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Incorporation of the β3 subunit has a dominant negative effect on the function of recombinant central-type neuronal nicotinic receptors

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Running title: *β3 expression reduces neuronal nicotinic responses*

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nAChR, nicotinic acetylcholine receptor; P_{open}, channel open probability

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Abstract

The β3 neuronal nicotinic subunit is localised in dopaminergic areas of the central nervous system, where many other neuronal nicotinic subunits are expressed. So far, β3 has only been shown to form functional receptors when expressed together with the α3 and β4 subunits. We have systematically tested in *Xenopus* oocytes the effects of co-expressing human β3 with every pairwise functional combination of neuronal nicotinic subunits likely to be relevant to the CNS. Expression of α7 homomers or α/β pairs (α2, α3, α4 or α6 together with β2 or β4) produced robust nicotinic currents for all combinations, save α6β2 and α6β4. Co-expression of wild-type β3 led to a nearly complete loss of function (measured as maximum current response to ACh) for α7 and for all functional α/β pairs except α3β4. This effect was also seen in hippocampal neurons in culture, which lost their robust α7-like responses when transfected with β3. The level of surface expression of nicotinic binding sites (α3β4, α4β2 and α7) in tsA201 cells was only marginally affected by β3 expression. Furthermore, the dominant-negative effect of β3 was abolished by a VS mutation in the 9′ position of the second transmembrane domain of β3, a mutation thought to facilitate channel gating. Our results show that incorporation of β3 into neuronal nicotinic receptors other than α3β4 has a powerful dominant negative effect, probably due to impairment in gating. This raises the possibility of a novel regulatory role for the β3 subunit on neuronal nicotinic signalling in the central nervous system.

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Introduction

The function of the β3 subunit has been a puzzle since it was cloned more than 15 years ago (Deneris et al., 1989). β3 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, β3 is restricted to nAChRs on dopaminergic terminals (Zoli et al., 2002). Data from β3 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 β3-containing receptors have a specific role, possibly in locomotor control, we know little of the effects of β3 incorporation into functional nAChRs. β3 does not form functional nAChRs when expressed with a "typical" α (α2-α4) or with a β-type subunit (β2 or β4) (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* oocytes, β3 does assemble together with α3 and β4 into a functional receptor that contains two copies each of α3 and β4 and one of β3 (Boorman et al., 2000). The presence of β3 is hard to detect, as only single-channel conductance and kinetics are affected (Boorman et al., 2003). Because of the relatively restricted expression of the α3 and β4 subunits in the central nervous system, this combination (α3β4 + β3) is likely to be relevant only to habenular nAChRs (Sheffield et al., 2000), and other subunit combinations may predominate in most areas

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that express β3, such as *substantia nigra* and the ventral tegmentum area. Here a wide subset of nicotinic 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 co-expression 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 downregulation of responses from receptors that incorporate this subunit.

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Materials and Methods

Human Neuronal Nicotinic Subunit cDNAs

Sequences for nicotinic subunits are as deposited in Genbank, 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 linearised 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 Xenopus Oocytes

Female *Xenopus laevis* frogs were anaesthetized by immersion in neutralized ethyl m-aminobenzoate solution (tricaine, methanesulphonate salt; 0.2% solution w/v), and killed by decapitation and destruction of the brain and spinal cord (in accordance to Home Office guidelines) before removal of ovarian lobes to sterile modified Barth's solution of composition (in mM): NaCl 88; KCl 1; $MgCl_2$ 0.82; $CaCl_2$ 0.77; $NaHCO_3$ 2.4; Tris-HCl 15; with 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Paisley, UK); pH 7.4 adjusted with NaOH. Mature oocytes were manually defolliculated after collagenase IA treatment (see Boorman et al., 2000 for further details) before cRNA was injected, at a ratio of 1:1 for pair receptors, and 1:1:20 for triplet receptors. The total

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amount of cRNA for each combination was determined empirically, with the aim of achieving a maximum ACh-evoked current of 1 – 2 μA, and was 0.25 – 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 Ca^{2+}-free Ringer (mM: NaCl 150, KCl 2.8, HEPES 10, $MgCl_2$ 2, atropine sulphate 0.5 μM, 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 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 response amplitude and reduce inter-oocyte variability (Zwart et al., 2002). Chemicals 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). In order 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 Hill equation:

$$I = I_{\max} \frac{[\mathrm{A}]^{n_\mathrm{H}}}{[\mathrm{A}]^{n_\mathrm{H}} + EC_{50}{}^{n_\mathrm{H}}} \qquad (1)$$

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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 by the program CVFIT, courtesy of D. Colquhoun & I. Vais, available from http://www.ucl.ac.uk/Pharmacology/dc.html. Each curve was fitted separately, individual responses being equally weighted, in order to obtain estimates for I_{max}, EC_{50} and n_H. For display purposes, datapoints were normalised 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 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, while 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, pp200-214; GraphPad Prism, version 4.00 for Windows, GraphPad Software, San Diego). For all comparisons, we also carried out randomization tests (Colquhoun, 1971; RANTEST, 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% CO_2 in Dulbecco's Modified Eagle's Medium containing Glutamax (Invitrogen, Paisley, UK), plus 10%

