4^{LT}\beta3^{VT}$. ACh was bath-applied to oocytes clamped at −70 mV. *B* and *C* show ACh concentration–response curves pooled from experiments such as the ones shown in *A* ($n = 4$ oocytes): each response was normalised to the maximum current evoked by ACh in that oocyte (see Methods). Pooled normalised results were fitted with the Hill equation either as free fit (*C*) or under the constraint of equal slopes (*B*) in order to estimate the horizontal distance between the curves as a dose ratio (see Table 1). The concentration–response curves shown refer to oocytes injected with $\alpha3\beta4^{LT}\beta3^{VL}$ (■) or $\alpha3\beta4^{LT}\beta3^{VT}$ (▲). *B* and *C* also show for reference (curves with no symbols, reproduced from Fig. 2) the concentration–response curves relating to 9′ Thr mutations in α3 and/or β3. Note that the effect of mutating β4 is similar to that of mutating α3.

Mutating $\beta4$ is equivalent to mutating $\alpha3$ in $\alpha3\beta4\beta3$ receptors

If the $\alpha3\beta4\beta3$ receptor contains two copies of $\alpha3$ and one of $\beta3$, it follows that the receptor should also contain two copies of $\beta4$, in order to make up the total subunit number of five. This would predict that mutating $\beta4$ should have the same effect as mutating $\alpha3$. This question was addressed by the next set of experiments, which are shown in Fig. 3.

Introducing $\beta4^{LT}$ (alone or with $\beta3^{VT}$) in the $\alpha3\beta4\beta3^{VL}$ receptor had the same effect as introducing $\alpha3^{LT}$; the concentration–response curves for the $\beta4^{LT}$-containing receptors (Fig. 3*B* and *C*) were very close to those for the $\alpha3^{LT}$-containing receptors (shown for reference as dashed curves in Fig. 3*B* and *C*); indeed the 2-unit likelihood intervals for the EC_{50} values (Table 1) overlap both for $\alpha3^{LT}$ or $\beta4^{LT}$ alone and for $\alpha3^{LT}$ or $\beta4^{LT}$ together with $\beta3^{VT}$. Again the simplest explanation for these results is that $\alpha3\beta4\beta3$ receptors contain two copies of $\alpha3$ and $\beta4$, but only one copy of the $\beta3$ subunit.

Mutating $\beta4$ is not equivalent to mutating $\alpha3$ in $\alpha3\beta4$ receptors

Further confirmation that the effect of the mutation on ACh sensitivity only depends on the number of mutant subunits in a nicotinic receptor comes from another series of experiments. Here the reporter mutation 9′ Thr was introduced in either subunit of pair $\alpha3\beta4$ receptors. The rationale for this is that pair neuronal nicotinic receptors are known to contain α:β subunits in a 2:3 ratio (see for chick $\alpha4\beta2$ receptors, Anand *et al.* 1991; Cooper *et al.* 1991). If our hypothesis is correct and the effect of the mutation is independent of which subunit type carries it,

