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*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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**Running title:**  *$\beta$ 3 expression reduces neuronal nicotinic responses*

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

## Abstract

The  $\beta 3$  neuronal nicotinic subunit is localised in dopaminergic areas of the central nervous system, where 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* oocytes the effects of co-expressing human  $\beta 3$  with every pairwise functional combination of neuronal nicotinic subunits likely to be relevant to the CNS. Expression of  $\alpha 7$  homomers or  $\alpha/\beta$  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  $\alpha 6\beta 4$ . Co-expression of wild-type  $\beta 3$  led to a nearly complete loss of function (measured as maximum current response to ACh) for  $\alpha 7$  and for all functional  $\alpha/\beta$  pairs except  $\alpha 3\beta 4$ . 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 VS mutation in the 9' position of the second transmembrane domain of  $\beta 3$ , a mutation thought 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 signalling in the central nervous system.

## Introduction

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  $\alpha$ -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”  $\alpha$  ( $\alpha 2$ - $\alpha 4$ ) or with a  $\beta$ -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”  $\alpha$  and a “typical”  $\beta$  subunit (Groot-Kormelink et al., 1998). In *Xenopus* 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, as 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 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  $\beta 3$  subunit can form functional receptors with combinations other than  $\alpha 3\beta 4$  and, if so, how it changes the properties of the receptor. One exception is that of  $\alpha 7$ : it has been reported that  $\beta 3$  forms ‘silent’ heteromeric receptors with  $\alpha 7$  subunits (Palma et al., 1999). There is also a brief report that  $\beta 3$  may change the macroscopic time course of  $\alpha 3\beta 2$  currents (McIntosh *et al.*, 2000), but a detailed study of  $\alpha 3\beta 2\beta 3$  receptors is not available in the literature.

We systematically examined  $\beta 3$  incorporation by co-expression in oocytes and found that  $\beta 3$  profoundly reduces responses produced by all pairwise functional nAChR subunit combinations known to date, with the exception of  $\alpha 3\beta 4$ . This dominant negative effect is not due to a change in receptor numbers and disappears when a gain-of-function mutant  $\beta 3$  is expressed rather than wild-type  $\beta 3$ . This, together with the evidence for  $\beta 3$  incorporation, suggests that  $\beta 3$  reduces the maximum receptor open probability ( $P_{\text{open}}$ ). This effect of  $\beta 3$  was also observed in the native  $\alpha 7$ -like nAChRs of hippocampal neurons in culture. Expression of  $\beta 3$  can exert a novel effect on neuronal nAChRs, which results in the functional downregulation 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, Accession Numbers Y16281 ( $\alpha$ 2), Y08418 ( $\alpha$ 3), Y08421 ( $\alpha$ 4), Y08419 ( $\alpha$ 5), Y16282 ( $\alpha$ 6), Y08420 ( $\alpha$ 7), Y08415 ( $\beta$ 2), Y08417 ( $\beta$ 3) and Y08416 ( $\beta$ 4). 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  $\beta$ -globin sequence. Capped cRNA was transcribed using the SP6 mMessage mMachin™ 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<sub>2</sub> 0.82; CaCl<sub>2</sub> 0.77; NaHCO<sub>3</sub> 2.4; Tris-HCl 15; with 50 units/ml penicillin and 50  $\mu$ g/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

amount of cRNA for each combination was determined empirically, with the aim of achieving a maximum ACh-evoked current of 1 – 2  $\mu$ A, 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  $\text{Ca}^{2+}$ -free Ringer (mM: NaCl 150, KCl 2.8, HEPES 10,  $\text{MgCl}_2$  2, atropine sulphate 0.5  $\mu$ M, 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 M $\Omega$  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  $\alpha 7$ -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:

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

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 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<sub>2</sub> in Dulbecco's Modified Eagle's Medium containing Glutamax (Invitrogen, Paisley, UK), plus 10%



foetal calf serum, 100units/ml penicillin and 100 $\mu$ g/ml streptomycin (all Sigma Aldrich, Gillingham, UK). Sub-confluent cultures were transiently transfected overnight with a total of 0.6  $\mu$ g of cDNA per 10cm dish, using the Effectene<sup>TM</sup> 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  $\alpha$ 7 experiments, cells were transfected with cDNA for human RIC-3 protein (subcloned into pcDNA3 and transfected in equimolar amount with  $\alpha$ 7 cDNA) to ensure  $\alpha$ 7 surface expression (Williams *et al.*, 2005).

