

Incorporation of the $\beta 3$ Subunit Has a Dominant-Negative Effect on the Function of Recombinant Central-Type Neuronal Nicotinic Receptors

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ABSTRACT

The $\beta 3$ neuronal nicotinic subunit is localized in dopaminergic areas of the central nervous system, in which many other neuronal nicotinic subunits are expressed. So far, $\beta 3$ has only been shown to form functional receptors when expressed together with the $\alpha 3$ and $\beta 4$ subunits. We have systematically tested in *Xenopus laevis* oocytes the effects of coexpressing human $\beta 3$ with every pairwise functional combination of neuronal nicotinic subunits likely to be relevant to the central nervous system. Expression of $\alpha 7$ homomers or α/β pairs ($\alpha 2$, $\alpha 3$, $\alpha 4$, or $\alpha 6$ together with $\beta 2$ or $\beta 4$) produced robust nicotinic currents for all combinations, save $\alpha 6\beta 2$ and $\alpha 6\beta 4$. Coexpression of wild-type $\beta 3$ led to a nearly complete loss of function (measured as maximum current response to acetylcholine) for $\alpha 7$ and for all functional α/β pairs except for $\alpha 3\beta 4$. This effect

was also seen in hippocampal neurons in culture, which lost their robust $\alpha 7$ -like responses when transfected with $\beta 3$. The level of surface expression of nicotinic binding sites ($\alpha 3\beta 4$, $\alpha 4\beta 2$, and $\alpha 7$) in tsA201 cells was only marginally affected by $\beta 3$ expression. Furthermore, the dominant-negative effect of $\beta 3$ was abolished by a valine-serine mutation in the 9' position of the second transmembrane domain of $\beta 3$, a mutation believed to facilitate channel gating. Our results show that incorporation of $\beta 3$ into neuronal nicotinic receptors other than $\alpha 3\beta 4$ has a powerful dominant-negative effect, probably due to impairment in gating. This raises the possibility of a novel regulatory role for the $\beta 3$ subunit on neuronal nicotinic signaling in the central nervous system.

The function of the $\beta 3$ subunit has been a puzzle since it was cloned more than 15 years ago (Deneris et al., 1989). $\beta 3$ is present in substantia nigra, ventral tegmentum, and medial habenula (Deneris et al., 1989), and it is incorporated into nicotinic ACh receptors (nAChRs) in the cerebellum (Forsayeth and Kobrin, 1997), retina (Vailati et al., 2000), and striatum (Zoli et al., 2002), as shown by immunoprecipitation. In the striatum, $\beta 3$ is restricted to nAChRs on dopaminergic terminals (Zoli et al., 2002). Data from $\beta 3$ -null mutant mice suggest that this is a distinct receptor population that binds α -conotoxin MII with high affinity (Cui et al., 2003).

Despite these indications that $\beta 3$ -containing receptors

have a specific role, possibly in locomotor control, we know little of the effects of $\beta 3$ incorporation into functional nAChRs. $\beta 3$ does not form functional nAChRs when expressed with a "typical" α ($\alpha 2$ – $\alpha 4$) or with a β -type subunit ($\beta 2$ or $\beta 4$) (Deneris et al., 1989; Boorman et al., 2000; Colquhoun et al., 2003), but it only incorporates into nAChRs as the third subunit of a "triplet" (i.e., in receptors that also contain a "typical" α and a "typical" β subunit) (Groot-Kormelink et al., 1998). In *Xenopus laevis* oocytes, $\beta 3$ does assemble together with $\alpha 3$ and $\beta 4$ into a functional receptor that contains two copies each of $\alpha 3$ and $\beta 4$ and one of $\beta 3$ (Boorman et al., 2000). The presence of $\beta 3$ is hard to detect, because only single-channel conductance and kinetics are affected (Boorman et al., 2003). Because of the relatively restricted expression of the $\alpha 3$ and $\beta 4$ subunits in the central nervous system, this combination ($\alpha 3\beta 4 + \beta 3$) is likely to be relevant only to habenular nAChRs (Sheffield et al., 2000), and other subunit combinations may predominate in most areas that express $\beta 3$, such as substantia nigra and the ventral tegmentum area. Here, a wide subset of nicotinic

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; P_{open} , channel open probability; CNS, central nervous system; ANOVA, analysis of variance; MLA, methyllycaconitine.

subunits is present in a manner typical of many CNS areas (Le Novère et al., 1996; Azam et al., 2002).

So far, there are few detailed studies in the literature testing whether the β3 subunit can form functional receptors with combinations other than α3β4 and, if so, how it changes the properties of the receptor. One exception is that of α7: it has been reported that β3 forms “silent” heteromeric receptors with α7 subunits (Palma et al., 1999). There is also a brief report that β3 may change the macroscopic time course of α3β2 currents (McIntosh et al., 2000), but a detailed study of α3β2β3 receptors is not available in the literature.

We systematically examined β3 incorporation by coexpression in oocytes and found that β3 profoundly reduces responses produced by all pairwise functional nAChR subunit combinations known to date, with the exception of α3β4. This dominant-negative effect is not due to a change in receptor numbers and disappears when a gain-of-function mutant β3 is expressed rather than wild-type β3. This, together with the evidence for β3 incorporation, suggests that β3 reduces the maximum receptor open probability (P_{open}). This effect of β3 was also observed in the native α7-like nAChRs of hippocampal neurons in culture. Expression of β3 can exert a novel effect on neuronal nAChRs, which results in the functional down-regulation of responses from receptors that incorporate this subunit.

Materials and Methods

Human Neuronal Nicotinic Subunit cDNAs. Sequences for nicotinic subunits are as deposited in GenBank, with accession numbers Y16281 (α2), Y08418 (α3), Y08421 (α4), Y08419 (α5), Y16282 (α6), Y08420 (α7), Y08415 (β2), Y08417 (β3), and Y08416 (β4). Subunits contained only coding sequences and an added Kozak consensus sequence (GCCACC) immediately upstream of the start codon and were subcloned into pcDNA3.1 (Invitrogen, Breda, The Netherlands) or pSP64GL (for oocyte expression). Mutations were introduced using the QuikChange Kit (Stratagene, Amsterdam, The Netherlands), and full-length sequence was verified. All pSP64GL plasmids were linearized immediately downstream of the 3'-untranslated β-globin sequence. Capped cRNA was transcribed using the SP6 mMessage mMachine Kit (Ambion, Cambridge, UK) and checked by RNA electrophoresis.