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foetal calf serum, 100units/ml penicillin and 100μg/ml streptomycin (all Sigma Aldrich, Gillingham, UK). Sub-confluent cultures were transiently transfected overnight with a total of 0.6 μg of cDNA per 10cm dish, using the Effectene™ reagent kit (Qiagen, Crawley, UK). Cells were harvested 40 to 44 hours later and resuspended in HBSS (Hanks' Buffered Saline Solution; Invitrogen, Paisley, UK) for assay. Amounts of total cellular protein were determined by a Bio-Rad protein assay 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).

[^{3}H]-epibatidine binding Samples were incubated on ice for 2 hours with a single saturating dose of [^{3}H]-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 non-specific binding. Internal binding was estimated by blocking external binding sites using an excess of the non-permeant ligand ACh (10 mM) and the number of external sites was computed by subtraction.

[^{125}I]-α-bungarotoxin binding Samples were incubated at room temperature for 2 hours with a single saturating concentration of [^{125}I]-α-bungarotoxin (6-10 nM; GE Healthcare UK Ltd, Chalfont St. Giles, UK) in the presence of 1% BSA to minimize non-specific binding. Receptor-bound ligand was isolated as above. Cell surface binding was

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measured in intact cells either in the presence of buffer alone, or in the presence of excess nicotine and carbachol (1 mM each) in order to define non-specific binding.

Patch-Clamp Recording of Primary Hippocampal Neurons

Hippocampal neurons were cultured from E18 rat embryos (Thomas et al., 2005): the hippocampus was incubated in trypsin (0.25 % w/v, Invitrogen, Paisley, UK) for 15 min before washing in Hank's medium (Invitrogen), and dissociation 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 a week before transfection with cDNA for EGFP-c1 (BD Biosciences, Oxford, UK) and either α7, β3 or $\beta3^{V273S}$ (1:1 ratio, 0.4 μg/μl per 35 mm-dish) using Effectene™ (Qiagen, Crawley, UK). 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 (in mM): NaCl 150, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 5; atropine (1 μM) and tetrodotoxin (TTX, 0.3 μM); pH adjusted to 7.3 with NaOH. The pipette solution consisted of (in mM): CsCl 147, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 10, HEPES 10, pH adjusted to 7.3 with CsOH. Pipettes were pulled from borosilicate glass (GC150-TF, Harvard Apparatus, Edenbridge, UK) to a resistance of 1-3 MΩ. Series resistance (4-8 MΩ) 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 10-90%

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exchange time was less than 1 ms were included. In experiments with methyllycaconitine (Tocris Cookson, Bristol, UK), the antagonist was added to the bath perfusion (2 minutes 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.

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Results

Expression of the β3 subunit, but not of $\beta3^{VS}$, abolishes the functional responses of most recombinant neuronal nicotinic subunit combinations

The traces in Figure 1 (top) show that co-expression of the β3 subunit effectively suppressed responses evoked by 1 mM ACh from neuronal nicotinic receptors expressed in oocytes from α/β subunit "pair" combinations, α2β2, α2β4, α3β2, α3β4, α4β2 and α4β4. With the exception of the α3β4 combination, adding β3 to the subunits expressed completely abolished functional nicotinic current responses. We repeated the measurement in several oocyte batches, obtaining consistent results. We have previously shown in experiments with mutant β3, that expressing α3β4 and β3 in a 1:1:20 ratio is necessary to ensure that the majority of receptors contain β3 (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 β3 was not due to a decrease in ACh sensitivity in β3-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 α3β4 combination was somewhat spared by co-expression with β3, with a reduction of approximately two-thirds (from 1.9 ± 0.36 to 0.71 ± 0.13 μA, n =10 and 12, respectively).

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In order to test whether the striking dominant negative effect of β3 is a specific effect that requires the incorporation of β3 in the receptor pentamer, we repeated these experiments, expressing a point mutant of β3, V237S β3 (which we will refer to as $\beta3^{VS}$) 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 thought to facilitate channel gating by destabilising part of the hydrophobic girdle that keeps the channel closed (Miyazawa et al., 2003). The reason for doing this experiment is that this sort of point mutation could change the effect of β3 only if this effect is mediated by the incorporation of the subunit into the pentamer, rather than by a non-specific effect of β3 on subunit production, receptor assembly or trafficking. We found that introducing this mutation in the β3 subunit abolished the dominant negative effect of the wild-type subunit. Thus, expression of $\beta3^{VS}$ did not suppress functional expression of any of the subunit combinations tested (see 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 β3 is due to a specific reduction in the P_{open} of receptors that incorporate this subunit. When the $\beta3^{VS}$ subunit is incorporated instead of β3, the facilitation in gating produced by the mutation is such that the decrease in P_{open} seen with wild type β3 does not take place or is much reduced and receptor function is preserved.