*[<sup>3</sup>H]-epibatidine binding* Samples were incubated on ice for 2 hours with a single saturating dose of [<sup>3</sup>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.

*[<sup>125</sup>I]- $\alpha$ -bungarotoxin binding* Samples were incubated at room temperature for 2 hours with a single saturating concentration of [<sup>125</sup>I]- $\alpha$ -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

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  $\alpha 7$ ,  $\beta 3$  or  $\beta 3^{V273S}$  (1:1 ratio, 0.4  $\mu\text{g}/\mu\text{l}$  per 35 mm-dish) using Effectene<sup>TM</sup> (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<sub>2</sub> 2, MgCl<sub>2</sub> 2, glucose 10, HEPES 5; atropine (1  $\mu\text{M}$ ) and tetrodotoxin (TTX, 0.3  $\mu\text{M}$ ); pH adjusted to 7.3 with NaOH. The pipette solution consisted of (in mM): CsCl 147, MgCl<sub>2</sub> 2, CaCl<sub>2</sub> 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 M $\Omega$ . Series resistance (4-8 M $\Omega$ ) 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%

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.

## 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 Figure 1 (top) show that co-expression of the  $\beta 3$  subunit effectively suppressed responses evoked by 1 mM ACh from neuronal nicotinic receptors expressed in oocytes from  $\alpha/\beta$  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 previously shown 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  $\alpha 3\beta 4$  combination was somewhat spared by co-expression with  $\beta 3$ , with a reduction of approximately two-thirds (from  $1.9 \pm 0.36$  to  $0.71 \pm 0.13$   $\mu\text{A}$ ,  $n = 10$  and 12, respectively).

In order 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 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  $\beta 3$  only if this effect is mediated by the incorporation of the subunit into the pentamer, rather than by a non-specific 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 (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  $\beta 3$  is due to a specific reduction in the  $P_{\text{open}}$  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  $P_{\text{open}}$  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 ( $n = 10$ , 2 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 (see Boorman et al. 2000). Nevertheless, we tested this possibility, by characterising the ACh sensitivity of receptors expressed in oocytes by  $\alpha 4\beta 2 + \beta 3^{\text{VS}}$ . Figure 2 shows the dose-response curves of recombinant  $\alpha 4\beta 2$  receptors expressed alone (filled circles, continuous line) or together with  $\beta 3^{\text{VS}}$  (filled triangles, dashed 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  $160 \pm 14 \mu\text{M}$  ( $n\text{H}$  was  $0.78 \pm 0.09$  and  $2.4 \pm 0.2$ , 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  $\alpha$  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 co-expressed with  $\alpha 4$  and  $\beta 2$ , the ACh dose-response curve is shifted leftwards and appears to have only one component ( $EC_{50}$  of  $1.0 \pm 0.1 \mu\text{M}$ ,  $n\text{H}$   $1.1 \pm 0.1$ ,  $n = 4$ ), which is clearly different from either of the two components observed for  $\alpha 4\beta 2$  alone. These effects of  $\beta 3^{\text{VS}}$  co-expression 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  $\beta 3^{VS}$ . Similar results were obtained for the  $\alpha 3\beta 2$  combination expressed alone and with  $\beta 3^{VS}$  (data not shown).