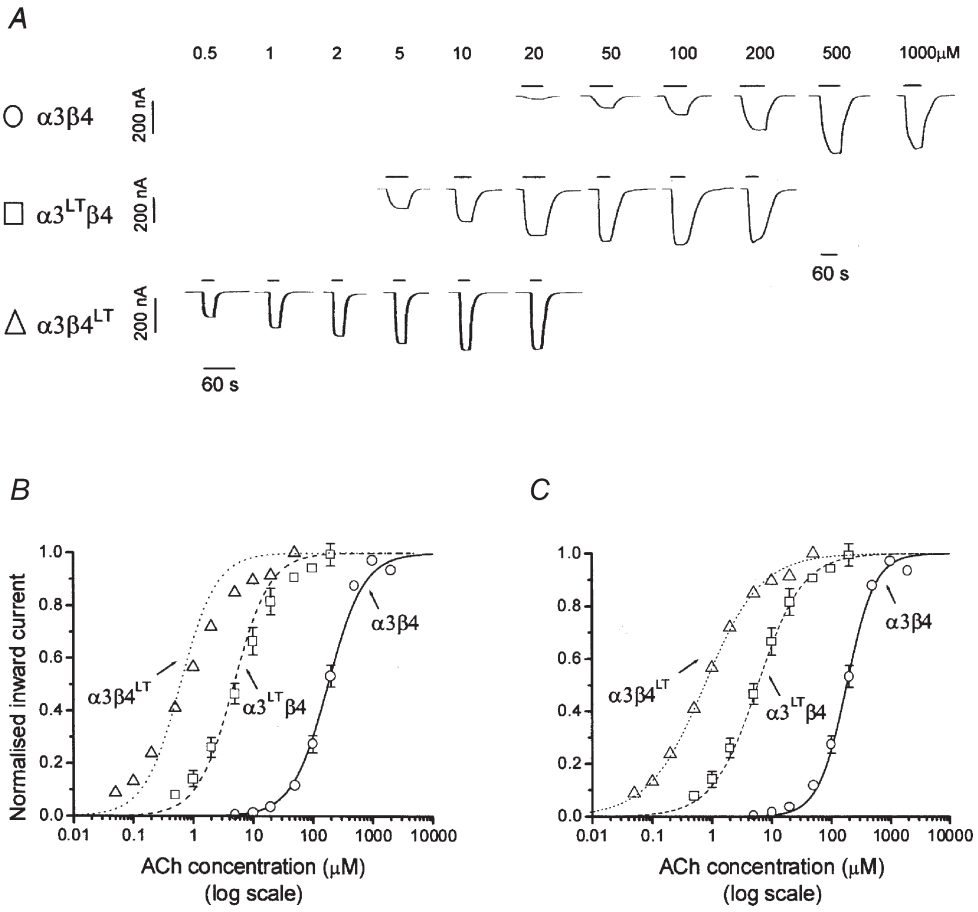

Figure 4. Agonist sensitivity of 'pair' $\alpha3\beta4$ nicotinic receptors containing 9′ Thr mutations in $\alpha3$ or $\beta4$

A, examples of inward currents elicited by bath-applied ACh in oocytes expressing $\alpha3\beta4$, $\alpha3^{LT}\beta4$ or $\alpha3\beta4^{LT}$. *B* and *C* show ACh concentration–response curves pooled from experiments such as the ones shown in *A* ($n = 4$–7 oocytes): each response was normalised to the maximum current evoked by ACh in that oocyte (see Methods). Pooled normalised results were fitted with the Hill equation either as free fit (*C*) or under the constraint of equal slopes (*B*) in order to estimate the horizontal distance between the curves as a dose ratio (see Table 1). The concentration–response curves refer to oocytes injected with $\alpha3\beta4$ (○), $\alpha3^{LT}\beta4$ (□) or $\alpha3\beta4^{LT}$ (△). Note that the greater increase in ACh sensitivity was produced by mutating $\beta4$.

the increase in ACh potency (compared with the wild-type pair receptors) should be greater when the mutation is introduced in $\beta4$ than when the mutation is introduced in $\alpha3$. This is indeed what was found, as shown in Fig. 4; $\alpha3\beta4^{LT}$ receptors were about sixfold more sensitive to ACh than $\alpha3^{LT}\beta4$ receptors, which in turn were 37-fold more sensitive than $\alpha3\beta4$ receptors (see dose ratio values in Table 1). This is consistent with the receptor containing one more copy of $\beta4$ than $\alpha3$, i.e. three copies of $\beta4$ to two copies of $\alpha3$ in the pentameric $\alpha3\beta4$ receptor.