Two-Electrode Voltage-Clamp Recording of *X. laevis* Oocytes. Female *X. laevis* frogs were anesthetized by immersion in neutralized ethyl *m*-aminobenzoate solution (tricaine, methanesulfonate salt; 0.2% solution, w/v) and were killed by decapitation and destruction of the brain and spinal cord (in accordance with Home Office guidelines) before removal of ovarian lobes to sterile modified Barth's solution consisting of 88 mM NaCl, 1 mM KCl, 0.82 mM MgCl₂, 0.77 mM CaCl₂, 2.4 mM NaHCO₃, and 15 mM Tris-HCl, with 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Paisley, UK); pH 7.4 was adjusted with NaOH. Mature oocytes were manually defolliculated after collagenase IA treatment (Boorman et al., 2000) before cRNA was injected at a ratio of 1:1 for pair receptors and 1:1:20 for triplet receptors. The total amount of cRNA for each combination was determined empirically, with the aim of achieving a maximum ACh-evoked current of 1 to 2 μA and was 0.25 to 10 ng per oocyte, depending on the combination.

Oocytes, held in a 0.2-ml bath, were perfused at 4.5 ml/min with modified, nominally Ca²⁺-free Ringer solution (150 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 2 mM MgCl₂, and 0.5 μM atropine sulfate, pH 7.2, adjusted with NaOH; 18–20°C) and voltage-clamped at –70 mV using the two-electrode clamp mode of an Axoclamp-2B amplifier (Molecular Devices, Union City, CA), and electrodes were filled with 3 M KCl (resistance, 0.5–1 MΩ on the current-passing side). Agonist

solution (acetylcholine chloride, freshly prepared from frozen stock aliquots) was applied via the bath perfusion at 5-min intervals. ACh responses from α7-expressing oocytes were recorded in the presence of 5 mM 5-hydroxyindole to enhance the response amplitude and reduce interoocyte variability (Zwart et al., 2002). Chemicals were from Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

A descending-dose protocol was used for dose-response curves. All data shown are compensated for response rundown (Boorman et al., 2000). To reassure ourselves that the lack of functional expression observed for some subunit combinations was true and not a false-negative due to oocyte health or expression problems contingent to a given batch, oocyte data were obtained from a minimum of two separate oocyte batches for each combination. In every experimental batch, at least one “control” highly expressing subunit combination was injected to check for expression efficiency.

Concentration-response curves were fitted with the following 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; least-squares fitting was performed with the use of the program CVFIT, courtesy of D. Colquhoun and I. Vais, available at <http://www.ucl.ac.uk/Pharmacology/dc.html>. Each curve was fitted separately, with individual responses being equally weighted, to obtain estimates for I_{max} , EC_{50} , and n_H . For display purposes, data points were normalized to the fitted maximum and pooled before fitting.

When two components were detected in the concentration-response curve, free fits of the individual curves were poorly defined because of the large number of parameters fitted. Good fits were obtained when all of the concentration-response curves for this combination were fitted simultaneously with EC_{50} and n_H values for the two components constrained to be equal across oocytes, whereas the proportion of current in the first component was allowed to vary from one oocyte to the other.

Because of the heteroscedasticity of the functional data, we used Kruskal-Wallis one-way nonparametric ANOVA followed by Dunn's post hoc multiple comparisons test (Daniel, 1978) (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA). For all comparisons, we also carried out randomization tests (Colquhoun, 1971; RANTEST, available at <http://www.ucl.ac.uk/Pharmacology/dc.html>), which gave similar results to the ANOVA/post hoc tests.

Radioligand Binding Studies. Mammalian tsA201 cells were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium containing Glutamax (Invitrogen), plus 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma-Aldrich). Subconfluent cultures were transiently transfected overnight with a total of 0.6 μg of cDNA per 10-cm dish, using the Effectene reagent kit (Qiagen, Crawley, UK). Cells were harvested 40 to 44 h later and resuspended in Hanks' buffered saline solution (HBSS) (Invitrogen) for assay. Amounts of total cellular protein were determined by a Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK) using bovine serum albumin standards. For the α7 experiments, cells were transfected with cDNA for human RIC-3 protein (subcloned into pcDNA3 and transfected in equimolar amount with α7 cDNA) to ensure α7 surface expression (Williams et al., 2005).

[³H]Epibatidine binding samples were incubated on ice for 2 h with a single saturating dose of [³H]epibatidine (3 nM; PerkinElmer LAS, Beaconsfield, UK), and receptor-bound ligand was isolated using Whatman GF/B filters presoaked in 0.5% polyethylenimine on a Brandel cell harvester (Semat, St. Albans, UK). Radioligand binding was measured in the presence of buffer alone, along with binding in the presence of buffer containing excess nicotine (1 mM) to define nonspecific binding. Internal binding was estimated by blocking

external binding sites using an excess of the nonpermeant ligand ACh (10 mM) and the number of external sites was computed by subtraction.

¹²⁵I- α -Bungarotoxin Binding. Samples were incubated at room temperature for 2 h with a single saturating concentration of ¹²⁵I- α -bungarotoxin (6–10 nM; GE Healthcare UK Ltd, Chalfont St. Giles, UK) in the presence of 1% bovine serum albumin to minimize non-specific binding. Receptor-bound ligand was isolated as above. Cell-surface binding was measured in intact cells either in the presence of buffer alone or in the presence of excess nicotine and carbachol (1 mM each) to define nonspecific binding.

Patch-Clamp Recording of Primary Hippocampal Neurons. Hippocampal neurons were cultured from embryonic day-18 rat embryos (Thomas et al., 2005): the hippocampus was incubated in trypsin (0.25% w/v; Invitrogen) for 15 min before washing in Hanks' medium (Invitrogen), and dissociation was performed using the polished tip of a Pasteur pipette. Suspended cells were plated onto 22-mm coverslips coated with 0.1 mg/ml poly(L-lysine) and maintained in B-27-Supplemented Neurobasal Medium (Invitrogen) for 1 week before transfection with cDNA for EGFP-c1 (BD Biosciences, Oxford, UK) and either $\alpha 7$, $\beta 3$, or $\beta 3^{V273S}$ (1:1 ratio, 0.4 μ g/ μ l per 35-mm dish) using Effectene (Qiagen). Whole-cell recordings were performed on the first and second day after transfection at a holding potential of -70 mV.