As a negative control, we systematically tested whether $\beta3^{VS}$ can produce functional receptors when expressed alone or together with one other subunit (i.e. α2, α3, α4, β2 or β4). None of these combinations ($\beta3^{VS}$, $\alpha2\beta3^{VS}$, $\alpha3\beta3^{VS}$, $\alpha4\beta3^{VS}$, $\beta2\beta3^{VS}$ and

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$\beta4\beta3^{VS}$) gave detectable functional responses to the application of 1 mM ACh (n = 10, 2 batches of oocytes for each combination).

The $\beta3^{VS}$ subunit is incorporated into functional nAChRs

One possible alternative explanation is that when the $\beta3^{VS}$ mutant is transfected, function remains because the mutant subunit, contrary to wild-type β3, 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 (see Boorman et al. 2000). Nevertheless, we tested this possibility, by characterising the ACh sensitivity of receptors expressed in oocytes by $\alpha4\beta2 + \beta3^{VS}$. Figure 2 shows the dose-response curves of recombinant α4β2 receptors expressed alone (filled circles, continuous line) or together with $\beta3^{VS}$ (filled triangles, dashed line). The α4β2 dose-response curve shows a typical feature of this receptor combination, in that it has two components, one at 6.6 ± 1.8 μM and one at 160 ± 14 μM (nH was 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 α4 (Nelson et al., 2003). When $\beta3^{VS}$ is co-expressed with α4 and β2, the ACh dose-response curve is shifted leftwards and appears to have only one component (EC_{50} of 1.0 ± 0.1 μM, nH 1.1 ± 0.1, n = 4), which is clearly different from either of the two components observed for α4β2 alone. These effects of $\beta3^{VS}$ co-expression show that the mutant subunit is incorporated in the α4β2-type receptor: in addition to that, the presence of only one component suggests that this new receptor can only take one stoichiometry,

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which is likely to include two copies each of α4 and β2 and one of $\beta3^{VS}$. Similar results were obtained for the α3β2 combination expressed alone and with $\beta3^{VS}$ (data not shown).

Combinations containing the α6 subunit

The expression of 'pair' α6 subunit combinations produced only zero 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 α3β4 combination). For α6β2 transfections, only 2 oocytes out of 8 injected with this cRNA amount responded to 1 mM ACh with an average 12 nA response. Responses were also poor, but more consistent for α6β4 (49 ± 15 nA, n = 8, 2 batches of oocytes). Co-expression of the β3 subunit in its wild-type form did not affect the amplitude of responses elicited from α6 combinations: only 2 out of 8 oocytes injected with α6β2β3 responded to ACh (average 4 nA), whereas 6 out of 8 oocytes injected with α6β4β3 responded to 1 mM ACh (18 ± 5.6 nA). Somewhat unexpectedly, co-expression of the gain-of-function mutant $\beta3^{VS}$ significantly increased nicotinic responses in both combinations to 120 ± 17 nA and 560 ± 140nA for $\alpha6\beta2\beta3^{VS}$ and $\alpha6\beta4\beta3^{VS}$, respectively (8 out of 8 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 β3 suppresses functional ACh responses by native nicotinic receptors in hippocampal neurons in culture

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The powerful and consistent dominant negative effect of β3 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 β3. We chose hippocampal primary cultures, as hippocampal neurons have robust responses to nicotinic agonists, but do not express the β3 subunit (Deneris et al., 1989;Cui et al., 2003; see however Sudweeks and Yakel, 2000).

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 out of 27 neurons tested). As previously reported by Albuquerque and co-authors (1997), these responses are likely to be mediated by α7 nicotinic receptors (note their fast time course and the fast and complete sag in the response with sustained ACh application). The α7-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 α7 receptors (Alkondon and Albuquerque, 1993), MLA completely blocked the response to 3 mM ACh in 14 out of 14 neurons (Fig 3A). We also transfected hippocampal neurons with the α7 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 out 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 non-responders). Having established that nicotinic responses in the preparation are robust, consistent and α7-like, we proceeded to test the effect of transfection with β3. Transfection with wild-type β3 had a striking dominant negative effect: 34 out of 38

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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 4 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 $\beta3^{VS}$ subunit failed to produce the dominant negative effect observed for the wild-type transfections. As shown in Fig 3A, almost all neurons transfected with $\beta3^{VS}$ responded to 3 mM ACh with inward currents comparable in amplitude with those measured in control neurons in the same dishes (240 ± 36 pA, 48 out 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 α7 subunit: co-expression of the β3 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 $\beta3^{VS}$ subunit was co-expressed, instead of wild-type β3, 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 β3 on function.