#### *Combinations containing the $\alpha 6$ subunit*

The expression of 'pair'  $\alpha 6$  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  $\alpha 3\beta 4$  combination). For  $\alpha 6\beta 2$  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  $\alpha 6\beta 4$  ( $49 \pm 15$  nA,  $n = 8$ , 2 batches of oocytes). Co-expression of the  $\beta 3$  subunit in its wild-type form did not affect the amplitude of responses elicited from  $\alpha 6$  combinations: only 2 out of 8 oocytes injected with  $\alpha 6\beta 2\beta 3$  responded to ACh (average 4 nA), whereas 6 out of 8 oocytes injected with  $\alpha 6\beta 4\beta 3$  responded to 1 mM ACh ( $18 \pm 5.6$  nA). Somewhat unexpectedly, co-expression of the gain-of-function mutant  $\beta 3^{VS}$  significantly increased nicotinic responses in both combinations to  $120 \pm 17$  nA and  $560 \pm 140$  nA for  $\alpha 6\beta 2\beta 3^{VS}$  and  $\alpha 6\beta 4\beta 3^{VS}$ , 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  $\beta 3$  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, as hippocampal neurons have robust responses to nicotinic agonists, but do not express the  $\beta 3$  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 ( $250 \pm 54$  pA, 25 out of 27 neurons tested). As previously reported by Albuquerque and co-authors (1997), these responses are likely to be mediated by  $\alpha 7$  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 out 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 ( $3800 \pm 510$  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  $\alpha 7$ -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 out 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 4 neurons, currents similar to those recorded in untransfected neurons were observed ( $490 \pm 240$  pA,  $n=4$ ).

As in oocyte-expressed recombinant receptors, transfection of hippocampal neurons with the mutant  $\beta 3^{\text{VS}}$  subunit failed to produce the dominant negative effect observed for the wild-type transfections. As shown in Fig 3A, almost all neurons transfected with  $\beta 3^{\text{VS}}$  responded to 3 mM ACh with inward currents comparable in amplitude with those measured in control neurons in the same dishes ( $240 \pm 36$  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  $\alpha 7$  subunit: co-expression of the  $\beta 3$  wild-type subunit produced an 88% decrease in the responses to 1 mM ACh (from  $0.97 \pm 0.15$  to  $0.10 \pm 0.027$   $\mu\text{A}$ ,  $n = 8$  and 14, respectively), as shown by the middle trace in Fig 3B. If the mutant  $\beta 3^{\text{VS}}$  subunit was co-expressed, instead of wild-type  $\beta 3$ , the decrease was by 60% (to  $0.38 \pm 0.057$   $\mu\text{A}$ ,  $n = 13$ ; 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  $\beta 3^{VS}$ . The ligand was [ $^3\text{H}$ ]-epibatidine for the  $\alpha 3\beta 4^*$  and  $\alpha 4\beta 2^*$  sites and [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin for  $\alpha 7^*$  receptors. In the  $\alpha 7$  experiments tsA201 cells were transfected with both  $\alpha 7$  and the human RIC-3 protein in order to ensure reliable surface expression of the  $\alpha 7$  receptor (Williams *et al.*, 2005).

As shown in Figure 4, co-expression of wild-type  $\beta 3$  did not abolish surface expression of nAChR. After co-expression of  $\beta 3^{WT}$ , 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. 4A, cfr. 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. 4B; 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. 4C; cfr. the 88% suppression in functional ACh responses for the same combination, Fig. 3B) by the co-expression of  $\beta 3$  wild-type, but not by co-expression of  $\beta 3^{VS}$  ( $86 \pm 14\%$  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 co-workers (1999) that chick  $\beta 3$  and  $\alpha 7$  subunits co-assemble into non-functional 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  $\beta 3^{VS}$  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  $P_{open}$  for the agonist, and we shall examine these factors in turn.

Our data show that the effect of co-expressing  $\beta 3$  or  $\beta 3^{VS}$  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).

Agonist responses will also be affected by the size of the single-channel current. The nearly complete suppression of 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 which form three rings of charges.  $\beta 3$  has a negatively-charged glutamate in the external 20' ring, where other neuronal  $\beta$  subunits have a positively-charged lysine. As  $\beta 3$  takes the place of a  $\beta$  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  $P_{\text{open}}$  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  $P_{\text{open}}$  is given by

$E/(E+1)$  (where  $E = \beta/\alpha$  is the gating equilibrium constant for the fully-liganded channel,  $\beta$  and  $\alpha$  being the opening and closing rate constants, respectively). The effect of reducing  $E$  on the maximum  $P_{\text{open}}$  will be relatively small if  $E$  is large in the first place. Hence,  $\alpha3\beta4$  function would be relatively spared by  $\beta3$  if  $\alpha3\beta4$  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<sub>A</sub> receptors, Jones and Westbrook, 1995).