Plated cells were superfused (3 ml/min) with an extracellular solution containing 150 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, 5 mM HEPES, 1 μ M atropine, and 0.3 μ M tetrodotoxin, with pH adjusted to 7.3 with NaOH. The pipette solution consisted of 147 mM CsCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, and 10 mM HEPES, with pH adjusted to 7.3 with CsOH. Pipettes were pulled from borosilicate glass (GC150-TF; Harvard Apparatus, Edenbridge, UK) to a resistance of 1 to 3 M Ω . Series resistance (4–8 M Ω) was compensated between 60 and 90%.

ACh (3 mM) was applied via a modified U-tube. Exchange time was tested by the application of an 80% diluted extracellular solution both before obtaining the seal and after the end of recording and rupture of the seal. Only neurons in which the 10 to 90% exchange time was less than 1 ms were included. In experiments with meth-

yllycaconitine (Tocris Cookson, Bristol, UK), the antagonist was added to the bath perfusion (2 min before the application of ACh) and to the ACh solution in the U-tube. Recordings were acquired using a Digidata 1322A with Clampex software (Molecular Devices, Union City, CA), filtered at 1 kHz with an 8-pole Bessel filter (built in-house), and digitized at 10 kHz.

Results

Expression of the $\beta 3$ Subunit, but Not of $\beta 3^{VS}$, Abolishes the Functional Responses of Most Recombinant Neuronal Nicotinic Subunit Combinations. The traces in Fig. 1 (top) show that coexpression of the $\beta 3$ subunit effectively suppressed responses evoked by 1 mM ACh from neuronal nicotinic receptors expressed in oocytes from α/β subunit "pair" combinations, $\alpha 2\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, and $\alpha 4\beta 4$. With the exception of the $\alpha 3\beta 4$ combination, adding $\beta 3$ to the subunits expressed completely abolished functional nicotinic current responses. We repeated the measurement in several oocyte batches, obtaining consistent results. We have shown previously in experiments with mutant $\beta 3$ that expressing $\alpha 3\beta 4$ and $\beta 3$ in a 1:1:20 ratio is necessary to ensure that the majority of receptors contain $\beta 3$ (Groot-Kormelink et al., 1998). In the present series of experiments, the decrease in ACh currents was nearly complete when this injection ratio was used (i.e., 90–100% depending on the combination). Smaller but substantial decreases in functional responses were observed for less extreme transfection ratios, such as 1:1:1 (data not shown). We checked that the effect of $\beta 3$ was not due to a decrease in ACh sensitivity in $\beta 3$ -containing receptors by testing higher ACh concentrations (up to 20 mM, data not shown) but failed to see any increase in responses with increases in concentration.

As shown by the summary of the data in the bar charts in Fig. 1 (bottom), functional expression of the $\alpha 3\beta 4$ combina-

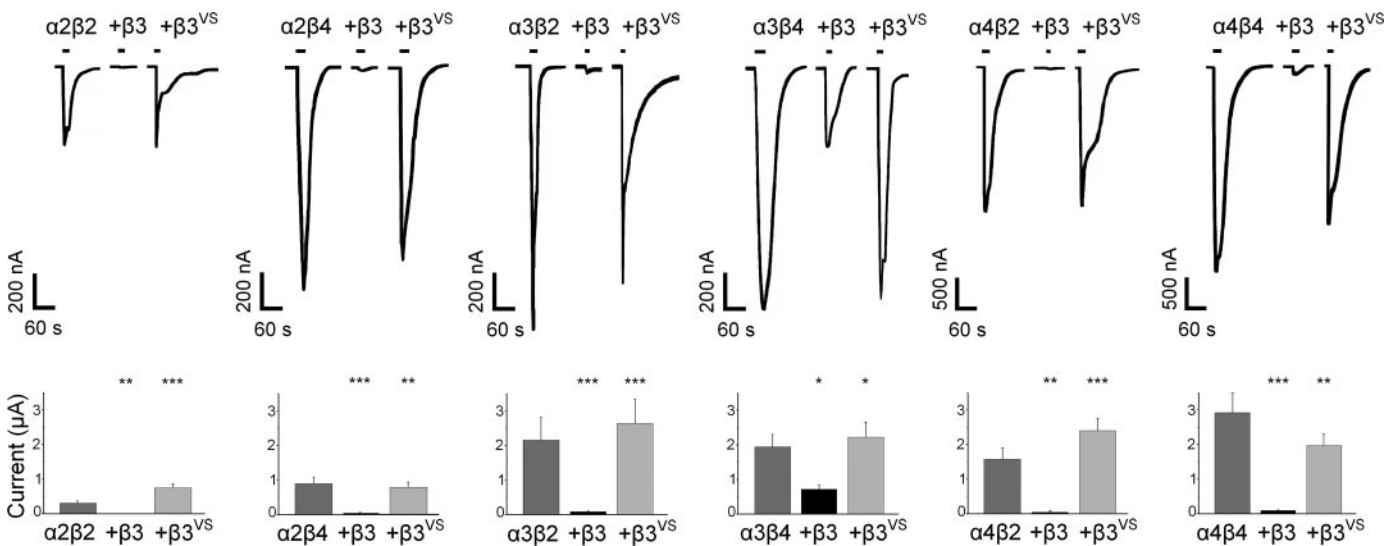


Fig. 1. The dominant-negative effect of coexpressing $\beta 3$ with α/β pair receptors is reversed by the valine-serine mutation in position 9' of the second transmembrane domain of $\beta 3$. Top, traces are inward currents recorded at -70 mV in response to the application of 1 mM ACh (black bars above the traces) to oocytes expressing the base α/β pair alone (first trace in each group of three), together with the wild-type $\beta 3$ subunit (middle trace) or together with the $\beta 3^{VS}$ mutant subunit (last trace in each group). Bottom, average inward currents (in microamperes) for the same subunit combinations. Each bar represents the mean (\pm S.D. of the mean) of 7 to 17 oocytes. Note that coexpression of $\beta 3$ completely suppressed functional ACh responses in all pair combinations except $\alpha 3\beta 4$, which is relatively spared, retaining approximately a third of control current responses. The dominant-negative effect was not observed when the mutant $\beta 3^{VS}$ was expressed. Neither $\beta 3$ nor $\beta 3^{VS}$ formed functional receptors when expressed alone or together with any single "pair-forming" receptor subunit (i.e., $\alpha 2$, $\alpha 3$, $\alpha 4$, $\beta 2$, or $\beta 4$; $n = 10$ –21). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Kruskal-Wallis one-way nonparametric ANOVA followed by Dunn's post hoc multiple comparisons test; comparisons were with control (i.e., pair alone) for the $+\beta 3^{WT}$ data (middle column) and with $+\beta 3^{VS}$ for the $+\beta 3^{VS}$ data (columns on the right).

tion was somewhat spared by coexpression with $\beta 3$, with a reduction of approximately two thirds (from 1.9 ± 0.36 to $0.71 \pm 0.13 \mu\text{M}$, $n = 10$ and 12 , respectively).