The effect of expressing $\beta3^{VS}$ is already a strong indication that β3 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 α3β4, α4β2 or α7

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alone or together with either wild-type β3 or with $\beta3^{VS}$. The ligand was [^{3}H]-epibatidine for the α3β4* and α4β2* sites and [^{125}I]-α-bungarotoxin for α7* receptors. In the α7 experiments tsA201 cells were transfected with both α7 and the human RIC-3 protein in order to ensure reliable surface expression of the α7 receptor (Williams *et al.*, 2005).

As shown in Figure 4, co-expression of wild-type β3 did not abolish surface expression of nAChR. After co-expression of $\beta3^{WT}$, there were relatively small but significant changes in the number of surface binding sites. The number of α3β4β3 sites was 65.0 ± 7.8% of the number of α3β4 sites (Fig. 4A, cfr. the 63% reduction in maximum ACh response shown in Fig. 1), whereas the number of α4β2β3 sites was somewhat increased with respect to the number of α4β2 sites (140 ± 16%, n = 7, Fig. 4B; cfr. the 97% reduction in maximum ACh response for the same combination; Fig. 1). α7 sites were approximately halved (to 52 ± 16% of control, n=6, Fig. 4C; cfr. the 88% suppression in functional ACh responses for the same combination, Fig. 3B) by the co-expression of β3 wild-type, but not by co-expression of $\beta3^{VS}$ (86 ± 14% of control). Thus the wild type form of β3 halved the surface expression of α7 receptors, but effectively suppressed (by 88%) their functional responses. Clearly the dominant negative effect of β3 cannot be explained by a change in the number of receptors on the surface.

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Discussion

The β3 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 β3 together with every known type of pairwise functional recombinant neuronal nicotinic combination (except for α3β4) abolished functional nicotinic responses. Our data confirm and substantially extend those of Palma and co-workers (1999) that chick β3 and α7 subunits co-assemble into non-functional receptors. We found that the dominant negative effect of β3 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 β3.

A mechanism for the dominant negative effect of β3 on nicotinic function

The changes in the α4β2 dose-response curves produced by $\beta3^{VS}$ and our previous data on α3β4β3 receptors (Boorman et al., 2000; 2003) show that β3 is incorporated into nAChRs. It follows that the suppression of nicotinic responses must result mainly from impaired function of β3-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} for the agonist, and we shall examine these factors in turn.

Our data show that the effect of co-expressing β3 or $\beta3^{VS}$ 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

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number of α3β4-type sites (cfr. a two-thirds decrease in α3β4 currents), no change in the number of α4β2 sites and a halving of α7 sites, in contrast with the nearly complete abolition of α4β2 and α7 responses. This agrees with the finding that β3 only slightly reduced surface α7 sites (Palma et al., 1999).

Agonist responses will also be affected by the size of the single-channel current. The nearly complete suppression of functional responses by β3 makes it difficult to measure the single-channel conductance of most β3-containing receptors, but we already know that β3 *increases* α3β4 channel conductance (Boorman et al., 2003). In nAChRs, conductance is determined by conserved pore-lining domain residues which form three rings of charges. β3 has a negatively-charged glutamate in the external 20′ ring, where other neuronal β subunits have a positively-charged lysine. As β3 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 β3 differs depending on subunit combination.

This leaves us with the possibility that $\beta3^{WT}$ reduces the maximum channel P_{open} by reducing gating efficacy, *E*. This would explain why the dominant negative effect is suppressed (or counterbalanced) by the V9′S mutation, which is thought to facilitate gating (Labarca et al., 1995). For many simple mechanisms, maximum P_{open} is given by

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$E/(E+1)$ (where $E = \beta/\alpha$ is the gating equilibrium constant for the fully-liganded channel, β and α being the opening and closing rate constants, respectively). The effect of reducing E on the maximum P_{open} will be relatively small if E is large in the first place. Hence, α3β4 function would be relatively spared by β3 if α3β4 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 β3 produces such a complete suppression of nicotinic currents, obtaining direct estimates of the maximum P_{open} by single-channel measurements is not feasible for most β3-containing combinations. The exception is α3β4β3, and our single-channel data from this combination are consistent with the hypothesis that β3 primarily impairs gating. Incorporation of β3 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; Beato and Sivilotti, unpublished), but does not affect the macroscopic EC_{50} of α3β4 nAChRs (Groot-Kormelink et al., 1998). The mean number of openings, m, in an activation is

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

where β is the opening rate constant and k_{off} is the dissociation rate constant. If β3 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} is linearly related to

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the dissociation equilibrium constant. In the simplest case of a receptor opened by two agonist molecules (Colquhoun, 1998)

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

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 doesn't consider the possibility that β3 changes the proportion of missed single-channel events), but corroborates indications from the other experiments (i.e. binding assays and the effect of the VS gain-of-function mutation).