If the effect of each hydrophobic to hydrophilic substitution in 9′ is independent of position and multiplicative on the ACh EC_{50} (i.e. additive in terms of free energy), then plotting the logarithm of the ACh EC_{50} against the presumed number of mutations in each pentamer channel should yield a linear plot. Figure 5 shows that this is indeed the case both for the $\alpha3\beta4$ pair (Fig. 5*B*) and for the $\alpha3\beta4\beta3$ triplet receptors (Fig. 5*A*), both of which show an approximately linear relation between log EC_{50} and the putative number of mutations in the receptor. Figure 5 also shows clearly that the effect of the mutation did not saturate up to a presumed mutation copy number of three (note, however, the apparent shallowing of the effect in the triplet receptor passing from two to three mutations).

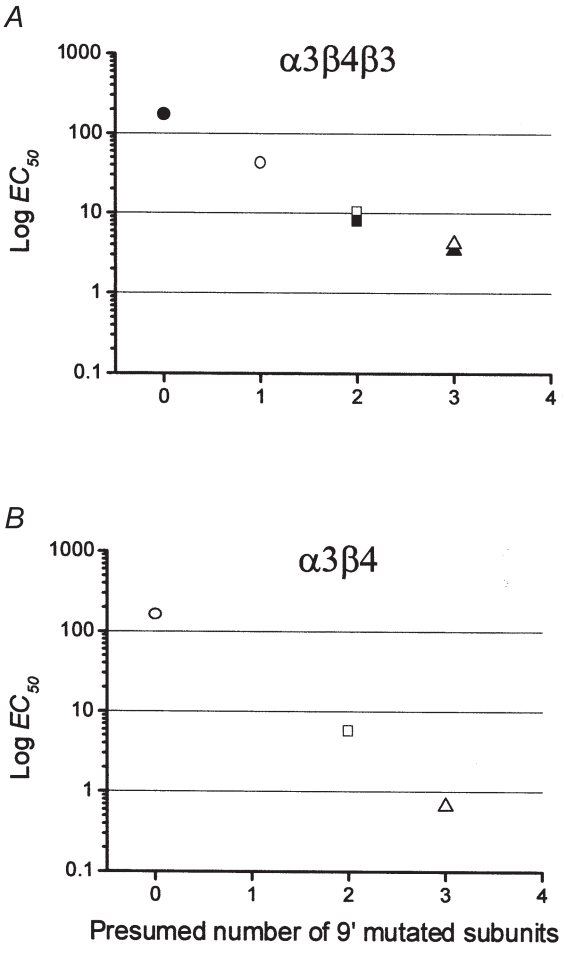

Figure 5. Relation between the ACh EC_{50} of the different combinations and their putative number of 9′ Thr mutations

EC_{50} values are plotted on a log scale against the number of mutant subunits in each combination. This number was calculated on the basis of a subunit stoichiometry of 2:2:1 for the triplet $\alpha3\beta4\beta3$ (*A*) and 2:3 for the pair $\alpha3\beta4$ (*B*). Symbols as in Figs 2–4. Note the approximate linearity of the decline in EC_{50} for both types of receptor.

DISCUSSION

The data presented above show that introducing a hydrophobic to hydrophilic mutation in the 9′ position of TM2 of neuronal nicotinic subunits increases the agonist sensitivity of the resulting nAChR. When the receptor expressed is an $\alpha3\beta4\beta3$ triplet, mutations in $\alpha3$ or $\beta4$ are equivalent in effect, and they shift the ACh dose–response curve to the left by an amount $\log(r)$ (where r is the dose ratio of Table 1) that is approximately twice the shift observed if $\beta3$ is mutated. If the mutation is inserted in *both* the $\beta3$ *and* the $\alpha3$ (or $\beta4$) subunits, the magnitude of the resulting $\log(r)$ shift is approximately the sum of the shifts caused by each mutation separately. These results suggest that, in the triplet nAChR $\alpha3\beta4\beta3$, the $\alpha3$, $\beta4$ and $\beta3$ subunits are present in a 2:2:1 ratio.