Because  $\beta3$  produces such a complete suppression of nicotinic currents, obtaining direct estimates of the maximum  $P_{\text{open}}$  by single-channel measurements is not feasible for most  $\beta3$ -containing combinations. The exception is  $\alpha3\beta4\beta3$ , and our single-channel data from this combination are consistent with the hypothesis that  $\beta3$  primarily impairs gating. Incorporation of  $\beta3$  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  $38.3 \pm 11.3$  to  $2.9 \pm 0.31$  gaps per burst,  $n=10$  and  $5$ , respectively; Beato and Sivilotti, unpublished), but does not affect the macroscopic  $EC_{50}$  of  $\alpha3\beta4$  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  $\beta$  is the opening rate constant and  $k_{\text{off}}$  is the dissociation rate constant. If  $\beta3$  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

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

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

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 doesn't consider the possibility that  $\beta_3$  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 $\beta_3$ 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 signalling 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, see for instance Gerzanich *et al.*, 1995), the predominance of  $\alpha_3\beta_4$  responses may mean that higher ACh levels are needed to produce nicotinic responses where  $\beta_3$  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  $\alpha/\beta$  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, as 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 proved very hard to characterize because of low functional expression. In our hands, 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  $\beta 3^{VS}$  was co-expressed. 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. 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).

The disappearance of a specific striatal  $\alpha 6$ -containing receptor ( $\alpha$ -conotoxinMII-sensitive) in  $\beta 3$ -null mice has been taken to mean that efficient formation of this receptor requires  $\beta 3$ . However,  $\beta 3$  deletion *increased* another type of nicotinic response, i.e. the  $\alpha$ -conotoxinMII-resistant component of dopamine release produced by nicotine (Cui et al., 2003). Hence, this distinct receptor population was thought not to contain  $\beta 3$ . However, their enhanced function after  $\beta 3$  deletion could be explained if the receptors normally do contain  $\beta 3$  and if their function is reduced by the presence of  $\beta 3$ . The precise subunit composition of the receptor could thus determine whether  $\beta 3$  stabilises the receptor or reduces its function.

$\beta 3$  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  $\beta 3$  with  $\alpha/\beta$  pair receptors is reversed by the VS 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  $\alpha/\beta$  pair alone (first trace in each group of three), together with the wild type  $\beta 3$  subunit (middle trace) or together with the  $\beta 3^{\text{VS}}$  mutant subunit (last trace in each group).

Bottom: Average inward currents ( $\mu\text{A}$ ) for the same subunit combinations. Each bar represents the mean ( $\pm$  standard deviation of the mean) of 7-17 oocytes.

Note that co-expression 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  $\beta 3^{\text{VS}}$  was expressed.

Neither  $\beta 3$  nor  $\beta 3^{\text{VS}}$  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  $+\beta 3^{\text{WT}}$  data (middle column) and with  $+\beta 3^{\text{WT}}$  for the  $+\beta 3^{\text{VS}}$  data (columns on the right).

**Figure 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 (black bars) 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  $6.56 \pm 1.82$  and  $159 \pm 14.3 \mu\text{M}$  ( $n = 4$ ; filled circles, continuous line shows fit of a two-component Hill equation). When  $\beta 3^{\text{VS}}$  is co-expressed with  $\alpha 4$  and  $\beta 2$  (filled triangles, dashed line), these two components are replaced by a single component with high ACh sensitivity ( $EC_{50}$   $1.02 \pm 0.12 \mu\text{M}$ ,  $nH$   $1.12 \pm 0.10$ ,  $n = 4$ ).