Implications for native receptors containing the β3 subunit

β3-containing nAChRs, whether formed by a subunit pair with β3 or by α7 with β3 (including native hippocampal α7-like nAChRs) have profoundly reduced function, except for α3β4-type receptors. This "sparing" of α3β4 receptor function may mean that, in a typical CNS neuron that expresses a wide range of subunits together with β3, nicotinic responses would have a predominantly α3β4-type profile. The precise functional consequences for nicotinic signalling would depend on the physiological ACh concentrations that activate these central nAChRs. Because α3β4 receptors are less sensitive to ACh than the α4β2-type (by up to 100-fold, see for instance Gerzanich *et al.*, 1995), the predominance of α3β4 responses may mean that higher ACh levels are needed to produce nicotinic responses where β3 is expressed. This might be important

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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 β3. We do not know whether native receptors can have this sort of composition, or contain more than three different subunits. Furthermore, β3 expression is often associated with α6 expression (Le Novère et al., 1996) and β3 may facilitate α6* receptor trafficking, as it increases functional expression of α6/α3 β2 chimeric receptors (Kuryatov *et al.*, 2000; McIntosh *et al.*, 2004). Nevertheless, α6-containing receptors have proved very hard to characterize because of low functional expression. In our hands, expression of α6β2 or α6β4 produced at best very small functional responses, which were not increased by β3 co-expression. Robust responses were observed only when the gain-of-function mutant $\beta3^{VS}$ was co-expressed. A further complication is that efficient surface expression of α6β4 ligand binding sites may require *both* the β3 and the α5 subunit (Grinevich et al., 2005).

Results from knockout mice suggest that β3-containing nAChRs on striatal dopaminergic terminals are either α6β2β3 or α6α4β2β3 (Luetje, 2004; Salminen et al., 2004). At present, we cannot determine what the effect of β3 would be on this complex α6-containing receptor combination, because of the difficulty of expressing receptors that contain four different subunits when subsets of these four are also functional. In order 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).

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The disappearance of a specific striatal α6-containing receptor (α-conotoxinMII-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-conotoxinMII -resistant component of dopamine release produced by nicotine (Cui et al., 2003). Hence, this distinct receptor population was thought 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 stabilises 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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Legends for figures

Figure 1 The dominant negative effect of co-expressing β3 with α/β pair receptors is reversed by the VS mutation in position 9′ of the second transmembrane domain of β3.

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 β3 subunit (middle trace) or together with the $\beta3^{VS}$ mutant subunit (last trace in each group).

Bottom: Average inward currents (μA) for the same subunit combinations. Each bar represents the mean (± standard deviation of the mean) of 7-17 oocytes.

Note that co-expression of β3 completely suppressed functional ACh responses in all pair combinations except α3β4, which is relatively spared, retaining approximately a third of control current responses. The dominant negative effect was not observed when the mutant $\beta3^{VS}$ was expressed.

Neither β3 nor $\beta3^{VS}$ formed functional receptors when expressed alone or together with any single "pair-forming" receptor subunit (i.e. α2, α3, α4, β2 or β4; 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 $+\beta3^{WT}$ data (middle column) and with $+\beta3^{WT}$ for the $+\beta3^{VS}$ data (columns on the right).

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Figure 2 The mutant $\beta3^{VS}$ subunit is incorporated into functional nAChRs containing α4 and β2.

Top: traces are inward currents evoked by the application of ACh (black bars) to oocytes expressing α4β2 or α4β2 + $\beta3^{VS}$

The concentration-response curves of these two combinations are shown in the bottom graphs. Note that the ACh concentration-response curve of α4β2 receptors has two distinct components with EC_{50} values of 6.56 ± 1.82 and 159 ± 14.3 μM (n = 4; filled circles, continuous line shows fit of a two-component Hill equation). When $\beta3^{VS}$ is co-expressed with α4 and β2 (filled triangles, dashed line), these two components are replaced by a single component with high ACh sensitivity (EC_{50} 1.02 ± 0.12 μM, nH 1.12 ± 0.10, n = 4).

Figure 3 Nicotinic responses in hippocampal cultures are suppressed by β3 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 β3 or the $\beta3^{VS}$ subunits. Transfected neurons were identified by their green fluorescence, due to expression of EGFP-c1. Control ACh responses were completely abolished by the co-application of the nicotinic blocker methyllycaconitine (MLA, grey trace) at a concentration low enough to be selective for α7 receptors (1 nM, n=14). Bottom bar chart shows the amplitude of ACh responses in neurons in the different categories. The control and the +$\beta3^{VS}$ columns show the average current to 3 mM ACh in responding neurons (25 out of 27 and 48 out of 53, respectively). Most β3 transfected neurons (34 out of 38) did not respond to ACh at all; the remaining 4 neurons

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had a normal response (see Results; statistical tests incorporated all neurons tested – responders and non-responders).

B Top traces show responses to bath-applied 1 mM ACh in oocytes expressing α7 alone or together with $\beta3^{WT}$ or $\beta3^{VS}$, 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 non-parametric ANOVA followed by Dunn's *post hoc* multiple comparisons test; responses in cells transfected with $\beta3^{WT}$ were compared with controls and responses in cells transfected with $\beta3^{VS}$ were compared with those in cells transfected with $\beta3^{WT}$.