Assumptions of the reporter mutation approach

The first condition for the validity of our interpretation of the data is that the reporter mutation should not affect the correct assembly and therefore the subunit stoichiometry of the neuronal nAChR. The reporter mutation we chose is in TM2, i.e. in the pore-lining region. Mutations in this area are not expected to influence assembly directly, since the sequence determinants for subunit assembly are not in the pore-lining region, but in the NH_2-terminal domain preceding the first transmembrane domain (Gu *et al.* 1991; Yu & Hall, 1991; Sumikawa, 1992; Kreienkamp *et al.* 1995). An indirect, long-range effect of a channel mutation on the assembly cassette cannot in principle be excluded, but would appear to be unlikely, as no such effect was reported by the numerous studies which have mutated TM2 residues in order to establish which amino acids are accessible from the open or closed pore and how these residues influence nAChR channel conductance and ionic permeability. For instance, if muscle nAChR carrying 9′ mutations had a stoichiometry different from the normal (2:1:1:1 for $\alpha:\beta:\gamma:\delta$, respectively), detectable changes in single-channel conductance would be expected, because of the differences in the TM2 domains of the α, β, γ, δ-subunits (see Imoto *et al.* 1988): these are not seen when 9′ is mutated to Ser, Thr or isomers of Leu (Labarca *et al.* 1995; Filatov & White, 1995; Kearney *et al.* 1996). Evidence that the 9′ Thr mutation does not significantly affect the ligand-binding site is provided by the observation that

the binding of the competitive antagonist (+)-tubocurarine to recombinant muscle nAChRs is not affected by introducing the 9′ Thr mutation in the γ-subunit (Filatov & White, 1995).

In order to be able to interpret our results reliably, in terms of subunit stoichiometry, other conditions must also be verified. In particular, the overall effect of the reporter mutation must depend only on the number of mutations incorporated in the channel, not on the type of subunit that carries the mutation. Secondly, the relation between number of mutants and magnitude of the observed change in ACh potency must be a simple one, the ideal case being that the effect of each additional mutation is independent of the number of mutations already in the channel. Thirdly, the receptors considered must have uniform stoichiometry.

Comparison with other receptors in the nicotinic superfamily

These criteria have been shown to hold true for the muscle nAChR: in this receptor (which has a stoichiometry of 2:1:1:1 for α:β:γ:δ, respectively) the introduction of a progressively greater number of 9′ Ser or 9′ Thr mutations has a consistent effect on ACh EC_{50}, an effect which is independent and similar for each additional mutation. Each Ser in 9′ produced roughly a 10-fold shift in EC_{50}: there was no overlap between the range of EC_{50} observed for combinations with the different mutation copy numbers (Labarca *et al.* 1995). In a further analysis of muscle nAChR, Kearney *et al.* (1996) have investigated the lack of full subunit equivalence (probably due to asymmetry in the pore and interactions between adjacent subunits) by introducing in 9′ a wide range of unnatural amino acid residues with progressive increments in side-chain length or branching.

The situation appears quite different for the $GABA_A$ receptor. Introduction of 9′ Leu to Ser mutations in $\alpha1\beta2\gamma2$ $GABA_A$ recombinant receptors is very effective in increasing the receptor sensitivity to GABA, but the effectiveness of each mutation depends on which subunit type carries it. Consequently, stoichiometry could not be deduced from the EC_{50} shifts directly, but had to be confirmed by ascertaining how many dose–response components result from injecting mixtures of cRNA for the wild-type and mutant subunits (Chang *et al.* 1996). In $GABA_A$ receptors, a further complication is that the shift in EC_{50} saturates when more than two subunits carry the mutation (Chang & Weiss, 1999).

There is another confounding factor, which in principle may impinge differently on different receptors (and perhaps explain some of the discrepancies observed). The linearity and multiplicativity of the effect of each mutated subunit that we observed would be expected if the mutation produces linearly additive changes in the free energy of one or more steps of channel activation. That is because free energy values are related to the logarithm of rate (and equilibrium) constants. However, 9′ hydrophilic mutations are also known to affect desensitisation: changes in desensitisation should also alter the observed agonist EC_{50} to an extent which may depend on the impact of desensitisation on the wild-type dose–response curve. Since the extent to which desensitisation affects an agonist dose–response curve is difficult to determine macroscopically, this additional action of the mutation could make the final effect of the mutation unpredictable and estimates of stoichiometry unreliable.