**Figure 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  $\beta 3^{\text{VS}}$  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  $\alpha 7$  receptors (1 nM,  $n=14$ ). Bottom bar chart shows the amplitude of ACh responses in neurons in the different categories. The control and the  $+\beta 3^{\text{VS}}$  columns show the average current to 3 mM ACh in responding neurons (25 out of 27 and 48 out of 53, respectively). Most  $\beta 3$  transfected neurons (34 out of 38) did not respond to ACh at all; the remaining 4 neurons

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  $\alpha 7$  alone or together with  $\beta 3^{\text{WT}}$  or  $\beta 3^{\text{VS}}$ , 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  $\beta 3^{\text{WT}}$  were compared with controls and responses in cells transfected with  $\beta 3^{\text{VS}}$  were compared with those in cells transfected with  $\beta 3^{\text{WT}}$ .

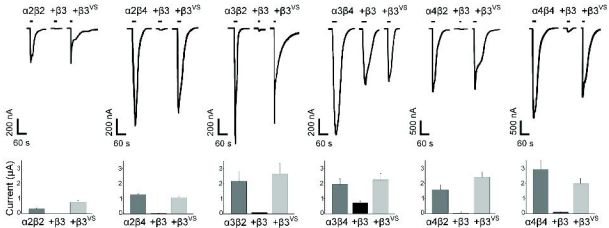
**Figure 4** Changes in the number of nAChRs on the cell surface cannot explain the dominant negative effect of  $\beta 3$  on ACh currents.

The bar charts show the number of nicotinic binding sites (measured by [ $^3\text{H}$ ]epibatidine, A and B or by [ $^{125}\text{I}$ ]- $\alpha$ -bungarotoxin in C) on the surface of tsA201 cells transiently transfected with a base combination ( $\alpha 3\beta 4$  in A,  $\alpha 4\beta 2$  in B,  $\alpha 7$  in C), 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 ( $416 \pm 92.0$ ,  $197 \pm 63.1$  and  $151 \pm 29.6$  fmol/mg of protein for  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$  and  $\alpha 7$ , respectively).

Note that co-transfection 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  $\beta 3$  co-expression.

\* $p < 0.05$ ; \*\* $p < 0.01$ , 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  $\beta 3^{\text{WT}}$ .