Figure 4 Changes in the number of nAChRs on the cell surface cannot explain the dominant negative effect of β3 on ACh currents.

The bar charts show the number of nicotinic binding sites (measured by [^{3}H]epibatidine, A and B or by [^{125}I]-α-bungarotoxin in C) on the surface of tsA201 cells transiently transfected with a base combination (α3β4 in A, α4β2 in B, α7 in C), alone or with β3, either as wild-type or as $\beta3^{VS}$ mutant (n =6-7). Numbers were normalized to the number of binding sites in the absence of β3 in each experiment (416 ± 92.0, 197 ± 63.1 and 151 ± 29.6 fmol/mg of protein for α3β4, α4β2 and α7, respectively).

Note that co-transfection with β3 does not suppress surface nAChR expression even for the α4β2 receptor, whose functional responses were reduced by 93% by β3 co-expression.

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*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 $\beta3^{WT}$ were compared with controls and cells transfected with $\beta3^{VS}$ were compared with cells transfected with $\beta3^{WT}$.

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Fig. 1

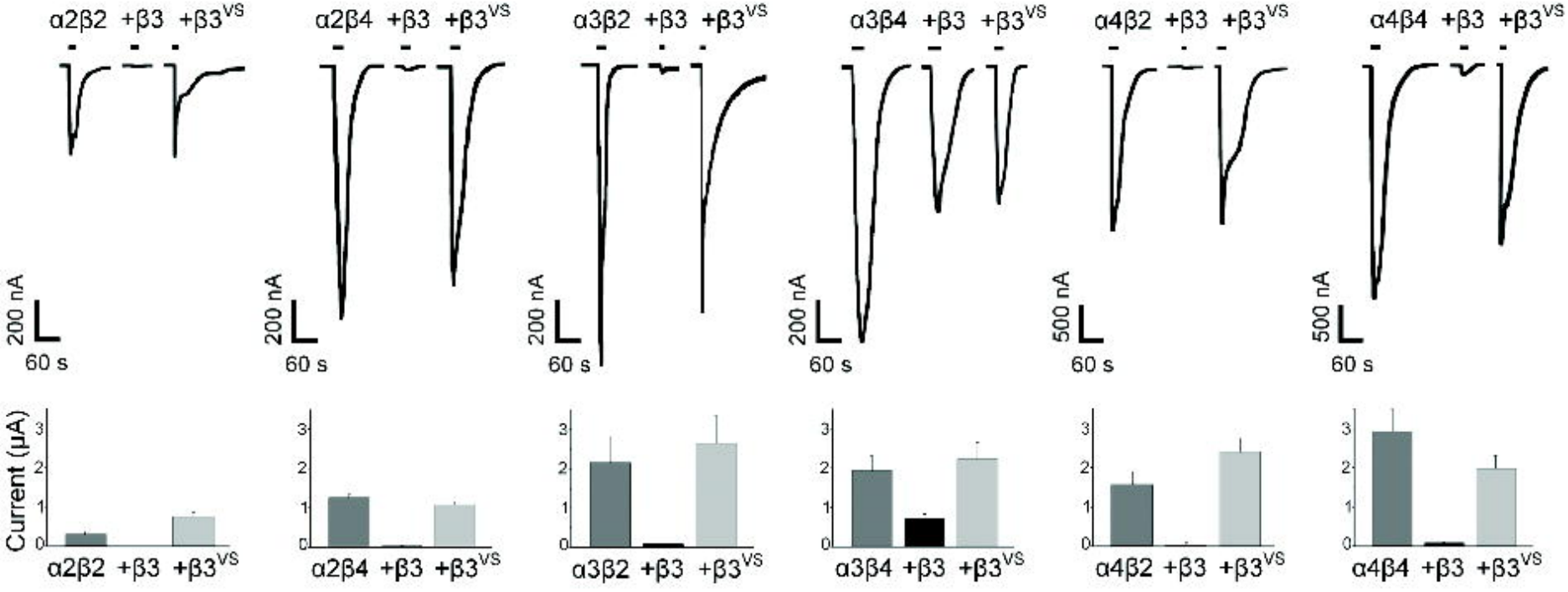
α2β2 +β3 +β3VS
α2β4 +β3 +β3VS
α3β2 +β3 +β3VS
α3β4 +β3 +β3VS
α4β2 +β3 +β3VS
α4β4 +β3 +β3VS
200 nA
500 nA
60 s
Current (μA)
0
1
2
3