Neuronal nicotinic receptor stoichiometry

There is little evidence to tell us whether the effects of 9′ hydrophilic mutations on neuronal nAChR are predictable like those described for muscle nAChR or resemble those described for $GABA_A$ receptors. 9′ hydrophilic TM2 mutations in a neuronal nAChR have been extensively investigated (see Revah *et al.* 1991) and found to enhance the potency of ACh on $\alpha7$ receptors. As recombinant $\alpha7$ receptors are homomeric, these findings cannot address the relation between the magnitude of the shift and the number of mutated subunits. We have therefore ourselves obtained corroborating evidence that in neuronal nAChR the effect of each copy of the 9′ mutation can be approximated as being independent and equivalent. First of all, we have introduced the reporter mutation in pair nAChR; a similar pair combination (chick $\alpha4\beta2$) is the only neuronal nicotinic receptor whose stoichiometry has been investigated and is known to contain α and β subunits in a 2:3 ratio (Anand *et al.* 1991; Cooper *et al.* 1991). It would seem reasonable to assume that pair combinations of related subunits have the same stoichiometry, irrespective of the species from which they were cloned. We found that in $\alpha3\beta4$ receptors, the increase in ACh potency produced by mutating α (which we term $r_{\alpha3}$) is 37.3-fold (see dose ratio values in Table 1), whereas the increase in ACh potency produced by mutating β ($r_{\beta4}$) is 292-fold (see Table 1). It can therefore be seen that:

$$(r_{\alpha3})^{1/2} \approx (r_{\beta4})^{1/3}.$$

The actual values observed were $(37.3)^{1/2} = 6.11$ and $(292)^{1/3} = 6.63$, respectively, in good agreement with the prediction. This is what we expect if the effect of each copy of the reporter mutation is equivalent, irrespective of whether it is carried by the α or the β subunits. The graphical counterpart of the numerical example above is the near-linearity of Fig. 5*B*, which shows the plot of the log(EC_{50}) for ACh against the putative number of mutant subunits, calculated on the basis of a subunit stoichiometry of 2:3 for $\alpha3\beta4$.

Further evidence that each additional mutation has a similar effect in neuronal nAChR (irrespective of the number of mutations already incorporated in the pore) comes from another set of our experiments. In $\alpha3\beta4\beta3$ triplets the combined effect of a mutation in $\beta3$ together

with a mutation in either $\alpha 3$ or $\beta 4$ is approximately equal to the product of the effect of the two mutations alone (again see the linearity of plot in Fig. 5*A*).

Neuronal nicotinic receptors commonly display multiple conductance levels (Papke, 1993). In native receptors different levels are linked by direct transitions and therefore are likely to be produced by different states of the same molecular complex (Sivilotti *et al.* 1997). An alternative explanation is that each level is produced by a receptor with a different stoichiometry; this could account for the differences in conductances observed for the rat $\alpha 2\beta 2$ combination expressed in oocytes using 1:9 and 9:1 α to β ratios (Papke *et al.* 1989). It is not known whether such receptor mosaics are produced by the combinations we studied; conclusive evidence on this point could come from single-channel recording and the analysis of sublevel transitions within bursts or within clusters of openings separated by desensitised periods. We have, however, assessed the likely effect of the presence of different receptor stoichiometries in the pair receptor by modelling the dose–response relation of such complexes (assuming only that the EC_{50} is determined by the number of 9′ Thr mutations in the receptor complex). While modelling suggests that such receptor heterogeneity could explain the shallower Hill slope observed in mutant receptors (see below), it also predicted that in this case the EC_{50} shift produced by mutating $\alpha 3$ should be similar to that produced by mutating $\beta 4$. This is in clear contrast with our findings that the $\alpha 3\beta 4^{LT}$ receptor complex is approximately sixfold more sensitive to ACh than $\alpha 3^{LT}\beta 4$ (Fig. 4*B* and *C*).