To test whether the striking dominant-negative effect of $\beta 3$ is a specific effect that requires the incorporation of $\beta 3$ in the receptor pentamer, we repeated these experiments, expressing a point mutant of $\beta 3$, V237S $\beta 3$ (which we will refer to as $\beta 3^{\text{VS}}$), instead of the wild-type subunit. This mutant carries in position 9' of the second, pore-lining transmembrane domain a hydrophilic residue, serine, instead of the hydrophobic amino acid (leucine or valine) present in this position in all subunits of the nicotinic superfamily. This type of mutation (Labarca et al., 1995) is believed to facilitate channel gating by destabilizing part of the hydrophobic girdle that keeps the channel closed (Miyazawa et al., 2003). The reason for performing this experiment is that this sort of point mutation could change the effect of $\beta 3$ only if this effect is mediated by the incorporation of the subunit into the pentamer rather than by a nonspecific effect of $\beta 3$ on subunit production, receptor assembly, or trafficking. We found that introducing this mutation in the $\beta 3$ subunit abolished the dominant-negative effect of the wild-type subunit. Thus, expression of $\beta 3^{\text{VS}}$ did not suppress functional expression of any of the subunit combinations tested (Fig. 1), where functional expression was measured as the response to 1 mM ACh. This result strongly supports the conclusion that the effect of wild-type $\beta 3$ is due to a specific reduction in the P_{open} value of receptors that incorporate this subunit. When the $\beta 3^{\text{VS}}$ subunit is incorporated instead of $\beta 3$, the facilitation in gating produced by the mutation is such that the decrease in P_{open} seen with wild-type $\beta 3$ does not take place or is much reduced, and receptor function is preserved.

As a negative control, we systematically tested whether $\beta 3^{\text{VS}}$ can produce functional receptors when expressed alone or together with one other subunit (i.e., $\alpha 2$, $\alpha 3$, $\alpha 4$, $\beta 2$, or $\beta 4$). None of these combinations ($\beta 3^{\text{VS}}$, $\alpha 2\beta 3^{\text{VS}}$, $\alpha 3\beta 3^{\text{VS}}$, $\alpha 4\beta 3^{\text{VS}}$, $\beta 2\beta 3^{\text{VS}}$, and $\beta 4\beta 3^{\text{VS}}$) gave detectable functional responses to the application of 1 mM ACh ($n = 10$, two batches of oocytes for each combination).

The $\beta 3^{\text{VS}}$ Subunit Is Incorporated into Functional nAChRs. One possible alternative explanation is that when the $\beta 3^{\text{VS}}$ mutant is transfected, function remains because the mutant subunit, contrary to wild-type $\beta 3$, cannot participate in receptor formation. In the nicotinic superfamily, channel pore 9' mutants have been widely used in stoichiometry studies, and no effects on subunit incorporation have been reported (Boorman et al., 2000). Nevertheless, we tested this possibility by characterizing the ACh sensitivity of receptors expressed in oocytes from $\alpha 4\beta 2 + \beta 3^{\text{VS}}$ cRNA. Figure 2 shows the dose-response curves of recombinant $\alpha 4\beta 2$ receptors expressed alone (●, solid line) or together with $\beta 3^{\text{VS}}$ (▲, broken line). The $\alpha 4\beta 2$ dose-response curve shows a typical feature of this receptor combination, in that it has two components, one at $6.6 \pm 1.8 \mu\text{M}$ and one at $160 \pm 14 \mu\text{M}$ (n_{H} values were 0.78 ± 0.09 and 2.4 ± 0.2 , respectively; $n = 4$). The presence of these two components has been ascribed to the existence of two distinct receptors that differ in the number of α subunits contained in the pentamer, the high-sensitivity form of the receptor containing two copies of $\alpha 4$ (Nelson et al., 2003). When $\beta 3^{\text{VS}}$ is coexpressed with $\alpha 4$ and $\beta 2$, the ACh dose-response curve is shifted leftward and seems to have only one component (EC_{50} of $1.0 \pm 0.1 \mu\text{M}$, n_{H} of 1.1 ± 0.1 , $n = 4$),

which is clearly different from either of the two components observed for $\alpha 4\beta 2$ alone. These effects of $\beta 3^{\text{VS}}$ coexpression show that the mutant subunit is incorporated in the $\alpha 4\beta 2$ -type receptor; in addition to that, the presence of only one component suggests that this new receptor can only take one stoichiometry, which is likely to include two copies each of $\alpha 4$ and $\beta 2$ and one of $\beta 3^{\text{VS}}$. Similar results were obtained for the $\alpha 3\beta 2$ combination expressed alone and with $\beta 3^{\text{VS}}$ (data not shown).

Combinations Containing the $\alpha 6$ Subunit. The expression of "pair" $\alpha 6$ subunit combinations produced only 0 or very small nicotinic responses in oocytes, even when relatively large amounts of cRNA were injected, up to 1 ng per subunit (i.e., 0.92 ng, namely 80 times more than was injected for the $\alpha 3\beta 4$ combination). For $\alpha 6\beta 2$ transfections, only two oocytes of eight injected with this cRNA amount responded to 1 mM ACh with an average 12-nA response. Responses were also poor but were more consistent for $\alpha 6\beta 4$ ($49 \pm 15 \text{ nA}$, $n = 8$, two batches of oocytes). Coexpression of the $\beta 3$ subunit in its wild-type form did not affect the amplitude of responses elicited from $\alpha 6$ combinations: only two of