Fig. 1



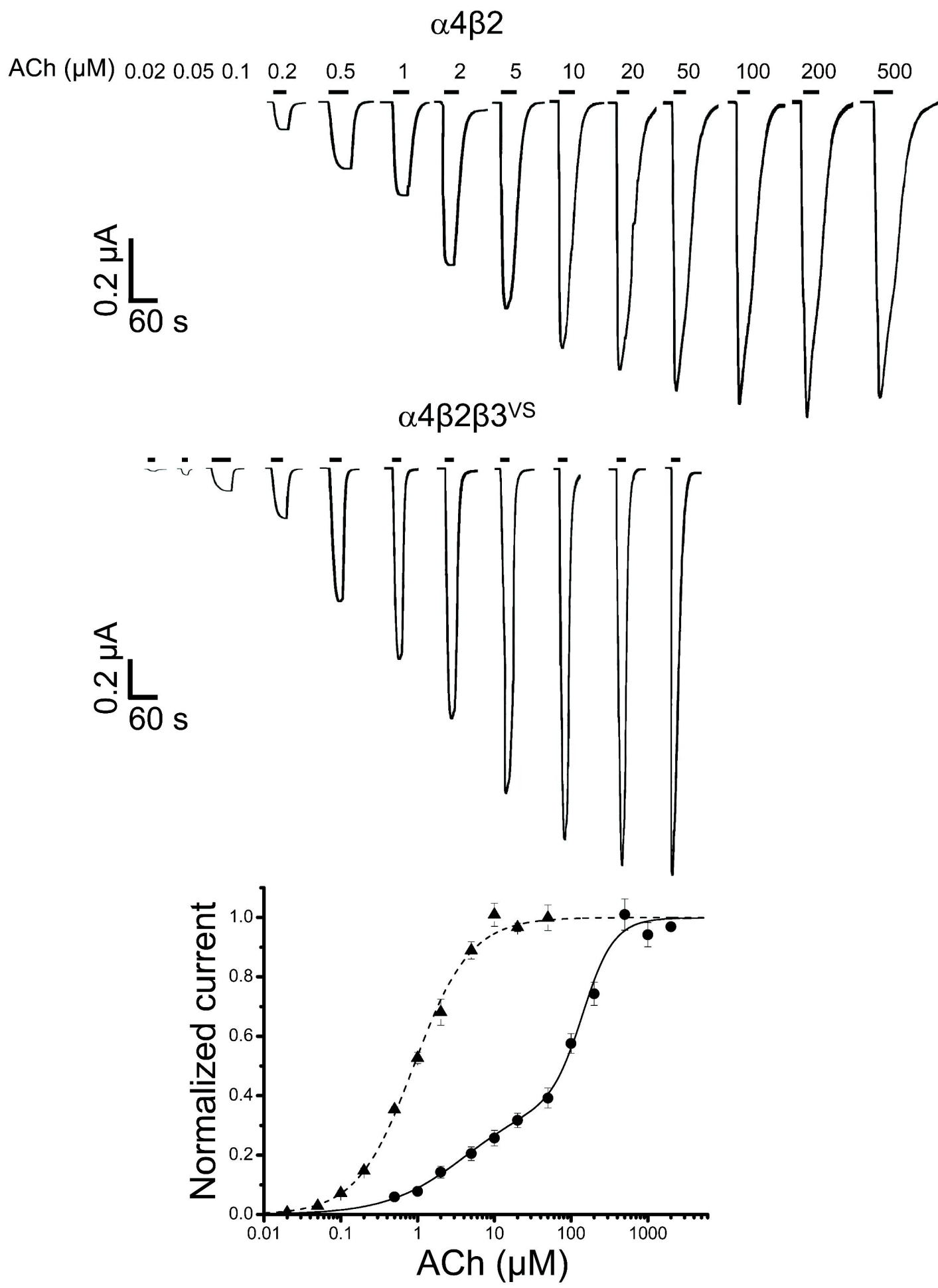
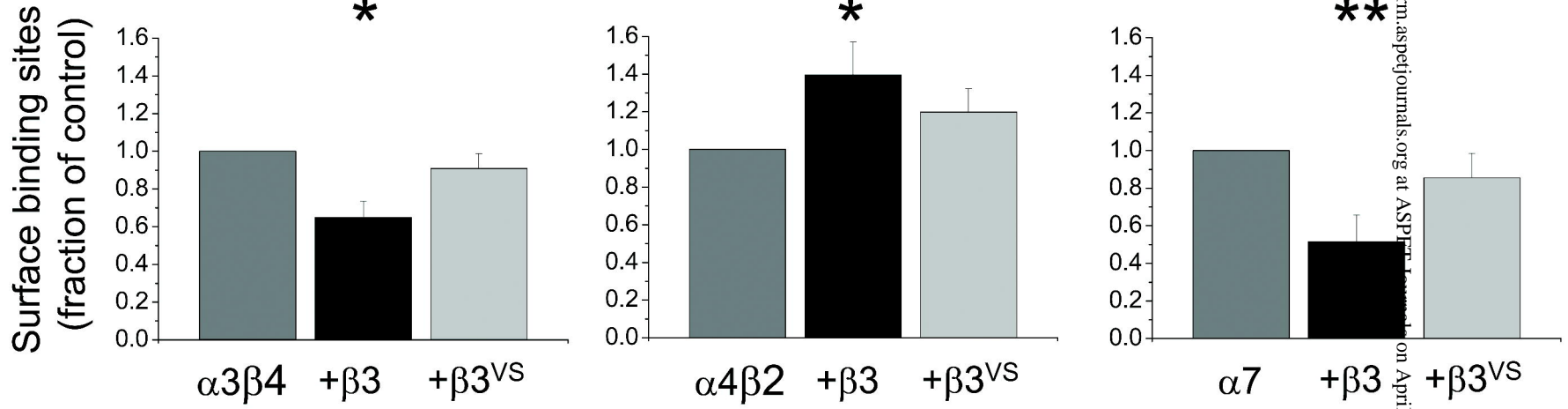