These observations substantiate our conclusions that the subunit stoichiometry in the $\alpha 3\beta 4\beta 3$ triplet is indeed 2:2:1. This stoichiometry fits in well with what is known of non-homomeric neuronal nAChRs, namely that their pentameric structure contains at least two agonist binding sites, each requiring the presence of an α subunit; as in the muscle receptor (Colquhoun & Sakmann, 1985), it is likely that both binding sites must be occupied for channel opening to occur effectively. The presence of at least two binding sites (which on average must be occupied for the channel to open) is in good accord with the observation of a Hill slope greater than 1 (1.42 for the wild-type triplet). It is unlikely that $\beta 3$ can play the role of an α subunit with respect to agonist binding, given that the sequence of the $\beta 3$ presumed binding domains is very similar to that of the other β subunits.

The slope of the ACh concentration–response curves in mutant receptors

The greatest shifts in agonist sensitivity (i.e. those observed in $\alpha 3^{LT}\beta 4\beta 3^{VT}$, $\alpha 3\beta 4^{LT}\beta 3^{VT}$ and $\alpha 3\beta 4^{LT}$) were associated with a decrease in the slope of the concentration–response curves. Such changes often result from receptor heterogeneity (the EC_{50} values for two receptor types have to differ by something approaching 100-fold before the presence of two components becomes detectable in dose–response curves, if the Hill coefficient values are similar to those observed for this type of receptor). While this explanation cannot be discounted in principle for our data, it is difficult to see why receptor heterogeneity should increase with the increase in the number of mutant subunits in the receptor, particularly since the decrease in slope is not limited to the triplet receptors, but can be observed for the $\alpha 3\beta 4$ pair receptors (see the $\alpha 3\beta 4^{LT}$ curve, Fig. 4*C*). Another more likely explanation is that the slope decreases because monoliganded openings contribute more current as the proportion of mutant subunits in the receptor rises. Monoliganded openings are known to occur in muscle nicotinic receptors (although they are relatively rare at all but the lowest concentrations, Colquhoun & Sakmann, 1985); their contribution to the total current can be expected to increase with mutation number, if the reporter mutation does indeed facilitate gating. A receptor which needs the binding of only a single agonist molecule to open can be expected to give rise to an equilibrium dose–response curve with a slope of 1. Hence, a greater contribution of monoliganded openings can be expected to make the curve shallower. Similar effects were observed by Chang & Weiss (1999) in $GABA_A$ receptors.

Relevance to native nAChR

For our finding on the stoichiometry of recombinant $\beta 3$-containing receptors to be relevant to native nAChR, two questions must be answered; firstly whether we can extrapolate from the oocyte expression system to a mammalian neurone and secondly whether the $\beta 3$ subunit is incorporated into native nAChR together with the $\alpha 3$ and $\beta 4$ subunits.

On the first point, oocytes do express muscle-type nAChR with an accurate native stoichiometry (Anand *et al.* 1991). No comparative data are available for neuronal nAChR; nevertheless, the fact that the $\alpha 4\beta 2$ nAChR expressed in oocytes contains two copies of the α subunit (Cooper *et al.* 1991) does suggest that recombinant heteromeric nAChRs contain two agonist binding sites. The presence of two binding sites in the oocyte-expressed receptor is in agreement with the fact that Hill slope values measured for native nAChR indicate that on average at least two agonist molecules need to bind in order for the channel to open (Covernton *et al.* 1994). At single-channel level most of oocyte-expressed $\alpha 3\beta 4$ nAChRs differ in conductance and kinetics from native ganglionic receptors (Sivilotti *et al.* 1997; Lewis *et al.* 1997; but see Nelson & Lindstrom, 1999). We assume here that whatever it may be that causes discrepancies in conductances and kinetics does not have a major effect on relative potencies or stoichiometry. In support of this view, we have found that, at whole-cell level, oocytes do reproduce the relative agonist potencies seen for native nicotinic receptors with reasonable accuracy (Covernton *et al.* 1994).