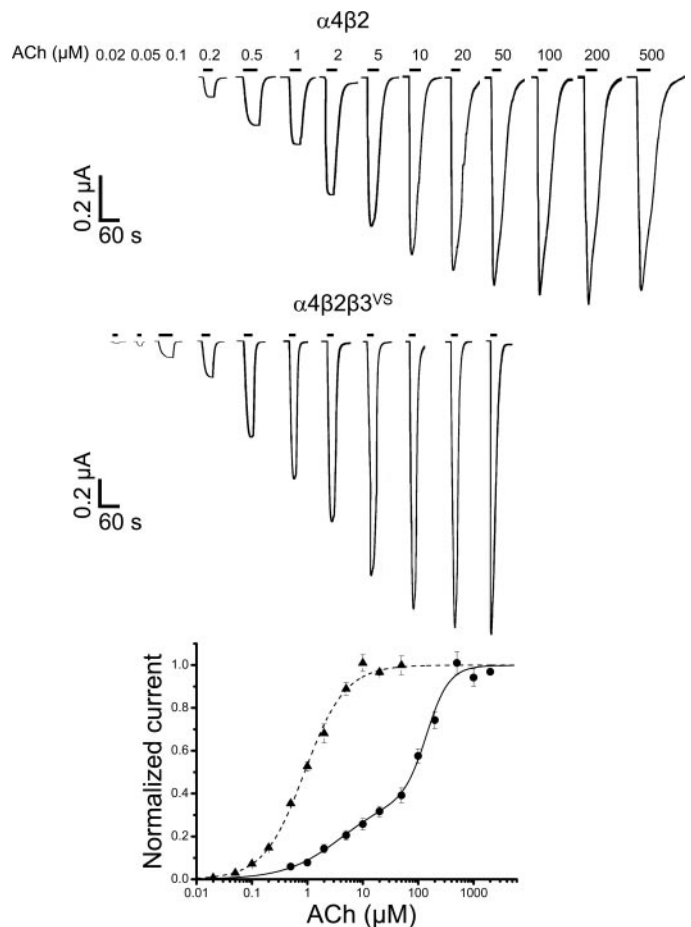


Fig. 2. The mutant $\beta 3^{\text{VS}}$ subunit is incorporated into functional nAChRs containing $\alpha 4$ and $\beta 2$. Top, traces are inward currents evoked by the application of ACh (solid bar) to oocytes expressing $\alpha 4\beta 2$ or $\alpha 4\beta 2 + \beta 3^{\text{VS}}$. The concentration-response curves of these two combinations are shown in the bottom graphs. Note that the ACh concentration-response curve of $\alpha 4\beta 2$ receptors has two distinct components with EC_{50} values of 6.6 ± 1.8 and $160 \pm 14 \mu\text{M}$ ($n = 4$; ●, solid line shows fit of a two-component Hill equation). When $\beta 3^{\text{VS}}$ is coexpressed with $\alpha 4$ and $\beta 2$ (▲, broken line), these two components are replaced by a single component with high ACh sensitivity ($\text{EC}_{50} = 1.0 \pm 0.1 \mu\text{M}$, $n_{\text{H}} = 1.1 \pm 0.1$, $n = 4$).

eight oocytes injected with $\alpha 6\beta 2\beta 3$ responded to ACh (average, 4 nA), whereas six of eight oocytes injected with $\alpha 6\beta 4\beta 3$ responded to 1 mM ACh (18 ± 5.6 nA). Somewhat unexpectedly, coexpression of the gain-of-function mutant $\beta 3^{VS}$ significantly increased nicotinic responses in both combinations to 120 ± 17 and 560 ± 140 nA for $\alpha 6\beta 2\beta 3^{VS}$ and $\alpha 6\beta 4\beta 3^{VS}$, respectively (eight of eight injected oocytes for both combinations; $p < 0.001$, Kruskal-Wallis one-way nonparametric ANOVA followed by Dunn's post hoc multiple comparisons test).

Transfection with $\beta 3$ Suppresses Functional ACh Responses by Native Nicotinic Receptors in Hippocampal Neurons in Culture. The powerful and consistent dominant-negative effect of $\beta 3$ on recombinant receptors raises the question of whether this effect is relevant for native receptors in neurons. The best way to test this is to transfect neurons in primary culture with $\beta 3$. We chose hippocampal primary cultures, because hippocampal neurons have robust responses to nicotinic agonists but do not express the $\beta 3$ subunit (Deneris et al., 1989; Sudweeks and Yakel, 2000; Cui et al., 2003).

Almost all hippocampal neurons tested responded to the U-tube application of 3 mM ACh with fast inward currents as shown in Fig. 3A (250 ± 54 pA, 25 of 27 neurons tested). As reported previously by Albuquerque et al. (1997), these responses are likely to be mediated by $\alpha 7$ nicotinic receptors (note their fast time course and the fast and complete sag in the response with sustained ACh application). The $\alpha 7$ -like

nature of these responses was confirmed by their sensitivity to the application of the nicotinic blocker methyllycaconitine (MLA). At a concentration (1 nM) selective for $\alpha 7$ receptors (Alkondon and Albuquerque, 1993), MLA completely blocked the response to 3 mM ACh in 14 of 14 neurons (Fig. 3A). We also transfected hippocampal neurons with the $\alpha 7$ subunit together with EGFP-c1 as a marker: responses in transfected neurons (identified because of their green fluorescence) had the same time course as in control but were much bigger (3800 ± 510 pA, 26 of 26 neurons tested; $p < 0.001$ against the 27 control neurons; Kruskal-Wallis one-way nonparametric ANOVA followed by Dunn's post hoc multiple comparisons test; statistical tests were carried on all the neurons tested, including nonresponders). Having established that nicotinic responses in the preparation are robust, consistent, and $\alpha 7$ -like, we proceeded to test the effect of transfection with $\beta 3$. Transfection with wild-type $\beta 3$ had a striking dominant-negative effect: 34 of 38 transfected (i.e., fluorescing) neurons did not respond to 3 mM ACh at all, as shown in the middle trace of Fig. 3A. In the remaining four neurons, currents similar to those recorded in untransfected neurons were observed (490 ± 240 pA, $n = 4$).

As in oocyte-expressed recombinant receptors, transfection of hippocampal neurons with the mutant $\beta 3^{VS}$ subunit failed to produce the dominant-negative effect observed for the wild-type transfections. As shown in Fig. 3A, almost all neurons transfected with $\beta 3^{VS}$ responded to 3 mM ACh with inward currents comparable in amplitude with those mea-