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Fig.2

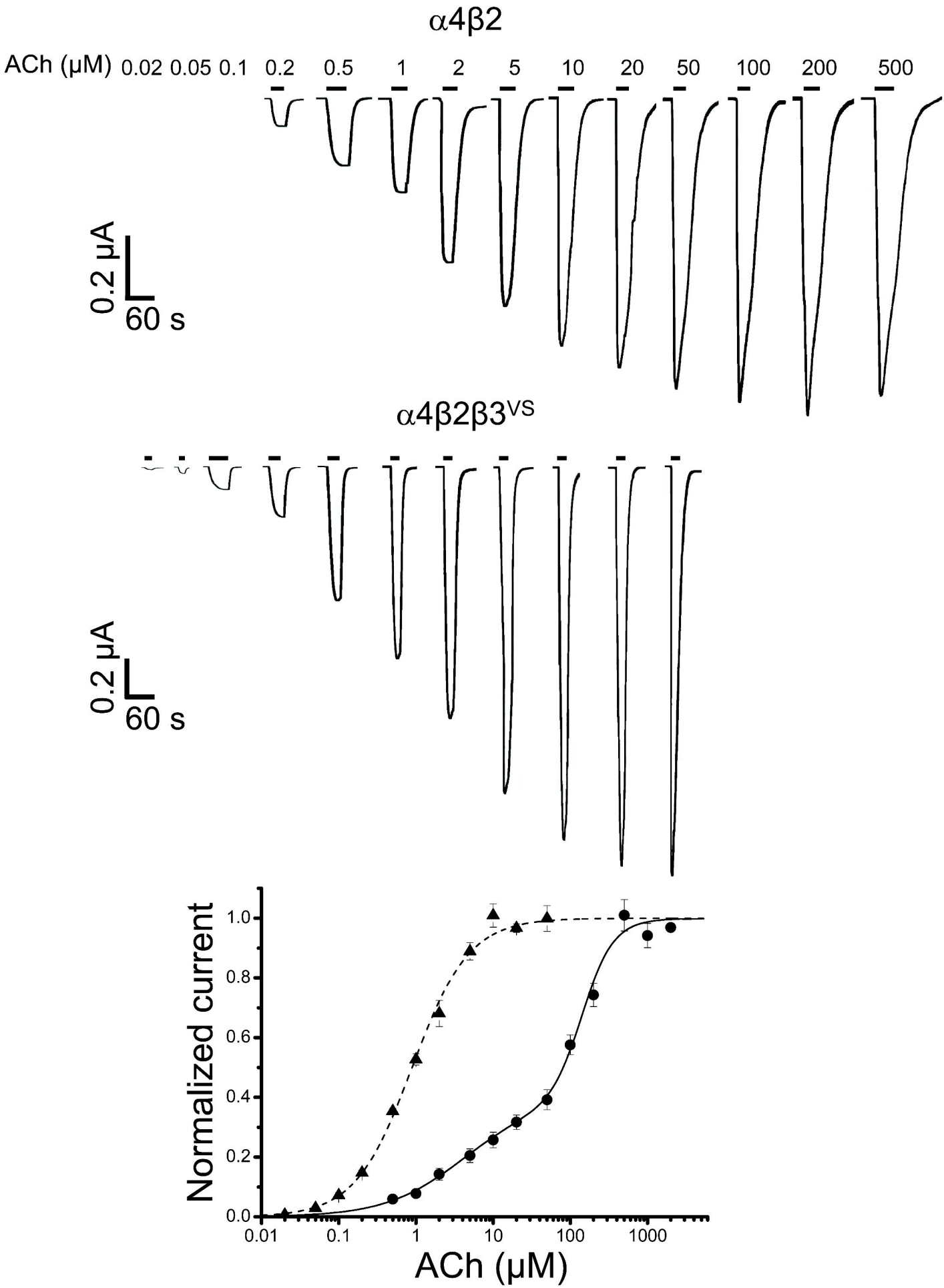
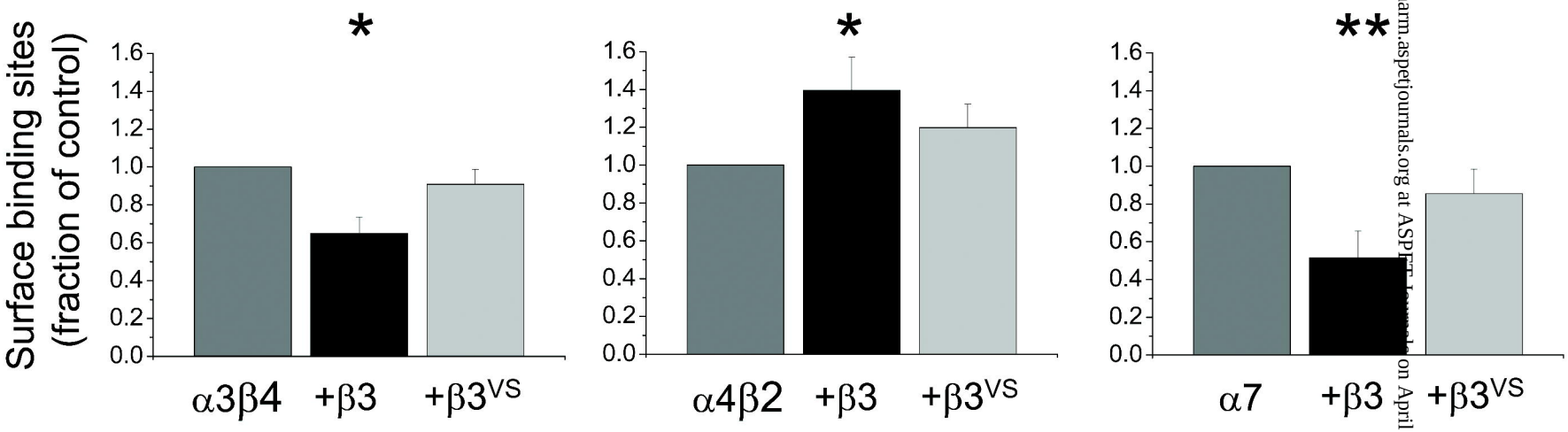