Receptors that contain the $\alpha3$ and $\beta4$ subunits are thought to be mainly expressed in the peripheral nervous system; co-assembly of these two subunits into the same receptor complex has been proved by immunoprecipitation in the chick ciliary ganglion (Vernallis *et al.* 1993; Conroy & Berg, 1995) and in adult rat trigeminal ganglia (Flores *et al.* 1996). In rat trigeminal ganglia mRNA for the $\beta3$ subunit is also present (Liu *et al.* 1998; for a review see McGehee & Role, 1995). In the central nervous system, receptors containing the $\alpha4$ and $\beta2$ subunits are likely to play a major role, but this is not exclusive. Indeed, mRNA for the $\alpha3$, $\beta4$ and $\beta3$ subunits has been detected in the rat brainstem, i.e. interpeduncular nucleus, thalamus, habenula (in particular in the ventromedial medial habenula, Le Novère *et al.* 1996; for a pharmacological analysis of these receptors see Quick *et al.* 1999) and in the locus coeruleus (Léna *et al.* 1999). Coincidence of expression of the mRNA for these subunits doesn't necessarily imply that they co-assemble into functional receptors, but unfortunately there are few studies examining this issue at protein level. The best evidence comes from the recent demonstration by sequential immunopurification (Vailati *et al.* 2000) that, in chick retina, the $\beta3$ subunit does assemble with a subset of subunits which include $\alpha3$ and $\beta4$. The expression of $\beta3$ protein has also been found to be widespread in the rat central nervous system (cerebral cortex, striatum, hippocampus, cerebellum and medulla, Forsayeth & Kobrin, 1997). In addition, immunoprecipitation of nicotinic receptors from the rat cerebellum reveals the presence of a receptor subpopulation containing *four* different subunits, including $\beta3$, namely $\alpha4\beta2\beta3\beta4$ (Forsayeth & Kobrin, 1997). Note that receptors that contain four different subunits are known to form in the chick ciliary ganglion (Conroy *et al.* 1995).

Although there is good evidence that $\beta3$-containing nAChRs exist in native tissue, it is not yet known whether these native receptors could be triplets (as the recombinant receptors we investigated), i.e. whether they contain only three different subunits instead of, for instance, four (as is the case for muscle). Nevertheless, the presence in triplet receptors of only one copy of $\beta3$ (which replaces one of the three $\beta4$ subunits in the pair receptors), does argue for the existence of constraints to the stoichiometry of neuronal nAChR, which may extend to 'quadruplet' receptors, particularly with regard to the ratio of α to β subunits.

In summary, our results confirm a 2:3 ratio for $\alpha3\beta4$ nAChR receptors and indicate that only one copy of $\beta3$ is present in $\alpha3\beta4\beta3$ receptors. Bearing in mind the sequence similarity between $\beta3$ and $\alpha5$, which has led to their inclusion in the same subunit tribe (Corringer *et al.* 2000), it will be important to test whether a 2:2:1 stoichiometry applies more generally, namely to all triplet neuronal nAChRs formed by the assembly of one subunit from tribe III-1 (i.e. $\alpha3$, but also $\alpha2$, $\alpha4$ or $\alpha6$), one subunit from tribe III-2 (such as $\beta4$ or $\beta2$) and one subunit from tribe III-3 ($\beta3$ or $\alpha5$). While our data do not shed light on the topology of $\beta3$-containing receptors, knowledge of subunit stoichiometry is essential to the investigation of what other $\beta3$-containing combinations are possible and of how $\beta3$ contributes to the nAChR structure and function.

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Acknowledgements

This work was supported by the Wellcome Trust (grant 055524), the Royal Society and the Millennium fund of the School of Pharmacy.

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