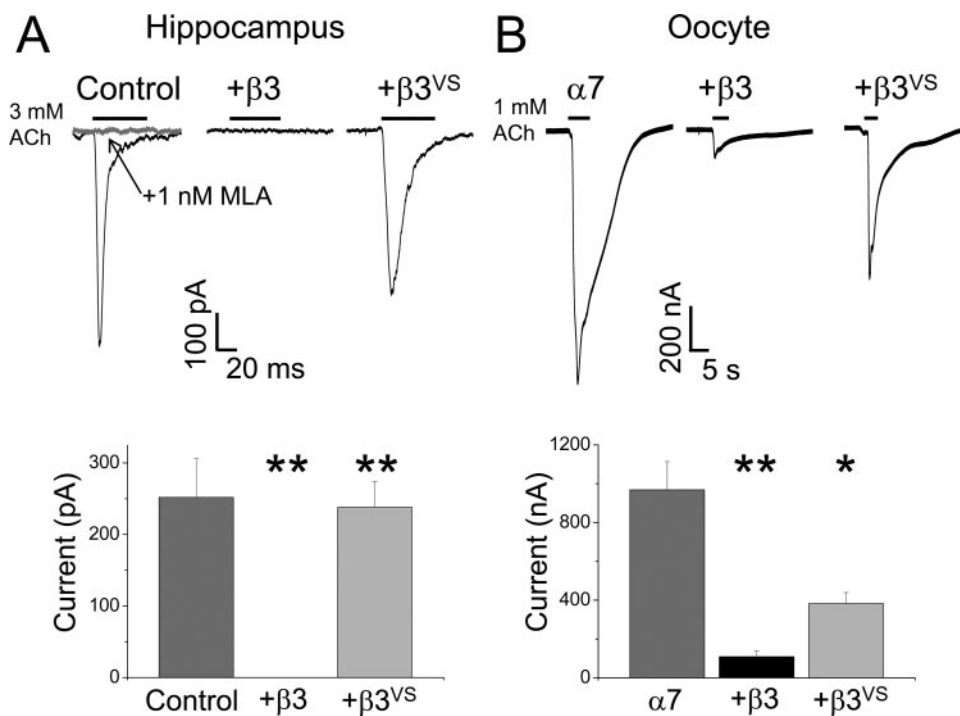


Fig. 3. Nicotinic responses in hippocampal cultures are suppressed by $\beta 3$ expression. A, top traces show responses to 3 mM ACh applied by U-tube to hippocampal neurons. Control responses are from untransfected neurons in the same dish as the neurons transfected with the $\beta 3$ or the $\beta 3^{VS}$ subunits. Transfected neurons were identified by their green fluorescence, due to the expression of EGFP-c1. Control ACh responses were completely abolished by the coapplication of the nicotinic blocker MLA (gray trace) at a concentration low enough to be selective for $\alpha 7$ receptors (1 nM, $n = 14$). Bottom bar chart shows the amplitude of ACh responses in neurons in the different categories. The control and the + $\beta 3^{VS}$ columns show the average current to 3 mM ACh in responding neurons (25 of 27 and 48 of 53, respectively). Most $\beta 3$ -transfected neurons (34 of 38) did not respond to ACh at all; the remaining four neurons had a normal response (see Results; statistical tests incorporated all neurons tested: responders and nonresponders). B, top traces show responses to bath-applied 1 mM ACh in oocytes expressing $\alpha 7$ alone or together with $\beta 3^{WT}$ or $\beta 3^{VS}$ in the presence of 5 mM 5-hydroxyindole (Zwart et al., 2002). Bottom bar chart shows average responses ($n = 8, 14,$ and 13 , respectively). *, $p < 0.01$; **, $p < 0.001$, Kruskal-Wallis nonparametric ANOVA followed by Dunn's post hoc multiple comparisons test; responses in cells transfected with $\beta 3^{WT}$ were compared with controls, and responses in cells transfected with $\beta 3^{VS}$ were compared with those in cells transfected with $\beta 3^{WT}$.

sured in control neurons in the same dishes (240 ± 36 pA, 48 of 53 neurons; 5 neurons did not respond to ACh), although the time course of these responses was somewhat slower than that of the untransfected controls.

We carried out a similar experiment in oocytes expressing the $\alpha 7$ subunit: coexpression of the $\beta 3$ wild-type subunit produced an 88% decrease in the responses to 1 mM ACh (from 0.97 ± 0.15 to 0.10 ± 0.027 μA , $n = 8$ and 14, respectively), as shown by the middle trace in Fig. 3B. If the mutant $\beta 3^{\text{VS}}$ subunit was coexpressed, instead of wild-type $\beta 3$, the decrease was by 60% (to 0.38 ± 0.057 μA , $n = 13$; last trace in Fig. 3B).

Changes in Receptor Surface Expression Cannot Account for the Dominant-Negative Effect of $\beta 3$ on Function. The effect of expressing $\beta 3^{\text{VS}}$ is already a strong indication that $\beta 3$ impairs receptor function at the level of the receptor molecule and reduces its open probability rather than reducing the number of receptors in the membrane. Nevertheless, we checked for that by carrying out a binding assay to measure the number of nicotinic sites expressed on the surface of tsA201 cells transiently transfected with $\alpha 3\beta 4$, $\alpha 4\beta 2$, or $\alpha 7$ alone or together with either wild-type $\beta 3$ or with $\beta 3^{\text{VS}}$. The ligand was [^3H]epibatidine for the $\alpha 3\beta 4^*$ and $\alpha 4\beta 2^*$ sites and ^{125}I - α -bungarotoxin for $\alpha 7^*$ receptors. In the $\alpha 7$ experiments, tsA201 cells were transfected with both $\alpha 7$ and the human RIC-3 protein to ensure reliable surface expression of the $\alpha 7$ receptor (Williams et al., 2005).

As shown in Fig. 4, coexpression of wild-type $\beta 3$ did not abolish surface expression of nAChR. After coexpression of $\beta 3^{\text{WT}}$, there were relatively small but significant changes in the number of surface binding sites. The number of $\alpha 3\beta 4\beta 3$ sites was $65.0 \pm 7.8\%$ of the number of $\alpha 3\beta 4$ sites (Fig. 4, left, see the 63% reduction in maximum ACh response shown in Fig. 1), whereas the number of $\alpha 4\beta 2\beta 3$ sites was somewhat increased with respect to the number of $\alpha 4\beta 2$ sites ($140 \pm 16\%$, $n = 7$, Fig. 4, middle; cfr. the 97% reduction in maximum ACh response for the same combination; Fig. 1). $\alpha 7$ Sites were approximately halved (to $52 \pm 16\%$ of control, $n = 6$, Fig. 4, right; cfr. the 88% suppression in functional ACh responses for the same combination, Fig. 3B) by the coexpression of $\beta 3$ wild type but not by coexpression of $\beta 3^{\text{VS}}$ ($86 \pm 14\%$ of control). Thus, the wild-type form of $\beta 3$ halved the surface

expression of $\alpha 7$ receptors but effectively suppressed (by 88%) their functional responses. Clearly the dominant-negative effect of $\beta 3$ cannot be explained by a change in the number of receptors on the surface.