Fig. 3



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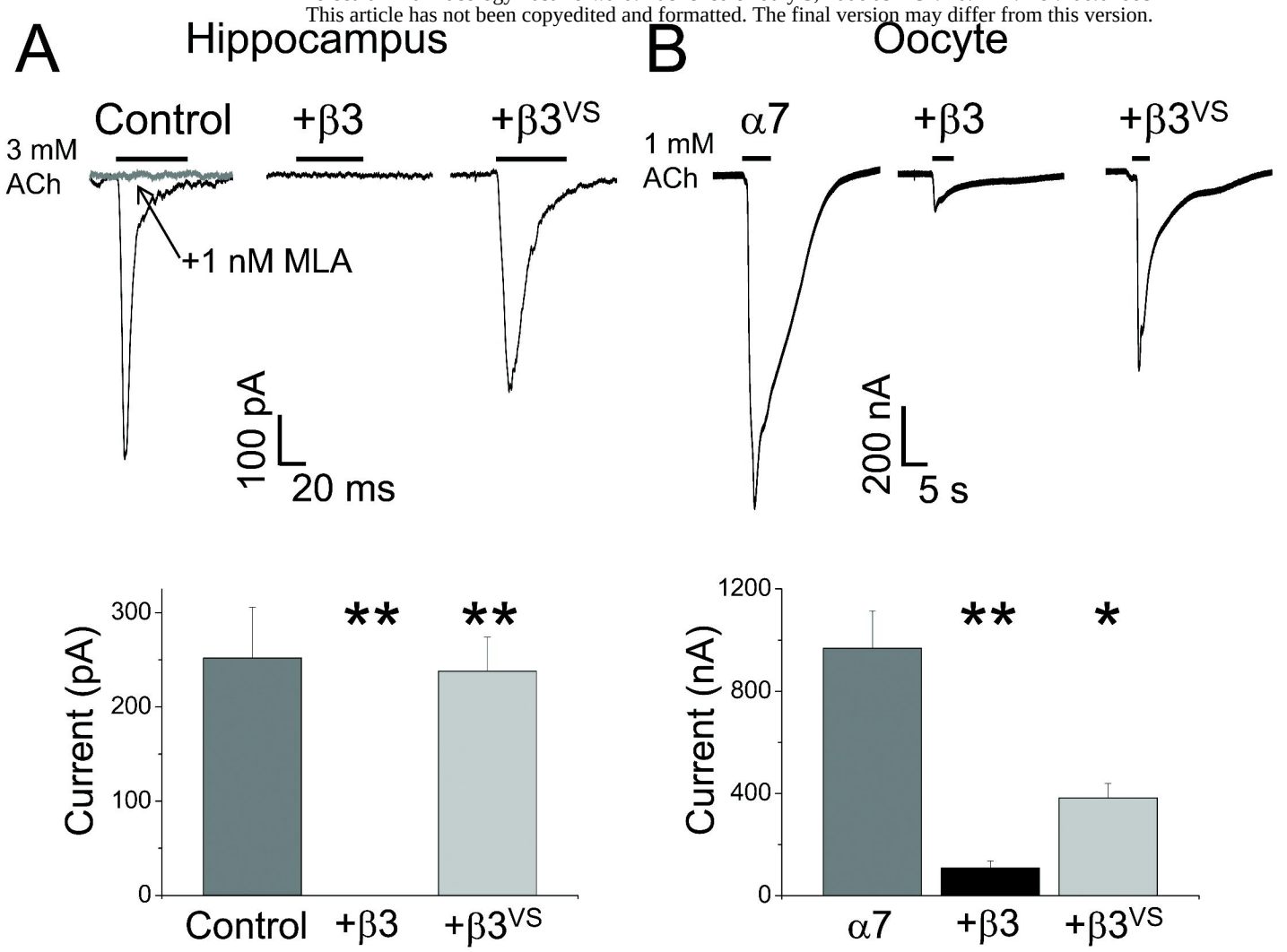

Fig.4