Fig. 3



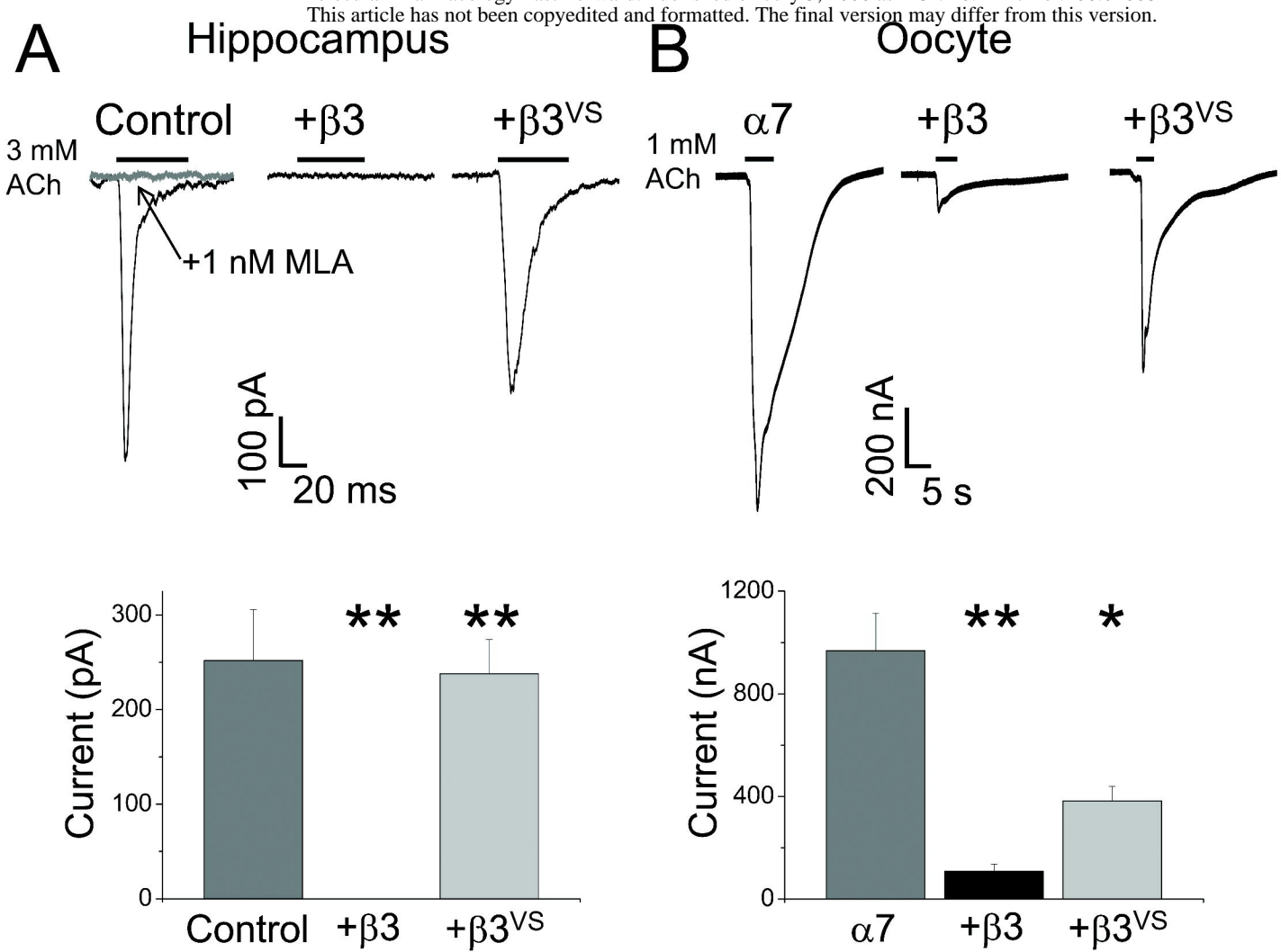


Fig.4