Discussion

The $\beta 3$ subunit is present at high levels in CNS regions that express many other nicotinic subunits (Deneris et al., 1989). To our surprise, we found that expressing $\beta 3$ together with every known type of pairwise functional recombinant neuronal nicotinic combination (except for $\alpha 3\beta 4$) abolished functional nicotinic responses. Our data confirm and substantially extend those of Palma and coworkers (1999) that chick $\beta 3$ and $\alpha 7$ subunits coassemble into nonfunctional receptors. We found that the dominant-negative effect of $\beta 3$ could not be accounted for by a reduction in the number of surface nAChRs and that it was reversed by a V9'S mutation in the second transmembrane domain of $\beta 3$.

A Mechanism for the Dominant-Negative Effect of $\beta 3$ on Nicotinic Function. The changes in the $\alpha 4\beta 2$ dose-response curves produced by $\beta 3^{\text{VS}}$ and our previous data on $\alpha 3\beta 4\beta 3$ receptors (Boorman et al., 2000, 2003) show that $\beta 3$ is incorporated into nAChRs. It follows that the suppression of nicotinic responses must result mainly from impaired function of $\beta 3$ -containing nAChRs. Function is not restored by increasing the ACh concentration, so the effect is not due to a shift in agonist sensitivity. The amplitude of the maximum agonist response of a ligand-gated ion channel is affected by the number of receptors, the unitary channel current, and the maximum channel P_{open} value for the agonist, and we shall examine these factors in turn.

Our data show that the effect of coexpressing $\beta 3$ or $\beta 3^{\text{VS}}$ on the number of surface receptors expressed in mammalian cells cannot account for the profound inhibition of functional responses we observe. There is a one-third reduction in the number of $\alpha 3\beta 4$ -type sites (cfr. a two-thirds decrease in $\alpha 3\beta 4$ currents), no change in the number of $\alpha 4\beta 2$ sites, and a halving of $\alpha 7$ sites, in contrast with the nearly complete abolition of $\alpha 4\beta 2$ and $\alpha 7$ responses. This agrees with the finding that $\beta 3$ only slightly reduced surface $\alpha 7$ sites (Palma et al., 1999).

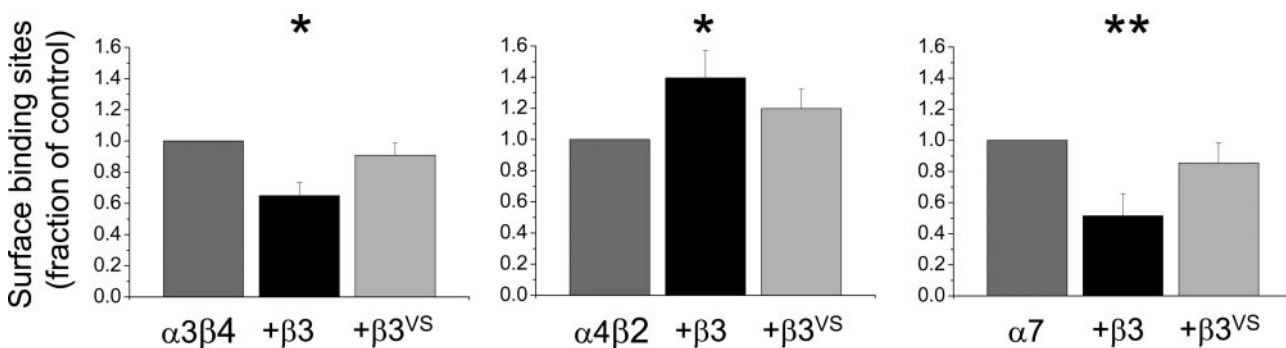


Fig. 4. Changes in the number of nAChRs on the cell surface cannot explain the dominant-negative effect of $\beta 3$ on ACh currents. The bar charts show the number of nicotinic binding sites (measured by [^3H]epibatidine or by ^{125}I - α -bungarotoxin for $\alpha 7$) on the surface of tsA201 cells transiently transfected with a base combination ($\alpha 3\beta 4$, $\alpha 4\beta 2$, or $\alpha 7$), alone or with $\beta 3$, either as wild-type or as $\beta 3^{\text{VS}}$ mutant ($n = 6-7$). Numbers were normalized to the number of binding sites in the absence of $\beta 3$ in each experiment (416 ± 92.0 , 197 ± 63.1 , and 151 ± 29.6 fmol/mg protein for $\alpha 3\beta 4$, $\alpha 4\beta 2$, and $\alpha 7$, respectively). Note that cotransfection with $\beta 3$ does not suppress surface nAChR expression even for the $\alpha 4\beta 2$ receptor, whose functional responses were reduced by 93% by $\beta 3$ coexpression. *, $p < 0.05$; **, $p < 0.01$, repeated-measures ANOVA followed by post hoc Bonferroni's test for multiple comparisons. The test was carried out on the data before normalization: cells transfected with $\beta 3^{\text{WT}}$ were compared with controls, and cells transfected with $\beta 3^{\text{VS}}$ were compared with cells transfected with $\beta 3^{\text{WT}}$.

Agonist responses will also be affected by the size of the single-channel current. The nearly complete suppression of the functional responses by $\beta 3$ makes it difficult to measure the single-channel conductance of most $\beta 3$ -containing receptors, but we already know that $\beta 3$ increases $\alpha 3\beta 4$ channel conductance (Boorman et al., 2003). In nAChRs, conductance is determined by conserved pore-lining domain residues that form three rings of charges. $\beta 3$ has a negatively charged glutamate in the external 20' ring, in which other neuronal β subunits have a positively charged lysine. Because $\beta 3$ takes the place of a β subunit (Boorman et al., 2000), its incorporation increases the negative charge in 20' and explains the observed increase in conductance (Imoto et al., 1988). In addition, it is hard to see how anything short of a complete loss of channel conductance could account for the near-total suppression of receptor function we observed. Furthermore, a conductance change would probably not be reversed by the 9' mutation, which does not affect single-channel conductance (Filatov and White, 1995). Finally, residues that determine conductance are highly conserved in neuronal nicotinic subunits, and it is hard to see why the effect of $\beta 3$ differs depending on subunit combination.

This leaves us with the possibility that $\beta 3^{\text{WT}}$ reduces the maximum channel P_{open} value by reducing gating efficacy, E . This would explain why the dominant-negative effect is suppressed (or counterbalanced) by the V9'S mutation, which is believed to facilitate gating (Labarca et al., 1995). For many simple mechanisms, maximum P_{open} value is given by $E/(E + 1)$ (where $E = \beta/\alpha$ is the gating equilibrium constant for the fully liganded channel, and β and α are the opening and closing rate constants, respectively). The effect of reducing E on the maximum P_{open} value will be relatively small if E is large in the first place. Hence, $\alpha 3\beta 4$ function would be relatively spared by $\beta 3$ if $\alpha 3\beta 4$ has a higher value of E than the other combinations. Unfortunately, nothing is known of the gating efficacy of neuronal nAChRs. Efficacy values in the nicotinic superfamily go from very high (muscle nAChRs, Colquhoun and Sakmann, 1985; and glycine receptors, Burzomato et al., 2004) to relatively low values (GABA_A receptors, Jones and Westbrook, 1995).

Because $\beta 3$ produces such a complete suppression of nicotinic currents, obtaining direct estimates of the maximum P_{open} value by single-channel measurements is not feasible for most $\beta 3$ -containing combinations. The exception is $\alpha 3\beta 4\beta 3$, and our single-channel data from this combination are consistent with the hypothesis that $\beta 3$ primarily impairs gating. Incorporation of $\beta 3$ greatly shortens the duration of bursts of openings at low agonist concentration (receptor activations; Boorman et al., 2003), mainly by reducing the number of events per burst (from 38.3 ± 11.3 to 2.9 ± 0.31 gaps per burst, $n = 10$ and 5 , respectively; M. Beato and L. Sivilotti, unpublished data), but does not affect the macroscopic EC_{50} value of $\alpha 3\beta 4$ nAChRs (Groot-Kormelink et al., 1998). The mean number of openings, m , in an activation is

$$m = 1 + \frac{\beta}{k_{\text{off}}} \quad (2)$$

where β is the opening rate constant, and k_{off} is the dissociation rate constant. If $\beta 3$ reduced the number of openings per burst primarily by increasing the dissociation rate constant,

we should also observe a change in EC_{50} , because the EC_{50} value is linearly related to the dissociation equilibrium constant. In the simplest case of a receptor opened by two agonist molecules (Colquhoun, 1998),

$$EC_{50} = \frac{k_{\text{off}}}{k_{\text{on}}} \frac{1 + \sqrt{2 + \frac{\beta}{\alpha}}}{1 + \frac{\beta}{\alpha}} \quad (3)$$

where k_{on} is the association rate constant. Equation 3 shows that changes in the gating constants have smaller effects on EC_{50} values, because EC_{50} is a function of the square root of the gating constants. This explanation is not unique (and does not consider the possibility that $\beta 3$ changes the proportion of missed single-channel events), but it corroborates indications from the other experiments (i.e., binding assays and the effect of the valine-serine gain-of-function mutation).

Implications for Native Receptors Containing the $\beta 3$ Subunit. $\beta 3$ -Containing nAChRs, whether formed by a subunit pair with $\beta 3$ or by $\alpha 7$ with $\beta 3$ (including native hippocampal $\alpha 7$ -like nAChRs), have profoundly reduced function, except for $\alpha 3\beta 4$ -type receptors. This "sparing" of $\alpha 3\beta 4$ receptor function may mean that, in a typical CNS neuron that expresses a wide range of subunits together with $\beta 3$, nicotinic responses would have a predominantly $\alpha 3\beta 4$ -type profile. The precise functional consequences for nicotinic signaling would depend on the physiological ACh concentrations that activate these central nAChRs. Because $\alpha 3\beta 4$ receptors are less sensitive to ACh than the $\alpha 4\beta 2$ -type (by up to 100-fold; Gerzanich et al., 1995), the predominance of $\alpha 3\beta 4$ responses may mean that higher ACh levels are needed to produce nicotinic responses in neurons in which $\beta 3$ is expressed. This might be important presynaptically, where transmitter levels may not reach the saturating concentrations at which peripheral fast synapses operate. Differences in the extent and rate of desensitization of the different receptors may also be important.

Caution must be exerted in extrapolating our findings to native receptors, because our results apply to relatively simple recombinant receptors (i.e., "triplet" receptors made of an α/β pair plus $\beta 3$). We do not know whether native receptors can have this sort of composition or contain more than three different subunits. Furthermore, $\beta 3$ expression is often associated with $\alpha 6$ expression (Le Novère et al., 1996), and $\beta 3$ may facilitate $\alpha 6^*$ receptor trafficking, because it increases functional expression of $\alpha 6/\alpha 3 \beta 2$ chimeric receptors (Kuryatov et al., 2000; McIntosh et al., 2004). Nevertheless, $\alpha 6$ -containing receptors have proven very hard to characterize because of low functional expression. In our hands, the expression of $\alpha 6\beta 2$ or $\alpha 6\beta 4$ produced at best very small functional responses which were not increased by $\beta 3$ co-expression. Robust responses were observed only when the gain-of-function mutant $\beta 3^{\text{VS}}$ was coexpressed. A further complication is that efficient surface expression of $\alpha 6\beta 4$ ligand binding sites may require both the $\beta 3$ and the $\alpha 5$ subunit (Grinevich et al., 2005).

Results from knockout mice suggest that $\beta 3$ -containing nAChRs on striatal dopaminergic terminals are either $\alpha 6\beta 2\beta 3$ or $\alpha 6\alpha 4\beta 2\beta 3$ (Luetje, 2004; Salminen et al., 2004). At present, we cannot determine what the effect of $\beta 3$ would be on this complex $\alpha 6$ -containing receptor combination because

of the difficulty of expressing receptors that contain four different subunits when subsets of these four are also functional. To obtain a pure population of these receptors, it may be necessary to use concatamer techniques (Zhou et al., 2003; Groot-Kormelink et al., 2004, 2006).

The disappearance of a specific striatal α6-containing receptor (α-conotoxin MII-sensitive) in β3-null mice has been taken to mean that efficient formation of this receptor requires β3. However, β3 deletion increased another type of nicotinic response (i.e., the α-conotoxin MII-resistant component of dopamine release produced by nicotine) (Cui et al., 2003). Hence, this distinct receptor population was believed not to contain β3. However, their enhanced function after β3 deletion could be explained if the receptors normally do contain β3 and if their function is reduced by the presence of β3. The precise subunit composition of the receptor could thus determine whether β3 stabilizes the receptor or reduces its function.

β3 expression profoundly reduces nAChR function in a variety of subunit combinations. The magnitude of this effect depends on the channel subunit composition and may result in the switching to a different profile of functional receptors.

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